Transcriptional Regulation of Cidea, Mitochondrial Cell Death-inducing DNA Fragmentation Factor α-Like Effector A, in Mouse Liver by Peroxisome Proliferator-activated Receptor α and γ*

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Cidea (cell death-inducing DNA fragmentation factor α-like effector A), a member of a novel family of proapoptotic proteins, is expressed abundantly in the brown adipose tissue of the mouse. Although Cidea mRNA is not detectable in the mouse liver, we now show that peroxisome proliferator-activated receptor (PPAR) α ligands Wy-14,643 and ciprofibrate increase the Cidea mRNA level in a PPARα-dependent manner, whereas Cidea induction in liver by PPARγ overexpression is PPARα independent. Increase in Cidea mRNA content in liver did not alter the expression of uncoupling protein 1 (Ucp1) gene, which regulates thermogenesis, lipolysis, and conservation of energy. Although Cidea is considered to be a proapoptotic factor, Cidea induction in liver did not result in increased apoptosis. To elucidate the mechanism by which PPARα and PPARγ regulate Cidea gene expression in the liver, we analyzed the promoter region of the Cidea gene. Three putative peroxisome proliferator response elements (PPREs) are found in the Cidea gene promoter. Transactivation, gel-shift, and chromatin immunoprecipitation assays indicated that the proximal PPRE in Cidea gene (Cidea-PPRE1 at ~680/−668) is functional for both PPARα and -γ. We conclude that Cidea is a novel target gene for both PPARα and -γ in the liver where these two transcription factors utilize the same PPRE region for dual regulation. The induction of Cidea in liver with these PPARα and -γ agonists suggests a possible role for Cidea in energy metabolism and a less likely role in hepatocyte apoptosis.

The peroxisome proliferator-activated receptor (PPAR)2 subfamily of nuclear receptors consists of three isoforms, namely PPARα(NR1C1), PPARβ/δ(NR1C2), and PPARγ(NR1C3) (1–3). These transcription factors serve as sensors for fatty acids and their metabolic intermediates to regulate the combustion and storage of dietary lipids (2). PPARα is the master regulator of fatty acid oxidation systems and energy combustion, predominantly in liver, and also in brown adipose tissue (BAT) and heart (1, 2, 4, 5). On the other hand, PPARγ, which is expressed at a relatively high level in both BAT and white adipose tissue (WAT), controls adipogenesis vis-à-vis energy conservation (6). PPARβ/δ is ubiquitously expressed and controls energy burning in extrahepatic tissues such as skeletal muscle (7). Despite the high levels of homologies at the protein level, PPARs generally display distinct and non-interchangeable functional roles in overseeing the processes controlling mammalian energy balance. PPARs heterodimerize with the 9-cis-retinoic acid receptors (RXRs) and the PPAR-RXR heterodimer binds to peroxisome proliferator response elements (PPREs), consisting of a direct repeat of the consensus half-site motif (AGGNCA) spaced by a single nucleotide (DR-1) (8). Unliganded PPAR-RXR heterodimers bind to these DNA elements in association with nuclear receptor corepressor molecules such as SMRT and N-CoR and repress transcription (9). Upon ligand binding, PPARs manifest conformational changes that facilitate the dissociation of corepressor molecules to enable a spatio-temporally orchestrated recruitment of coactivators and coactivator-associated proteins to initiate transcriptional activity (10, 11). Several genes that are selectively regulated by a given PPAR isotype have been identified over the years, and a majority of these genes is known to play a central role in energy metabolism (4, 5, 12–15). Microarray technology and genomewide identification of PPREs suggest the existence of many other target genes that are not previously known to be regulated by PPARs (12–15). In mouse liver, for example, studies with PPARα ligands, as well as overexpression of PPARγ yielded valuable novel information about putative target gene(s) for these two PPAR isoforms (12, 13). One of the genes identified by microarray analysis, called Cidea, a member of a novel family of cell death-inducing DFFA (DNA fragmentation factor-α)–like effectors (CIDEs) (17–20), is up-regulated in liver in response to PPARα ligands as well as PPARγ overexpression (12, 16).

Cides represent a novel family of proapoptotic proteins that consist of five known members in the transcriptome of humans and mice. These include Cidea, Cideb, Cidec (also known as...
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Cide-3, or FSP27/fat-specific protein 27), DFF40 and DFF45 (17–22). In the mouse, Cidec/FSP27 functions as an adipocyte-specific gene and its expression is regulated by C/EBP (CCAT enhancer-binding protein) and in liver by PPARγ, implying that Cidec and possibly other Cide family members participate in adiogenesis (20, 21). Recently, Cidea has received considerable attention because genetic deletion of this gene by homologous recombination resulted in increased lipolysis, heightened metabolic rate, and excess thermogenesis (19, 20). Mice deficient in Cidea are also resistant to high-fat diet-induced obesity suggesting that the absence of Cidea in BAT leads to up-regulation of thermogenic mitochondrial protein Ucp1 (19). From these observations it is concluded that Cidea functions as a negative regulator of Ucp1 activity in BAT (19, 20). In this study, we examined the proximal Cidea promoter region to elucidate the mechanism of increased Cidea transcriptional activation by PPARα and PPARγ. Cidea is undetectable in normal mouse liver but it is expressed at very high levels following treatment with PPARα ligands or with PPARγ overexpression.

We show that a Cidea gene promoter contains a functional PPRE that is responsive to both these PPAR isotypes. Cidea induction in liver caused by PPARα ligands is not associated with enhanced apoptosis.

EXPERIMENTAL PROCEDURES

Mice and Treatments—Wild-type (C57BL/6J) and PPARα−/− mice (3–4 month old) were kept on a 12-h light-dark cycle (23). They were maintained on powdered chow, with or without Wy-14,643 (0.125% w/w) or ciprofibrate (0.025% w/w) for 10 days. Aox gene knock-out (AOX−/−) mice were maintained on normal chow and killed at 3, 6, and 9 months of age (24, 25). Ad/PPARγ1 and Ad/LacZ were injected intravenously via tail vein, in a volume of 200 μl with 1 × 1011 virus particles and killed 6 days later (13, 16). PPARγ ligand troglitazone (TZD) was fed for 7 days at a concentration of 0.1% (w/w). For the induction of fatty liver, PPARα−/− mice were either fasted for 96 h or fed a choline-deficient diet (Dyets, Bethlehem, PA) for 15 days (13). Hepatocellular carcinomas (HCC) used in this study were as described before (25). Briefly, HCC were induced by feeding mice with Wy-14,643 (0.125% w/w) or ciprofibrate (0.025% w/w) for 1 year and liver tumors harvested for RNA isolation. AOX−/− mice develop liver tumors spontaneously by 12 months of age, due to sustained activation of PPARα by unmetabolized PPARα natural ligands (25, 26). Diethylthiourea was used for liver tumors induced by a genotoxic carcinogen (26). Each group consisted of at least 5 tumor-bearing animals. These animals had multiple liver tumors and one randomly selected tumor from each animal was used for Cidea expression. To assess whether Cidea induction in liver leads to increased apoptosis, groups of 5–8 wild-type mice were fed control diet or diet containing Wy-14,643 for 4 days and 4 weeks, then hematoxylin- and eosin-stained or TUNEL-stained (Roche Applied Science) liver sections were analyzed.

RNA Preparation, RT-PCR, and Northern Blotting—Total RNA was prepared from mouse tissues using TRIzol reagent (Invitrogen). RT-PCR was performed with 2 μg of total RNA with SuperScript™ III One-Step RT-PCR System using Platinum® Taq DNA polymerase (Invitrogen) to amplify Cidea and Ucp1 coding sequences. Specific primers used to amplify the full-length coding sequence were Cidea-F/Cidea-R and mUcp1-F/mUcp1-R (see sequences in Table I). Amplified cDNA was cloned into pCR4-TOPO vector (Invitrogen), sequenced, radiolabeled, and used as a probe. Quantitative PCR procedure was used for the Cidea, aP2, Ucp1, Ucp2, and Ucp3 using the primer sequences in Table 1. Total RNA (20 μg) was glyoxylated, electrophoresed on 0.9% agarose gel, transferred to nylon membrane, and hybridized at 65 °C in Rapid-hyb buffer (GE Healthcare) and probed with 32P-labeled cDNA. These membranes were also probed with glyceraldehyde-3-phosphate dehydrogenase cDNA to gauge equivalency of RNA loading.

Mapping the Cidea Transcription Start Site—The transcription start site of the Cidea gene was established by 5′ rapid amplification of cDNA ends (RACE) protocol. A 5′/3′ RACE kit, essentially as recommended by the manufacturer (Roche Applied Science) was used. Briefly, total RNA extracted from BAT was cleared of contaminating DNA by digestion with 5 units of RNase-free DNase (Ambion) for 1 h at 37 °C. Purified RNA (3 μg) was reverse transcribed at 55 °C with recombinant transcriptor reverse transcriptase using Cidea-specific primer Cide-A(RACE)-1, which anneals 106 bp downstream of the Cidea translational start site. A homopolymeric tail was appended to the 3′ end of the synthesized first-strand cDNA using terminal transferase and dATP by incubation at 37 °C for 20 min as described in the RACE kit protocol. The dA-tailed cDNA was PCR amplified using an oligo(dT)-anchor primer and another Cidea-specific primer Cide-A (RACE)-2, which anneals 79 bp downstream of the Cidea translational start site. The amplified products were purified using a High Pure PCR product purification kit (Roche Applied Science) and cloned into the pCR4-TOPO (Invitrogen) and sequenced to assign transcription start site.

Promoter Sequence Retrieval by Computational Analysis—From the NCBI BLAST Mouse Genome Sequence data base, we selected 3004 bp of sequence upstream to the translation initiation codon (ATG) of the Cidea gene, which also includes a 79-bp coding sequence (GenBank™ accession number EF451058). Transcription factor binding site analysis was performed using MatInspector tool from Genomatix Bibliosphere and the presence of PPRE in the Cidea promoter was detected.

Construction of Luciferase Deletion Plasmids—BAC clone (RP24-213P14), containing the full-length mouse Cidea genomic sequence was identified from the clone finder tool of Mouse Genome resources (NCBI) and obtained from the BACPAC resources, CHORI. The DNA fragment (~3 kb), containing the Cidea promoter was amplified using the Expand High Fidelity PCR system (Roche Applied Science) and designated pL-Cid1. Five different deletion fragments (see Fig. 3) were also generated using pL-Cid1 as template and subcloned into pGL3-basic vector (Promega). Restriction sites were incorporated into the primers used (Table 1, restriction sites are underlined). The primer pairs used for the amplification of all fragments were as follows: pL-Cid1, Cidea-1-Nhel-F and Cidea-2-XhoI-R (or 1 and 2), likewise pL-Cid2; 3 and 2, pL-Cid3; 4 and 2, pL-Cid4; 8 and 2, pL-Cid5; 5 and 6, and pL-Cid6; 5 and 7. All amplified DNA sequences were cloned into pCR4-TOPO. 
Regulation of Cidea by PPARα and PPARγ ligands.

Northern blot analysis of total RNA isolated from liver of PPARα+/+ (wild-type) and PPARα−/− mice using full-length RT-PCR amplified Cidea and Ucp1 coding sequence as probes (A). Mice were treated for 10 days with PPARα ligand Wy-14,643 (0.125% w/w), or ciprofibrate (Cip; 0.025% w/w) for Cidea and Ucp1 expression (B and C). Total RNA obtained from BAT of untreated mice was used as control for Cidea expression. D, quantitative analysis of Ucp1, Ucp2, and Ucp3 expression in PPARα−/− mouse liver. E and F, induction of Cidea in liver by adenoviral-mediated PPARγ overexpression (E) and (F) by PPARγ ligand TZD. PPARα−/− mice were infected with 1 × 10^13 Ad/PPARγ viral particles using tail vein injection and killed 6 days after infection. PPARα−/− mice injected with Ad/LacZ virus particles served as control (E). Induction of Cidea was also observed in the liver of wild-type mice fed a diet containing synthetic PPARγ ligand, TZD at 0.1% (w/w) for 7 days (F). RNA from two individual mice was loaded for each treatment group except RNA from BAT that was used as a positive control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a measure of RNA loading control. G and H, Northern blot and quantitative PCR data representing Cidea and aP2 expression in PPARα−/− mouse liver given TZD.

TOPO vector (Invitrogen) and sequenced to ensure fidelity of PCR amplification. All DNA fragments with deletions were then retrieved from the pCR4-TOPO and ligated into pGL3-basic vector (Promega) and designated pL-Cid2 to pL-Cid6. For transactivation assays, nuclear receptor proteins overexpressed from plasmids pCMX-PPARα, pCMX-PPARγ, and pCMX-RXRα were used.

Transient Transfection and Luciferase Assays—HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. For HepG2 cells this medium was supplemented with 10% fetal bovine serum, 1.5 g/liter sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. Cells were transfected using Lipofectamine 2000 (Invitrogen) with 1.5 μg of DNA and 0.04 μg of pCMV-RL (Renilla luciferase) to normalize for transfection efficiencies. For control, equimolar amounts of promoterless, enhancerless pGL3-basic vector was used along with pCMV-pRL construct. After transfection cells were incubated in the presence or absence of ligands, Wy-14,643 (10 μM), or troglitazone (10 μM) for 24 h. Transfections were performed in triplicate and repeated twice. Luciferase activity was performed 48 h after transfection using the Dual Luciferase assay kit, according to the manufacturer’s protocol (Promega). Luciferase activity of each construct was compared with that of the promoterless pGL3-Basic vector.

Electrophoretic Mobility Shift Assays—DNA-binding dynamics of PPARα and PPARγ on the Cidea-PPREs were studied by electrophoretic mobility shift assay (EMSA) with in vitro synthesized proteins and radiolabeled Cidea-PPRE double-stranded oligonucleotides. Mouse nuclear receptor proteins PPARα, PPARγ, and RXRα were synthesized using the TNT transcription/translation-coupled in vitro system (Promega). Oligonucleotides corresponding to the Cidea-PPRE1, -2, and -3 and for PPRE of peroxisomal bifunctional protein gene (HD-DR2) (see Table 1) were annealed and Klenow filled with dNTPs containing [α-32P]dCTP. Combinations of PPARα, PPARγ, and RXRα in unprogrammed reticulocyte lysate (as indicated) were incubated at 25°C for 10 min in binding buffer containing 50 mM Tris/HCl, pH 7.5, 250 mM NaCl, 5 mM MgCl2, 5 mM EDTA, 0.5 mM dithiothreitol, 250 μg/ml poly(dl-dC)-dl-dC), 5 μg of nonspecific competitor DNA, and 20% glycerol. Radiolabeled Cidea-PPRE or HD-DR2 probe (1 ng) were added to the reactions (total volume, 20 μl), and incubated for an additional 20 min. Reaction mixtures were analyzed by electrophoresis at 4°C on pre-run 3.5% polyacrylamide gels (30:1 acrylamide/N,N’-methylenebisacrylamide weight ratio) with 22 mM Tris base, 22 mM boric acid, 1 mM EDTA as running buffer.

Chromatin Immunoprecipitation Assay (ChIP)—Liver nuclei obtained from wild-type mice fed a control diet or a diet containing Wy-14,643 (0.125% w/w) for 4 days, and from PPARα−/− mice injected with Ad/mPPARγ for 6 days were cross-linked with 1% formaldehyde and used for extraction of soluble chromatin as described (27). Briefly, purified liver nuclei were fixed for 30 min in 1% formaldehyde to cross-link the DNA-binding proteins to cognate cis-acting elements.
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Nuclear homogenates were sonicated to shear the chromosomal DNA to an average length of ~1,000 bp. The chromatin was precleared by incubating with serum coupled to protein A-agarose beads saturated with bovine serum albumin (1 mg/ml) and salmon sperm DNA (0.4 mg/ml), immunoprecipitated with antibodies (1–2 μg/ChIP) specific to PPARα and PPARγ at 4 °C overnight. Protein-antibody complexes were pulled down with protein A beads coated with 1% bovine serum albumin, extensively washed sequentially by low salt wash buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 10 mM Tris-Cl, pH 8.1), eluted and reverse cross-linked. DNA in the immune complexes was extracted and used as the template in PCR amplification using primers Cidea-PPRE1(ChIP)-F and Cidea-PPRE1(ChIP)-R that encompass the putative peroxisome proliferator-response element of the mouse Cidea promoter region.

RESULTS

Cidea mRNA Is Induced in Mouse Liver by PPARα and γ Ligands—By using cDNA microarray, we noted the up-regulation of Cidea gene expression in wild-type mouse liver treated with PPARα ligands (12). Likewise, adenoviral-mediated overexpression of PPARγ ligand TZD in liver also resulted in a manifold increase in Cidea expression (13). To determine whether the Cidea gene contains a PPRE(s), which may be responsive to PPARα- and γ ligands, we first investigated the Cidea inductive effects in mouse liver following treatment with PPARα ligands Wy-14,643 and ciprofibrate, and PPARγ ligand TZD, using full-length RT-PCR amplified Cidea and Ucp1 cDNAs (Fig. 1A) for Northern blotting. All three ligands induced the expression of Cidea in wild-type mouse liver, but the induction was significantly greater with PPARα ligands compared with PPARγ ligand TZD (Fig. 1, B, C, and F). Cidea mRNA is not detectable by Northern blotting in untreated wild-type mouse liver, but when induced by PPARα ligands, the mRNA level appeared similar to that found in normal BAT (Fig. 1, B, C, F, and G). The induction of Cidea in liver by PPARα ligands is dependent upon the presence of PPARα in that PPARα−/− mice failed to respond to Wy-14,643 and ciprofibrate (Fig. 1, B, C, and E). Basal Cidea mRNA expression was also found to be negligible or undetectable in the liver of PPARα−/− mice on Northern blotting. These data clearly suggest that transcriptional enhancement of the Cidea gene by PPARα ligands is due to activation of nuclear receptor PPARα and the Cidea gene promoter would possess PPRE(s). However, the presence of PPARα is not a requirement with regard to the induction of Cidea mRNA by PPARγ overexpression (Fig. 1E). Although Cidea mRNA was not induced by TZD in PPARα−/− mouse liver (Fig. 1G), overexpression of PPARγ in these PPARα-deficient livers induced...
Cidea (Fig. 1E), implying that PPARγ is a limiting factor (28). We observed a robust expression of Cidea mRNA in liver of PPARα<sup>−/−</sup> mice 6 days after injection with 1 × 10<sup>11</sup> adenoviral-PPARγ particles intravenously, whereas untreated PPARα<sup>−/−</sup> mice or those given Ad/LacZ showed no Cidea mRNA expression (Fig. 1, E and G). Cidea protein interacts with Ucp1 and negatively regulates Ucp1 activity (19). Consequently, Cidea null mice exhibit enhanced uncoupling activity and increased thermogenic metabolism (19), suggesting that the expression profile of both genes in liver would possibly differ under the present experimental conditions. Ucp1 mRNA was also not detectable by Northern blotting in mouse liver and it was not induced by various treatments described above (Fig. 1, B–E), suggesting that Ucp1 regulation is independent of PPARα and -γ. As is known, Ucp2 and Ucp3 are expressed at relatively high levels in wild-type mouse livers (29) and PPARα deficiency did not affect the Ucp2 and Ucp3 levels in liver (Fig. 1D). TZD treatment resulted in an increase in Cidea mRNA level in PPARα<sup>+/+</sup> but not in PPARα<sup>−/−</sup> mouse liver (Fig. 1, F–H). Wild-type mice treated with TZD also showed induction in liver of adipocyte marker ap2 but not in PPARα<sup>−/−</sup> mice (Fig. 1, G and H).

**Stimulation of Cidea Expression in BAT and WAT with PPARα and -γ Ligands—PPARγ expression is more in BAT compared with WAT, whereas PPARα expression appears high in both BAT and WAT (18–23). We show that in response to PPARγ agonist Wy-14,643, there was nearly a 2-fold increase in Cidea mRNA in BAT (Fig. 2A). No change was observed for the Ucp1 expression in BAT (Fig. 2A). Mice treated with the PPARγ ligand troglitazone showed a minimal induction of the Cidea gene in WAT, and a modest increase in Cidea expression in BAT (Fig. 2B). The expression of the adipocyte marker ap2 in WAT and BAT appeared similar with no increase following TZD administration (Fig. 2B). Induction of Cidea also occurred in PPARα<sup>−/−</sup> mouse WAT and BAT following TZD treatment (Fig. 2C).**
Regulation of Cidea by PPARα and PPARγ

Because PPARα activation by exogenous ligands resulted in the induction of Cidea mRNA gene expression in liver, we decided to establish whether sustained activation of this receptor by endogenous ligands also leads to up-regulation of Cidea in liver. We analyzed total RNA extracted from the livers of 3-, 6-, and 9-month-old AOX−/− mice maintained on normal diet. Cidea mRNA levels increased in AOX−/− mouse liver at all age periods examined and the levels of expression appeared comparable with that occurring in response to exogenous ligands (Fig. 2D). PPARα is transcriptionally activated, in a sustained manner, in AOX−/− mouse liver due to unmetabolized substrate of AOX that function as endogenous ligands (agonists) of this transcription factor (25). Inability of AOX−/− mice to metabolize acyl-CoAs (most likely long chain and very long chain fatty acyl-CoAs) and other putative AOX ligands leads to sustained activation of PPARα and up-regulation of PPARα-regulated genes (25, 30).

Cidea gene is induced in livers with hepatic adiposis (adipogenic hepatic steatosis) resulting from PPARγ overexpression HepG2 did not express CIDEA and UCP1. Several factors can influence this non-response, in particular low levels of PPAR isoforms, and availability of RXR in this cell line or alterations in CIDEA promoter. It would be worthwhile to undertake additional studies designed to elucidate the effectiveness of overexpression of PPAR isoforms with or without their respective ligands on CIDEA induction.

Transcription Starting Site of Cidea—To identify the transcription start site of the Cidea gene, we searched for available mouse Cidea cDNA clones that contain potential full-length 5′-untranslated region. Alignment of the genomic sequence (NT_039674.6) with cDNA sequence (NM_007702) resulted in the identification of a 199-bp 5′-untranslated region, the longest sequence available in public databases. 5′ RACE analysis using total RNA isolated from mouse BAT identified a single DNA fragment of 824 bp (Fig. 3A). This was cloned into pCR4-TOPO vector (Invitrogen) and sequenced (Fig. 3B). The transcription start site was assigned to nucleotide A, located 745 bp upstream of the ATG start codon. Mapping of this 745-bp...
upstream region by NCBI tool, Spidey (32), and GT-AG rule for splicing confirmed the absence of an intron in the 5'-untranslated region and thus confirms that the sequence is part of exon 1.

Identification of the Mouse Cidea Promoter Region—After establishing the transcription start site position, we obtained a 3004-bp fragment of mouse genomic sequence that includes 39 bp downstream to the ATG translation start site (−2220/+784) (Fig. 3B; GenBank accession number EF451058). Transcription factor binding sites were identified by the MatInspector version 2.2 program from Genomatix (32). A core promoter of 80 bp was predicted from −424 to −503 bp that also contain putative TATA box motif (−474/−480 bp) upstream to the transcription start site identified by the 5'-RACE result (Fig. 3B). Several potential transcription factor binding sites were revealed in the Cidea promoter and of note is the presence of 3 putative PPREs (33, 34). The promoter also contains one or more vitamin D response elements (binding site for 1,25-dihydroxyvitamin D3 receptor/RXR heterodimer), sterol regulatory element-binding protein, GATA binding sites, and hepatocyte nuclear factor 4 and 1, among others (35–38).

Among all predicted transcription factor binding motifs in the Cidea promoter, we focused our efforts on the characterization of the three putative PPREs designated as Cidea-PPRE1, Cidea-PPRE2, and Cidea-PPRE3, which consists of an imperfect hexamer separated by 1 bp (DR1-like). The nucleotide sequences and positions of these putative PPREs are as follows: Cidea-PPRE1, 5'-TAGCCTTCCCCC-3' (−680/−668); Cidea-PPRE2, 5'-TGGCTTGCCACCC-3' (−1159/−1147); and Cidea-PPRE3, 5'-AAAGGCGAAGGCCCA-3' (−2102/−2090) (Figs. 3B and 4B). The sequence of Cidea-PPRE1 resembles more closely the PPRE of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene of a rat (5'-AGACCTTTGGC-3' (39).

Analysis of Mouse Cidea Promoter—To determine the functionality of PPREs in the Cidea gene promoter a series of 5' and 3' progressive deletions were generated by nested PCR using pL-Cid1 as a template, and their activities were measured by their ability to drive luciferase expression (Fig. 4A) in human embryonic kidney-derived HEK293 cells and hepatocellular carcinoma (HePG2). Nested deletions were created based on positions of PPRE and constructs containing one or more PPREs were prepared (Fig. 4A). The 5' deletions were designated as pL-Cid2 (−2130/+39), pL-Cid3 (−1633/+39), and pL-Cid4 (−1323/+39, minimal promoter) (Fig. 4B). pL-Cid2 and pL-Cid3 possess two and one proximal PPREs, respectively, whereas pL-Cid4 has no PPRE (minimal promoter). The 3' deletions, pL-Cid5 (−1358/−802) and pL-Cid6 (−2965/−1854) were designed to verify the activity exclusively from PPREs 2 and 3 (Fig. 4A). The Renilla expression vector, pCMV-RL, was used as an internal control for adjusting transfection efficiency. Both PPARα and PPARγ ligands enhanced the promoter activity of constructs pL-Cid1 (all three PPREs), pL-Cid2 (PPREs 1 and 2), and pL-Cid3 (PPRE1) in HepG2 (Fig. 4, B and C) and HEK293 cells (data not shown). In the presence of PPARγ or PPARα ligand, luciferase activity was maximal in the HepG2 cell line with pL-Cid3 (−1633/+39) that contains only PPRE1. pL-Cid5 and pL-Cid6 that contain PPRE2 and PPRE3, respectively, were found to be inactive (Fig. 4A). These cells express PPARα and PPARγ isoforms (Fig. 4D) necessary for PPRE activation. To determine whether the Cidea promoter responds to further stimulation, HEK293 cells were transfected with pL-Cid2 and promoterless pGL3-basic vector (minimum luciferase activity). These constructs were co-transfected with expression plasmid for PPARα/RXRα in the presence of Wy-14,643 (10 μM) or troglitazone (10 μM) were added to the medium 24 h after transfection. Cells were harvested 48 h after transfection. Luciferase activities were detected using the Promega Dual Luciferase Assay Kit (Promega) and represented as fold activity and given an arbitrarily value.

PPARα and RXRα Bind as Heterodimers to the Cidea-PPRE1—To determine whether α and γ isoforms of PPAR bind to the predicted Cidea PPRE(s) as heterodimers with RXRα, gel mobility shift assays were performed with three Cidea PPREs using in vitro translated PPARα, PPARγ, and RXRα proteins (Fig. 6, A and B). The double-stranded probe, encompassing individual PPRE (see Table 1), was Klenow filled with [α-32P]dCTP and incubated with in vitro translated proteins (Fig. 6A). This gel shift assay revealed an intense PPAR/RXR heterodimer complex with PPRE1 DNA but a weak complex with PPRE2 and PPRE3 (Fig. 6B). PPARγ/RXR complex with PPRE1 DNA appeared slightly more intense than that seen.

![FIGURE 5. Cidea cotransfection with PPARα or PPARγ expression vectors with appropriate ligand in HEK293 cells.](image-url)
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with PPARα/RXR (Fig. 6B). We used PRE of rat L-PBE (HD) as a positive control for the gel shift assay and as expected PPARα/RXR formed a protein complex on this PRE known to be regulated by PPARα (40). The interaction of Cidea-PPRE1 with PPAR subtype α and γ complexed with obligate heterodimer partner RXRα suggests that the proximal Cidea-PPRE1 is the functional PRE in the Cidea promoter sequence and Cidea-PPRE2 and the most distal Cidea-PPRE3 are likely redundant.

ChIP Assay and Mutational Analysis—To investigate whether PPARα/RXRα and PPARγ/RXRα are recruited to the Cidea promoter in vivo, ChIP assay was performed (27). Proteins were cross-linked to genomic DNA of liver cell nuclei of wild-type mouse treated with Wy-14,643 or from the liver of a PPARα+/− mouse injected intravenously with Ad/PPARγ1 and immunoprecipitated with normal rabbit IgG, or polyclonal antibodies to either PPARα or PPARγ. PCR with primers designed to amplify a 210-bp fragment (~780/-570) of the mouse Cidea-PPRE1 flanking region revealed significant amplification (Fig. 6C). Chromatin isolated from Wy-14,643-treated wild-type mice and immunoprecipitated with anti-PPARα resulted in the amplification of the Cidea promoter fragment containing PPRE1, but no binding was seen with Wy-14,643-treated PPARα−/− liver chromatin. When PPARγ antibody was used to pull down the protein-DNA immune complex, a very prominent amplification was obtained from the exogenously expressed PPARγ in the PPARα−/− mouse liver lysate (Fig. 6C). A weak signal was observed for the recruitment of PPARγ to the Cidea-PPRE1 in wild-type mice treated with PPARα ligand, Wy-14,643 (Fig. 6C). PPARα ligands are known to weakly trans-activate both PPARγ and PPARβ/δ and these two isoforms can diminish PPARα-mediated responsiveness to Wy-14,643 (2, 41). PPARγ ligands induce a limited binding or recruitment of PPARγ. To validate that the DNA fragment amplified was the expected 210-bp area of the mouse Cidea promoter region, this fragment was subcloned into TA cloning vector and then sequenced. Sequence data confirmed that the PCR product has the exact sequence of the Cidea promoter containing fragment, −780/−570 (data not shown). These results demonstrated the recruitment of PPARα and PPARγ transcripational factors to the mouse Cidea promoter.

Finally, to better delineate the observed DNA-protein interaction, several mutations were introduced in the Cidea-PPRE1 (Fig. 7A). All mutations in this functional PPRE1 abrogated PPARα/RXRα and PPARγ/RXRα binding except that PPARγ/RXRα binding was not completely abolished (Fig. 7B, data for m3–m5 not shown). To test the specificity of protein-DNA interaction, an excess of unlabeled oligonucleotide (Cidea-PPRE1) was added to the reactions. Binding of heterodimer PPARα/RXRα and PPARγ/RXRα to the Cidea-PPRE1 was specific because the radiolabeled complex was effectively competed out by the addition of unlabeled Cidea-PPRE1 (Fig. 7C). The PPARs/RXR complex generated by the normal Cidea-PPRE1 probe was partially dis-
placed by a 50-fold molar excess of the cold probe and almost entirely by a 100-fold excess.

Cidea Induction in Liver Does Not Lead to Increased Apoptosis—Control and Wy-14,643-treated wild-type mouse livers were analyzed for apoptosis. Apoptosis is a rare event in normal liver and treatment with Wy-14,643 for 4 days and 4 weeks caused no increase in apoptosis despite the increase in Cidea mRNA expression (Fig. 8). It appears that Cidea induction in liver does not lead to increased apoptosis. The functional significance of Cidea induction by PPARα and -γ ligands remains unclear.

DISCUSSION

Our results reveal high levels of expression of Cidea in mouse liver in response to activation of nuclear receptor PPARα by synthetic ligands such as Wy-14,643 and ciprofibrate but this induction did not occur in PPARα-deficient mice. The failure of Cidea induction by these peroxisome proliferators in PPARα−/− mouse liver suggests that the Cidea gene is possibly regulated by PPARα (23). This work also establishes that overexpression of PPARγ in liver, using the adenoviral approach, or treatment with PPARγ ligand TZD, leads to an increase in hepatic Cidea mRNA content independent of PPARα. Livers with PPARγ overexpression develop steatosis along with an increase in Cidea mRNA content. Such an increase in Cidea mRNA level was not observed in hepatic steatosis developing in mice fed a diet deficient in choline, which is mechanistically independent of PPARγ overexpression (13). Fatty liver induced by PPARα overexpression is unique in that such livers show activation of the adipogenic program in liver (13). Also of interest is the increase in Cidea mRNA content in HCC induced by sustained activation of PPARα but not in tumors induced by a genotoxic chemical carcinogen, confirming that HCC related PPAR activation constitute a unique set of neoplasms (26, 42).

The Cidea mRNA increase in the liver by PPARα and PPARγ activation is not associated with changes in hepatic Ucp1 levels. Ucp1, localized in BAT mitochondria, is responsible for energy utilization and thermogenesis as it functions to dissipate the proton electrochemical potential gradient into heat (19). Ucp1 is not normally expressed in liver and WAT, implying that these tissues do not contribute to thermogenesis as compared with Ucp1-rich BAT (19). Cidea, which is also localized to mitochondria, appears to suppress Ucp1 activity by forming a complex with Ucp1 (19, 29). Cidea-deficient mice are lean and resistant to diet-induced obesity possibly due to the elimination of a suppressive effect of this molecule on Ucp1 (19). The increased Ucp1 activity in Cidea null mice is likely responsible for excess energy burning and thermogenesis. Collectively, various observations point to a role for Cidea in the regulation of energy balance and adiposity (19, 22). Because Cidea gene disruption leads to an increase in Ucp1 level, it is likely that excess Cidea will deplete Ucp1 levels. This cannot be confirmed in liver under the present experimental conditions because Ucp1 mRNA is undetectable in normal mouse liver. Cidea has been first identified as a member of the proapoptotic Cide family of proteins (17, 19). Apoptosis is an evolutionarily conserved process to
eliminate unwanted or undesirable cells. The Cide group of proteins consists of five known members, namely DFF40 (also known as CPAN, CAD, or DFFB (DNA fragmentation factor, 40 kDa, β polypeptide)), DFF45 (also known as ICAD or DFFA (DNA fragmentation factor, 45 kDa, γ polypeptide)), Cidea, Cideb, and Cidec (also known as Cide3 or FSP27) (17, 18, 22, 43). The expression of Cide family genes appears to be tissue specific (17–19). Although Cidea and Cidec (FSP27) are not normally expressed in liver the expression of these two genes in liver is strongly induced by PPARα and PPARγ activation.

The massive increase in the Cidea mRNA level in liver in response to PPARα and PPARγ stimulation reported in this study, suggests that Cidea gene promoter activity is regulated via the PPRE (8). Although three divergent putative PPREs are located in the Cidea promoter (at −680/−668, −1159/−1147, and −2102/−2090), our data from promoter-deletion construct transfections in HEK293 and HepG2 cells, ChIP, EMSA, and EMSA with mutagenized PPRE demonstrate that both PPARγ and PPARα bind to proximal PPRE (Cidea-PPRE1 at −680/−668) in the Cidea gene. Cidea-PPRE2, and Cidea-PPRE3, appeared refractory to the binding of these nuclear receptors. Cidea, a mitochondrial protein, is encoded by a nuclear gene analogous to the PPAR-regulated mitochondrial HMG-CoA synthase (39), Cidea gene promoter contains an imperfect direct repeat (DR), TAGCCTTTCCCCC, in the Cidea-PPRE1 similar to the consensus half-site TGACCT needed for several nuclear hormone receptor-mediated gene transcriptions (8, 33). The functional Cidea-PPRE1 is composed of two hexameric DRs separated by one nucleotide (DR1). Mutations of either the 5’ half-site or the 3’ half-site or in both half-sites totally abolished the binding of PPARα/RXR heterodimers, and ChIP assay further confirmed the binding of
PPARα and PPARγ to the Cidea-PPRE1. These observations further establish the functionality of this PPRE in Cidea regulation in liver.

In addition to a PPRE that is functional with both PPARα and PPARγ, the Cidea gene promoter has several other transcription factor binding sites. The presence of 3 putative 1,25-dihydroxyvitamin D₃ receptor/RXR heterodimer binding sites in the Cidea promoter suggests that this gene may be regulated by vitamin D₃ (35). Vitamin D₃ plays a prominent role in the modulation of cell proliferation and the differentiation of several normal and malignant cells. Potent vitamin D₃ analogues induce growth inhibition of MCF-7 breast cancer cells by enhancing apoptosis (35). The presence of 1,25-dihydroxyvitamin D₃ receptor/RXR binding sites in the Cidea gene promoter supports the notion that certain nuclear receptor ligands induce apoptosis by increasing the amount of proapoptotic Cidea. Chronic exposure to PPARα ligands results in the development of liver tumors in rats and mice (44, 45). Sustained activation of PPARα by synthetic and natural ligands leads to liver tumor development possibly due to a confluence of several factors including liver cell proliferation, inhibition of apoptosis, and sustained oxidative DNA damage (30, 44–47). Although PPARα ligands induced Cidea mRNA in liver, this increase was not associated with enhanced apoptotic activity. PPARα ligands are known to inhibit hepatic apoptosis (46), but it is paradoxical that they increase Cidea, a proapoptotic molecule. Further studies are required to determine whether Cidea gene transcription is induced by vitamin D₃ and the role of nuclear receptor-induced Cidea in the maintenance of cell population dynamics. Cidea gene promoter also reveals six GATA binding sequences conforming to the WGATAR consensus sequence (in which W denotes A/T, and R indicates A/G) (37). GATA-1, GATA-2, and GATA-3 transcription factors are important regulators of erythroid and myeloid lineages and GATA regulation of the Cidea gene may be a safety for removing unwanted or defective cells during differentiation by inducing apoptosis.

In summary, our findings provide evidence for the first time that the Cidea gene is regulated by PPARα and PPARγ transcription factors. The Cidea gene promoter also reveals the presence of several other transcription factor binding sites and of note are 1,25-dihydroxyvitamin D₃ receptor/RXR, GATA, and HNF4 and HNF1 binding sites. We conclude that Cidea gene transcription is controlled by many transcription factors and this might explain the tissue and cell-specific effects.

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