The Insulin-like Growth Factor-binding Protein 1 Gene Is a Primary Target of Peroxisome Proliferator-activated Receptors

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Insulin-like growth factor-binding protein 1 (IGFBP-1) is a biomarker for metabolic and hyperproliferative diseases. At the same time, the nuclear receptors peroxisome proliferator-activated receptors (PPARs) are known for their critical role in the development of both the metabolic syndrome and various cancers. Here we demonstrate, in human hepatocellular carcinoma cells, that IGFBP-1 mRNA expression is under the primary control of PPAR ligands. We applied an improved in silico screening approach for PPAR response elements (PPREs) and identified five candidate PPREs located within 10 kb of the transcription start site (TSS) of the IGFBP-1 gene. Chromatin immunoprecipitation assays showed that, in living cells, the genomic region containing the most proximal PPRE, at position -1200 (relative to the TSS), preferentially associates with multiple PPAR subtypes and various other components of the transcriptional apparatus, which include their heterodimerizing partner, retinoid X receptor, as well as phosphorylated RNA polymerase II, co-repressor, co-activator, and mediator proteins. Moreover, further chromatin immunoprecipitation assays demonstrated that the TSS regions of the IGFBP-1 gene and those of the related IGFBP-2, -5, and -6, but not of IGFBP-3 and -4, genes bind PPARs as well. We also show that these additional PPAR binding genes contain a number of candidate PPREs and that their mRNA levels respond quickly to the presence of PPAR ligands, indicating that they are also primary PPAR target genes.

Lipid level dysregulation is a characteristic common to some of the most prevalent medical disorders, including obesity, cardiovascular disease, and type 2 diabetes (1). Nuclear receptor transcription factors may have important roles to play in these diseases, because many of them have lipophilic compounds as ligands, including fatty acids and their metabolic derivatives (2), which appear to be important in either the normal functioning or a role in the disease process affecting the metabolic pathways. For example, native and oxidized polyunsaturated fatty acids as well as arachidonic acid derivatives, such as prostaglan dins and prostacyclins, selectively bind the nuclear receptors peroxisome proliferator-activated receptors (PPARs) α, γ, and β/δ and stimulate their transcriptional activity (3). PPARs are prominent players in the metabolic syndrome, because they are important regulators of lipid storage and catabolism (4). However, PPARs also regulate cellular growth and differentiation and therefore have as well an impact on hyperproliferative diseases, such as cancer (5). PPARγ is the best characterized member of the subfamily due to its prominent role in the regulation of differentiation of cell types with active lipid metabolism, such as adipocytes and macrophage foam cells (6, 7). The importance of this receptor in lipid homeostasis and energy balance is accentuated by the widespread use of synthetic PPARγ ligands, such as the thiazolidinediones rosiglitazone, troglitazone, and pioglitazone, as anti-diabetic drugs (8).

In rodents a large number of significantly inducible PPAR target genes have been identified (9, 10), whereas in human cell lines only a few genes are activated more than 2-fold by PPAR ligands (11). An essential prerequisite for the direct modulation of transcription by PPAR ligands is the location of at least one activated PPAR protein close to the transcription start site (TSS) of the respective primary PPAR target gene. This is commonly achieved through the specific binding of PPARs to a PPAR response element (PPRE) (12). In detail, the DNA-binding domain of PPARs contact the major groove of a double-stranded hexameric DNA sequence with the optimal AGGTCA core binding sequence. PPARs bind to DNA as heterodimers with the nuclear receptor retinoid X receptor (RXR) (13). PPREs are therefore formed by two hexameric core binding motifs in a direct repeat orientation with an optimal spacing of one nucleotide (DR1), where PPAR occupies the 5′ motif (14).

3 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; FBS, fetal bovine serum; ChIP, chromatin immunoprecipitation; CPT 1, carnitine palmitoyl transferase I; DR1, direct repeat spaced by one nucleotide; GW501516, 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)methylsulfanyl)phenoxoacetic acid; GW7947, 2-(4-(2-(1-cyclohexanecbutyl)-3-cyclohexy lureido)ethyl)phenylthio)thiophene-2-methylpropionic acid; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; NCOR, nuclear corepressor; PGCG-1α, PPARγ co-activator 1α; pPol II, phosphorylated RNA polymerase II; PPRE, PPAR response element; RE, response element; rosiglitazone; 5-(4-(2-((methyl-2-pyridylamino)ethoxy)phenyl)methyl)-2,4-thiazolidinedione; RXR, retinoid X receptor; TRAP220, thyroid hormone receptor-associated protein 220; TSS, transcription start site; VDR, vitamin D receptor.
**PPAR Regulation of the IGFBP Gene Family**

In the absence of ligand, co-repressor proteins, such as nuclear co-repressor (NCoR), link DNA-bound nuclear receptors to enzymes with histone deacetylase activity that cause chromatin condensation (15). Binding of a ligand with agonistic properties to the nuclear receptors causes a conformational change within their ligand-binding domain that results in the replacement of co-repressors by co-activator proteins, such as receptor-associated co-activator 3 (16) or PPARγ co-activator 1α (PGC-1α) (17). These co-activators link ligand-activated nuclear receptors to enzymes displaying histone acetyltransferase activity that cause chromatin relaxation and thereby reverse the action of unliganded nuclear receptors. In a subsequent step, ligand-activated nuclear receptors exchange rapidly co-activator proteins for components of mediator complexes, such as thyroid hormone receptor-associated protein (TRAP)220/Med1 (18), which act as a bridge from the activated nuclear receptors to the basal transcriptional machinery. In this way ligand-activated nuclear receptors execute two tasks, the modification of chromatin and the regulation of transcription.

IGFBPs are a family of proteins that are multifunctional, having insulin-like growth factor (IGF)-independent actions as well as regulation of IGFs activity (19). The primary endocrine roles of IGFBP-1 appear to be the inhibition of the availability of IGFs as well as their hypoglycemic effect (20, 21). In addition, hepatic IGFBP-1 may play a paracrine role as a survival factor in liver (22). Recently, it was found that in the absence of insulin IGFBP-1 protein levels were induced 1.6-fold after the application of PPARγ ligands (23). The importance of nuclear receptors in the regulation of this gene is further supported by the fact that we have shown it to be a primary target of 1α,25-dihydroxyvitamin D₃ and its nuclear receptor, the vitamin D receptor (VDR) (24). In addition, an up-regulation of the IGFBP-1 gene by troglitazone could also be mediated by another nuclear receptor, the pregnane X receptor (25).

In this study, we demonstrate that the IGFBP-1 gene is a primary PPAR target in HepG2 human hepatocellular carcinoma cells as well as in normal mouse liver. We applied an improved in silico screening approach for DR1-type response elements (RES) and identified five candidate PPREs within 10 kb of the IGFBP-1 gene TSS. Chromatin immunoprecipitation (ChIP) assays showed that in living cells only the genomic region containing the most proximal PPRE (at position −1200) associated with PPARs as well as with their nuclear partners RXR, phosphorylated RNA polymerase II (pPol II), NCoR, receptor-associated co-activator 3, PGC-1α, and TRAP220. A second PPRE (at position −9400) showed also an association with PPARs and RXR. ChIP assays demonstrated that the PPARs are located on TSS regions of the IGFBP-1 gene and those of the related genes IGFBP-2, −5, and −6 but not on those of the genes IGFBP-3 and -4. The genes IGFBP-2, −5, and −6 each contain a number of candidate PPREs and responded in HepG2 and HEK293 human embryonal kidney cells to PPAR ligands. This suggests that they are also primary PPAR target genes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and in Vivo Experiments**—The human hepatocellular liver carcinoma cell line HepG2 and the human embryonal kidney cell line HEK293 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin in a humidified 95% air/5% CO₂ incubator. Before use, FBS was stripped of lipophilic compounds, such as endogenous nuclear receptor ligands, by stirring it with 5% activated charcoal (Sigma-Aldrich) for 3 h at room temperature. Charcoal was then removed by centrifugation and sterile filtration. Prior to mRNA or chromatin extraction, cells were grown overnight in phenol-red free DMEM supplemented with 5% charcoal-stripped FBS to reach a density of 50 – 60% confluency. Cells were then treated with either solvent (Me₃SO, 0.1% final concentration) or 100 nM of the PPARα agonist GW7647, 100 nM of the PPARγ agonist rosiglitazone or 100 nM of the PPARβ/δ agonist GW501516. GW7647 and GW501516 were purchased from Alexis Biochemicals (San Diego, CA), whereas rosiglitazone was kindly provided by Dr. Mogens Madsen (Leo Pharma, Ballerup, Denmark). The ligands were dissolved in Me₃SO.

8-Week-old male BALB/c × DAB2 mice (National Laboratory Animal Center, Kuopio, Finland) were housed in stainless steel metabolic cages under controlled temperature (21 – 23 °C) and light conditions (lights on 7 a.m. to 7 p.m.). Mice had free access to water and diet ad libitum (Altromin, Lage, Germany) for 14 days prior to initiation of treatment. All experiments were approved by the Committee for the Welfare of Laboratory Animals at the University of Kuopio and conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC. GW501516 was administered in saline by intraperitoneal injection (1 µg/g body weight). After 3 and 6 h the animals were sacrificed, and their livers were removed and shock frozen in liquid nitrogen.

**RNA Extraction and Real-time Quantitative PCR**—Total RNA was extracted using the Mini RNA Isolation II kit (Zymo Research, HjSS Diagnostics, Freiburg, Germany). For tissues, the samples were pre-homogenized in lysing matrix A tubes (Bio 101, Vista, CA) using a Fast Prep FP120 machine (Savant Instruments, Holbrook, NY). Samples were processed twice for 40 s at setting 6.0 with a 10-min cooling interval on ice. Afterward, the samples were cooled on ice for 10 min, spun down for 1 min at 2000 rpm in a bench-top centrifuge, and 600 µl of the cleared supernatant was transferred to the Mini RNA isolation kit columns for RNA extraction. The RNA was subsequently purified and eluted according to the manufacturer’s instructions (Zymo Research). From all RNA sources, cDNA synthesis was performed for 1 h at 37 °C using 1 µg of total RNA as a template, 100 pmol of oligo(dT)₁₅ primer, and 40 units of reverse transcriptase (Fermentas, Vilnius, Lithuania) in a 40-µl volume. Real-time quantitative PCR was performed in an IQ Cycler (Bio-Rad) using the dye Sybr Green I (Molecular Probes, Leiden, The Netherlands). Per reaction, 4 µl of the 1:10 dilution of the above cDNA, 1 unit of Hot Start Taq polymerase (Fermentas), and 3 mM MgCl₂, were used, and the PCR cycling conditions were: 45 cycles of 30 s at 95 °C, 30 s at 62 °C, and 40 s at 72 °C. The sequences of the gene-specific primer pairs for the human and mouse IGFBP genes, the reference gene carnitine palmitoyl transferase 1 (CPT 1), and the internal control gene acidic riboprotein P0 (ARP0, ArbP in mouse) are listed in sup-
ChIP Assays—Nuclear proteins were cross-linked to genomic DNA by adding formaldehyde for 10 min directly to the medium to a final concentration of 1% at room temperature. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating for 5 min at room temperature on a rocking platform. The medium was removed, and the cells were washed twice with ice-cold phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, 2H2O). The cells were collected by scraping into ice-cold phosphate-buffered saline containing Complete™ protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). After centrifugation the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors, 50 mM Tris-HCl, pH 8.1), and the lysates were sonicated to result in DNA fragments of 300–1000 bp in length. Cellular debris was removed by centrifugation, and the lysates were diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors, 16.7 mM Tris-HCl, pH 8.1). The samples were centrifuged, and the recovered chromatin solutions were incubated with 5 µl of indicated antibodies and 20 µl of sonicated salmon sperm (0.1 mg/ml) to remove unspecific background overnight at 4 °C with rotation. The antibodies against PPARα (sc-9000), PPARγ (sc-7196), PPARβ/δ (sc-7197), RXRα (sc-553), NCoR (sc-8994), RAC-3 (sc-7216), PGC-1α (sc-13067), TRAP220 (sc-5334), phosphorylated RNA polymerase II (pPol II, sc-13587), and control IgGs (sc-2027) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The immunocomplexes were collected by centrifugation of 1 ml of sonicated salmon sperm (0.1 mg/ml) to remove unspecific background overnight at 4 °C with rotation. The beads were pelleted by centrifugation for 1 min at 4 °C at 100 × g and washed sequentially with 5 min by rotation with 1 ml of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl, pH 8.1), and LiCl wash buffer (0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Finally, the beads were washed twice with 1 ml of TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The immunocomplexes were then eluted by adding 250 µl of elution buffer (1% SDS, 100 mM NaHCO3) and incubated for 15 min at room temperature with rotation. After centrifugation, the supernatant was collected, and the elution was repeated. The supernatants were combined, and the cross-linking was reversed by adding NaCl to a final concentration of 200 mM and incubated overnight at 65 °C.

ChIP Regulation of the IGFBP Gene Family

TABLE 1

Candidate PPREs of the human IGFBP-1 gene

The core sequences of the putative DR1-type PPREs in the human IGFBP-1 gene are indicated. Hexameric core binding motifs are in bold, and deviations from the consensus sequence (A/N)GGT(GA/ C)AGGTC (Forbes) are underlined. The 5′- and 3′-flanking sequences of all core sequences were ATTTCTAGA and TCTAGACCC, respectively.

| Response element | Locationa | Sequence | Class variation |
|------------------|------------|----------|-----------------|
| RE1              | −9446      | CATGCTCAAGGTCa | 1 × 1, 2 × II  |
| RE2              | −1178      | CATGCTCAAGGTCa | 2 × 1, 2 × II  |
| RE3              | +3601      | CATGCTCAAGGTCa | 1 × 1, 2 × II  |
| RE4              | +7059      | CATGCTCAAGGTCa | 1 × 1, 2 × II  |
| RE5              | +8574      | CATGCTCAAGGTCa | 1 × 1, 2 × II  |

a Relative to the TSS.
TABLE 2
Genomic PCR primers

Sequence and location of the primer pairs used to detect genomic regions containing putative REs 1–5 and a control region of the IGFBP-1 gene or the TSS of all six IGFBP genes.

| Region (gene) | Location | Primer sequences (5′-3′) |
|---------------|----------|-------------------------|
| 1 (IGFBP-1)   | −9536 to −9253 | GACTGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |
| 2 (IGFBP-1)   | −1197 to −1002 | GATCCAGGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |
| 3 (IGFBP-1)   | +3498 to +3691 | AGAGGAGACACATGAGAAAGAGCAG GTTATGCGTTCTTTTGAGGAGG |
| 4 (IGFBP-1)   | +6997 to +7370 | CTCCTCTGTTATGAGGCTGACGAG GTGATGTCGTTCTTTTGAGGAGG |
| 5 (IGFBP-1)   | +8341 to +8690 | CTCTCTGTTATGAGGCTGACGAG GTGATGTCGTTCTTTTGAGGAGG |
| Control (IGFBP-1) | −8869 to −8377 | ATGATGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |
| TSS (IGFBP-1) | −100 to +153 | GCAAGTCACTCCTCTTATCA |
| TSS (IGFBP-2) | −62 to +117 | CAGAGAAGGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |
| TSS (IGFBP-3) | −129 to +92 | GAACTCTGCACTCAAGAGGAGGAG GTGATGTCGTTCTTTTGAGGAGG |
| TSS (IGFBP-4) | −41 to +118 | CAGAGAAGGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |
| TSS (IGFBP-5) | −32 to +92 | CAGAGAAGGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |
| TSS (IGFBP-6) | −159 to +18 | CAGAGAAGGATGCTATTTAGGAAGCAG CGAAGTAGGAGAAATGGAAGAC |

* Relative to the TSS.

teins were digested by adding protease K (final concentration, 40 µg/ml) and incubation for 1 h at 45 °C. Genomic DNA fragments were recovered by phenol-chloroform extraction, followed by a salt-ethanol precipitation and subsequent resuspension in sterile H2O.

PCR of Chromatin Templates—For each of the five candidate RE-containing genomic regions and the control region of the IGFBP-1 gene as well as for the TSS of all six IGFBP genes, specific primer pairs were designed (Table 2), optimized, and controlled by running PCR reactions with 25 ng of genomic DNA (input) as a template. When running immunoprecipitated DNA (output) as a template, the following PCR profile was used: preincubation for 5 min at 95 °C, 50 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C and one final incubation for 10 min at 72 °C. The PCR products were separated by electrophoresis through 2% agarose gels. Gel images were scanned on a FLA3000 reader using ScienceLab99 software.

Transfection and Luciferase Reporter Gene Assays—HepG2 cells were seeded into 6-well plates (10^5 cells/ml) and grown overnight in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS. Polyethyleneimine transfections were performed by incubating a reporter plasmid and the expression vector for human PPARα, PPARγ, or PPARβ/δ (each 1 µg) with 50 µl of 150 mM NaCl for 15 min at room temperature. Simultaneously, 10 µg of polyethyleneimine (Sigma-Aldrich) in 50 µl of 150 mM NaCl was incubated for 15 min at room temperature. The two solutions were then combined and incubated for additional 15 min at room temperature. With dilution of 900 µl of phenol red-free DMEM, the mixture was added to the cells. 500 µl of phenol red-free DMEM, supplemented with 15% charcoal-stripped FBS, and the ligands were added 4 h after transfection. The cells were lysed 16 h later using reporter gene lysis buffer (Roche Diagnostics). The constant light signal luciferase reporter gene assay was performed as recommended by the supplier (Canberra-Packard, Groningen, The Netherlands). Luciferase activities were normalized with respect to protein concentration, and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

RESULTS

IGFBP-1 is a Primary PPAR Target Gene—RNA was extracted from HepG2 cells treated for 2, 4, and 6 h with 100 nM of each either of the PPARα agonist GW7647, or the PPARγ agonist rosiglitazone or the PPARβ/δ agonist GW501516. These early time points were chosen to detect primary effects of the PPAR ligands. The relative -fold induction of the mRNA amounts of the IGFBP-1 gene and of the known PPAR target gene CPT 1 were determined by real-time quantitative PCR (Fig. 1A). Interestingly, all three PPAR subtype-specific ligands were found to stimulate significantly IGFBP-1 mRNA expression. After 4 h the maximal mRNA induction factors were 2.1- to 2.9-fold. For the reference gene CPT 1 comparable stimulations of 1.6- to 2.8-fold were measured, but these maxima were reached 2 h later than with the IGFBP-1 gene. With the chosen ligand concentrations the pregnant X receptor was not activated (data not shown), and therefore we can exclude pregnant X receptor-dependent effects of rosiglitazone (25). The most responsive PPAR ligand, GW501516, was chosen for an in vivo experiment, in which 1 mg/kg body weight of the compound was injected intraperitoneally into mice (Fig. 1B). Livers were taken 3 and 6 h after ligand application, and the induction of murine Igfbp-1 and Cpt 1 mRNA expression was measured by real-time quantitative PCR. Igfbp-1 gene expression was induced 2.1-fold after 3 h and even 3.2-fold after 6-h ligand treatment, whereas in mice the orthologous gene reached its maximum after 6 h. Conversely, in human cells the CPT 1 gene...
expression induction was highest after 6 h, but in mouse liver it had peaked already after 3 h. Taken together, in human liver cells as well as in mouse liver tissue the IGFBP-1 gene was found to be significantly inducible after 2–3 h of treatment with PPAR ligands. This early induction profile was comparable, although not in the same time frame, to that of the human and mouse CPT 1 gene orthologues, suggesting that the genes of both humans and mice are responsible for producing the IGFBP-1 orthologue proteins are primary PPAR targets.

In Silico and in Vitro Screening of the IGFBP-1 Gene for Candidate PPREs—As a heterodimer with RXR, all three PPAR subtypes show comparable in vitro binding to a DR1-type PPRE of the consensus sequence AGGTCAAGGTCA (supplemental Fig. S1A). To generate an experimental basis for a more efficient in silico prediction of candidate PPREs, the binding of PPAR-RXR heterodimers to synthetic and natural DR1-type PPRE was analyzed. Firstly, all 13 nucleotides of the DR1 consensus sequence were individually varied to the three other nucleotides, and then the binding of in vitro translated PPAR-RXR heterodimers (PPARα-RXR, PPARγ-RXR, and PPARβ/δ-RXR) to these 39 synthetic PPREs was compared in gel-shift experiments (supplemental Fig. S1, B–D). With the exception of the mutation of the A at position 1 to a G and of the C at position 5 to a G, all PPRE variations significantly reduced the binding of PPAR-RXR heterodimers by 10–90%. For each of the three PPAR subtypes, the variations were grouped into the classes I to A, all PPRE variations significantly reduced the binding of PPAR-RXR heterodimers to RE1 to -5 showed levels of 2.8, 2.1, 1.5, 4.0, and 16.8%, respectively (Fig. 2C), higher levels than that of PPARα-RXR heterodimers (1.8, 1.1, 0, 1.5, and 6.0%, Fig. 2B) or PPARβ/δ-RXR heterodimers (1.6, 0, 0, 1.1, and 4.7%, Fig. 2D). In summary, in silico screening of the regulatory region of the IGFBP-1 gene suggested five candidate DR1-type PPREs, which are recognized in vitro most efficiently by PPARγ-RXR heterodimers.

PPAR Regulation of the IGFBP Gene Family

IGFBP-1 gene and identified five candidate DR1-type PPREs at approximate positions −9400 (RE1), −1200 (RE2), +3600 (RE3), +7100 (RE4), and +8600 (RE5) (Fig. 2A). Interestingly, although all five of these REs were predicted to be acceptable candidate PPREs for PPARγ, nearly all of them failed the stringent criteria for effective PPARα and PPARβ/δ binding. Accordingly, the relative in vitro binding of PPARγ-RXR heterodimers to RE1 to -5 showed levels of 2.8, 2.1, 1.5, 4.0, and 16.8%, respectively (Fig. 2C), higher levels than that of PPARα-RXR heterodimers (1.8, 1.1, 0, 1.5, and 6.0%, Fig. 2B) or PPARβ/δ-RXR heterodimers (1.6, 0, 0, 1.1, and 4.7%, Fig. 2D). In summary, in silico screening of the regulatory region of the IGFBP-1 gene suggested five candidate DR1-type PPREs, which are recognized in vitro most efficiently by PPARγ-RXR heterodimers.

Functionality of Candidate PPREs in the Chromatin Context of Living Cells—We next examined, in living cells, whether PPARα, PPARγ, PPARβ/δ, and RXR were located to the genomic regions containing these five REs. Chromatin was extracted from HepG2 cells, which had been treated for 120 min with solvent (Me2SO), 100 nM GW7647, 100 nM rosiglitazone, or 100 nM GW501516 and then cross-linked for 10 min in the presence of formaldehyde. ChIP experiments were performed for all three ligand treatments with anti-PPARα, anti-PPARγ, anti-PPARβ/δ, and anti-RXR antibodies. The genomic DNA fragments that were recovered from reverse-cross-linked chromatin served as templates for PCR reactions with primers specific for the regions containing the five candidate PPREs and for a control region (Table 2). Representative agarose gels of the PCR products from all treatment conditions are shown (Fig. 3A). The input lane serves as a reference for comparable detection sensitivity for the five genomic regions, and ChIP assays using IgGs served as specificity controls. In the absence of ligand region 2 was already strongly occupied by all three PPAR subtypes. The addition of subtype-specific ligands appears to shift the occupancy of region 2 by their respective PPAR subtype. However, PPARβ/δ did not dissociate from region 2 after GW7647 treatment. In contrast, region 1 was not preoccupied by either of the three PPAR subtypes. PPARα shows the tendency to be recruited by GW7647 over rosiglitazone and GW501516, whereas PPARγ is equally well recruited by GW7647 and rosiglitazone. Only weak association of PPARβ/δ to region 1 was observed, and no ligand preference was detected. RXR associated constantly under all conditions with both regions. In contrast, IgG background binding suggested that the few signals obtained in regions 3 and 4 are apparently nonspecific. Moreover, region 5, as well as the control region, was found to be blank concerning PPAR and RXR binding.

Because the strongest ChIP results were observed after treatment with the PPARα agonist GW7647, we stimulated HepG2 cells with this ligand for 0, 30, 60, 120, 180, and 240 min. ChIP assays were then performed with antibodies against PPARα, RXR, pPol II, the mediator protein TRAP220, the co-repressor NCoR, and the co-activators PGC-1α and receptor-associated co-activator 3 (Fig. 3B). Even in this larger panel the control region showed at no time any association with the seven nuclear proteins. Moreover, regions 3–5 displayed only a few unspecific bands. Region 1 showed at 120 min binding of PPARα, RXR, and pPol II, but none of the tested mediator, co-activator, or...
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A

Chromosome 7
from nucleotide 45091327 to 45711327

RE1
RE2
IGFBP-1
RE3
RE4
RE5

10000
5000
TSS
-5000
-10000

B

PPARα
RXR

relative % of shifted probe

CPTI
RE1
RE2
RE3
RE4
RE5

PPAR/α
RXR

NS
free probe
8 % non-denaturating gels

100 (7.4)
1.8 (0.6)
1.1 (0.3)
0
1.5 (0.1)
6.0 (1.3)

C

PPARγ
RXR

relative % of shifted probe

CPTI
RE1
RE2
RE3
RE4
RE5

PPAR/γ
RXR

NS
free probe
8 % non-denaturating gels

100 (8.6)
2.8 (0.8)
2.1 (0.5)
1.5 (0.6)
4.0 (0.3)
16.8 (1.8)

D

PPARδ/δ
RXR

relative % of shifted probe

CPTI
RE1
RE2
RE3
RE4
RE5

PPAR/δ
RXR

NS
free probe
8 % non-denaturating gels

100 (9.2)
1.6 (0.2)
0
0
1.1 (0.3)
4.7 (0.5)
PAN Co-activators have recently been reported and structurally characterized as nuclear proteins. Receptor-associated co-activator 3, associated with PPARs and RXR. Moreover, only region 2 displayed a ligand-dependent trafficking of PPAR-associated nuclear proteins.

Functionality of PPREs—The functionality of the PPRE within regions 1 and 2 was also tested by reporter gene assays in transiently transfected HepG2 cells (Fig. 4). The effect of PPAR subtype overexpression was 1.5- to 2-fold for region 1 and 3- to 4-fold for region 2, whereas the region of the CPT 1 control gene induced a 2- to 4-fold increase of reporter gene expression. The mutation in the PPRE of the CPT 1 gene promoter was at a less critical position (7th) and resulted only in a minor but statistically significant reduction in the activity of the promoter construct. In contrast, the PPREs in regions 1 and 2 were each mutated at the critical 6th position and resulted both in a significant loss of basal activity as well as of ligand inducibility of the respective reporter gene constructs. In summary, both RE1 in region 1 and RE2 in region 2 were found to contribute significantly to the functionality of the IGFBP-1 promoter, but RE2 had the more prominent effect on the basal activity.

In Silico and TSS ChIP Analysis of Other IGFBP Gene Family Members—To test whether any of the other five IGFBP gene family members may also be a primary PPAR target gene, we screened them in silico for candidate PPREs in the same way as we did for the IGFBP-1 gene (Fig. 2A), i.e. 10 kb of the genomic sequence up- and downstream of their respective TSS was analyzed (Fig. 5A). For the IGFBP-2 gene this in silico screening resulted in five candidate PPREs within its upstream region, for IGFBP-3 and IGFBP-4 in each one candidate PPRE within their
respective intron 1 and one in the upstream region of IGFBP-3, for IGFBP-5 in one candidate PPRE within the upstream region and two in intron 1, and for the IGFBP-6 gene in four candidate PPREs within the upstream region, two in intron 1 and one downstream of the gene. Based on the experience with the IGFBP-1 gene, where from five candidate PPREs two showed functional association with PPARs (Figs. 3 and 4), we expected that the IGFBP-6 gene is a PPAR target and that the genes IGFBP-2 and -5 could be predicted to respond to PPAR activation, whereas it seemed rather unlikely that the genes IGFBP-3 and -4 are PPAR targets.

To test our prediction about the PPAR responsiveness of the IGFBP gene family members, we stimulated HepG2 and HEK293 cells (please note, the IGFBP-5 gene is not expressed in HepG2 cells and the IGFBP-1 gene not in HEK293 cells, see Fig. 6A) 16 h later, and relative light units are indicated. Two-tailed Student’s t tests were performed to determine the significance of the ligand induction and the effects of the RE mutations within the promoter regions (*, p < 0.05; **, p < 0.01).
IGFBP gene family members were monitored by real-time quantitative PCR in relation to the control gene ARP0 in HepG2 and HEK293 cells (Fig. 6A) and the orthologous gene product, Arbp, in mouse liver (Fig. 6B). In mouse liver PPARα showed the highest relative expression, whereas in HepG2 cells PPARγ showed equally prominent expression levels. In HEK293 cells PPARγ mRNA expression was highest. However, in both cell lines, as well as in mouse liver the two other PPAR subtype genes were only between 2- and 21-fold lower expressed. Within the IGFBP gene family IGFBP-1 showed the highest relative basal expression, followed by IGFBP-4 (19-fold less), IGFBP-2 (38-fold less), IGFBP-6 (157-fold less), and IGFBP-3 (632-fold less). IGFBP-5 gene expression was not detectable in HepG2 cells. In HEK293 cells the IGFBP gene family showed a different ranking: IGFBP-2 was expressed highest, followed by IGFBP-4 (4-fold less), IGFBP-5 (12-fold less), IGFBP-6 (45-fold less), and IGFBP-3 (70-fold less). The IGFBP-1 gene is not expressed in HEK293 cells. In the mouse liver the Igfbp-4 gene showed the highest relative expression levels followed by the genes Igfbp-1 (19-fold less), Igfbp-3 (52-fold less), and Igfbp-2 (68-fold less). Igfbp-5 and Igfbp-6 mRNA expression could not be detected in mouse liver.

Next, we performed in HepG2 cells (Fig. 6C), in HEK293 cells (Fig. 6D), and in mouse liver (Fig. 6E) a time-course analysis of the early changes in the mRNA expression of the genes IGFBP-2 to -6, in response to the PPARα agonist GW7647, the PPARγ agonist rosiglitazone (both only for the human cell lines), or the PPAR/β/δ agonist GW501516. In confirmation of the results obtained by in silico and TSS ChIP analysis (Fig. 5), in HepG2 cells the IGFBP-2 and the IGFBP-6 mRNA levels responded to all three PPAR ligands, but for the IGFBP-2 gene a 1.6- to 1.8-fold induction and for the IGFBP-6 gene a 1.4- to 2.3-fold reduction were observed (Fig. 6C).

The IGFBP-3 and -4 showed no statistically significant response to the PPAR ligands, which is also in accordance with our prediction. The IGFBP-5 gene is not expressed in HepG2 cells, but in HEK293 cells all three PPAR ligands were found to stimulate the IGFBP-5 mRNA expression (Fig. 6D). This confirmed our prediction from in silico and TSS ChIP analysis. In addition, the data from HEK293 also confirmed the up-regulation of the IGFBP-2 gene, the down-regulation of the
IGFBP-6 gene, and the non-responsiveness of the genes IGFBP-3 and -4 against PPAR ligands. In mouse liver the Igfbp-2 gene also responded to PPAR ligand but, surprisingly, by a reduction of up to 2.1-fold (Fig. 6E). This non-responsiveness of the Igfbp-4 gene is consistent with the response of the human genes, but the slight down-regulation of the Igfbp-3 gene was not foreseen. Finally, for the genes Igfbp-5 and -6 no conclusions were possible, because they were not expressed in mouse liver. Taken together, the expression profile of the IGFBP gene family in HepG2 and HEK293 cells as well as in mouse liver fits well with our prediction that, in addition to the IGFBP-1 gene, the genes IGFBP-2, -5, and -6 are primary PPAR target genes as well.

DISCUSSION

This study describes the IGFBP-1 gene as a primary PPAR target. We could demonstrate up-regulation of IGFBP-1 mRNA in human hepatocellular cells as well as in mouse liver after 2–3 h of PPAR ligand treatment, which classifies the gene as a primary PPAR target. Interestingly, although the expression levels of the three PPAR subtypes differed, modest concentrations (100 nM) of all subtype-specific ligands resulted in HepG2 cells in similar effects on the induction of IGFBP-1 mRNA. With 2.1- to 2.9-fold inductions, these factors are comparable or even higher than those observed with a number of known human PPAR target genes, such as CPT 1. Although expression studies in mice sometimes showed higher values, we consider these changes in IGFBP-1 expression significant, in particular in relation to the high basal expression of the gene in human cells. Interestingly, we also found that PPARs were present in the absence of ligand, indicating that these nuclear receptors may have a role in this highly expressed genes basal expression. Alternatively, endogenous ligands may also be produced by the cells and activate the PPARs and allow for their association with the IGFBP-1 gene promoter. Our findings were supported by the in silico identification of five candidate DR1-type PPREs located within 10-kb distance to the IGFBP-1 gene TSS. However, only two of these candidate PPREs, RE1 at position −9400 and RE2 at position −1200, showed in living cells an association with all three PPARs, RXR, and activated RNA polymerase II. Moreover, only RE2 associated with the co-repressor, co-activator, and mediator proteins that we tested in this study. Importantly, also the TSS region of the IGFBP-1 gene showed in ChIP assays binding of PPARs. This suggests a functional protein bridge between the TSS and a PPRE, which we assume is preferentially RE2. This observation was also supported by reporter gene assays, where the promoter region containing RE2 showed stronger effects than the region with RE1.

Our experience with the IGFBP-1 gene, allowed us to draw up some minimal requirements for a primary PPAR target gene. These were (i) the early (2–3 h) modulation of its mRNA expression levels in response to modest concentrations of PPAR ligands, (ii) the existence of a few PPREs (with sequences that do not strongly deviate from the consensus DR1-type RE) located within 10 kb of a gene's TSS, and (iii) the association of PPAR proteins with the genomic region of its TSS. Upon testing the genes of the other human IGFBP family members according to these criteria, the genes IGFBP-2, -5, and -6 are primary PPAR targets but not IGFBP-3 and -4. Indeed, our preliminary investigations with both an examination of the response of the mRNA levels of these genes by quantitative real-time PCR and TSS-specific ChIP indicate that these predictions may be true. However these findings will need further validation, such as the analysis of the individual candidate PPREs in ChIP assays and the quantification protein expression levels in different cell lines and tissues. However, our approach may offer a quick route to the identification of in vivo relevant PPAR target genes, which is flexible enough to accommodate the analysis of specific single genes, whole chromosome areas, or even the human genome as a whole.

Previously, alignment of a number of natural REs provided experimental data (41–44) to create position-specific weight matrices that are the basis for commonly used programs for in silico screening of nuclear receptor REs, such as ConSite (45) and JASPAR (46). However, we found they do not consider sufficiently the individual binding-sequence preferences of the PPAR subtypes as well as their heterodimerizing partner RXR. Because position weight matrices create mathematical scores over the whole length of a sequence of defined size, they are prone to overcompensate for nucleotides that are totally unfavorable to transcription factor binding at one position with scores from other positions. This effect becomes more problematic with larger motifs, such as nuclear receptor REs composed of two half sites. Therefore, we applied a scoring system of three classes of half-site variations and rules, and how they can be combined without completely losing the affinity for PPAR-RXR heterodimers.

This approach resulted in five PPRE candidates for the 20-kb genomic sequence around the TSS of the human IGFBP-1 gene. According to in vitro gel-shift screening RE5, which is located 8600 bp downstream of the TSS, showed most efficient binding of PPAR-RXR heterodimers. However, ChIP analysis showed that none of the three REs downstream of the TSS associated in living cells with PPARs. Moreover, the far upstream candidate PPRE, RE1 at position −9400, showed PPAR, RXR, and pPol II binding but not of the other essential nuclear proteins, such as co-activators and mediators. This left the in vitro rather weak RE2 as the only fully functional PPRE within the human IGFBP-1 gene area in vivo. It is likely that this PPRE is func-

FIGURE 6. Expression profiling of the IGFBP gene family. Real-time quantitative PCR was used to determine the basal mRNA expression of the three PPAR genes and the six IGFBP genes, relative to the control gene ARP0, in HepG2 and HEK293 cells (A) and in mouse liver (B). A logarithmic scale is employed on the y-axis to better present the data. In the same two cell lines and in the mouse liver the inducibility of the genes IGFBP-2 to -6 was tested. The established PPAR target gene CPT 1 served as a positive control. HepG2 and HEK293 cells were stimulated for 2, 4, and 6 h with 100 nM GW7647 (a), 100 nM rosiglitazone (b), or 100 nM GW501516 (b/0). Mice were injected intraperitoneally with 1 mg/kg (body weight) GW501516 and livers were taken after 3- and 6-h exposures. Data points (A and B) and columns (C–E) represent the means of at least three independent treatments, and the bars represent standard deviations (in A and B they were too small to be displayed). Dashed lines indicate the threshold of 2-fold up- or down-regulation. Two-tailed Student's t tests were performed to determine the significance of the mRNA induction by PPAR agonists in reference to solvent controls (*, p < 0.05; **, p < 0.01; and $$$, p < 0.001). ND, non-detectable.
PPAR Regulation of the IGFBP Gene Family

tional, because it is located rather close to the TSS (position -1200), where PPAR may occupy it because of co-operative interactions with other transcription factors located to the same region of open chromatin. Moreover, a sequence alignment of the human and mouse IGFBP-1 gene indicated that the sequence around RE2 showed a much higher conservation than the regions of the four other REs.

We provide an extensive dataset of the individual binding preferences of the different PPAR subtypes that can be used to predict binding of putative REs in future studies. All five elements found by our search showed binding in gel shift with at least one PPAR subtype. With one fully functional RE out of five candidate PPREs, the method is more efficient than most other in silico screenings (47). We have observed that the in vitro binding score of a PPRE is not proportional to the functionality of the RE in the chromatin context. Therefore, we are presently developing a method that also takes the relative distance to the TSS, the number and location of flanking binding sites of other transcription factors, and the amount of sequence conservation between species into account.

IGFBP-1 has been linked with several nutrition-related diseases. Reduced IGFBP-1 protein levels correlate with the risk of type 2 diabetes (48). Furthermore, overexpression of IGFBP-1 in transgenic mice leads to a marked inhibition of hepatic neoplasia, possibly by decreasing the mitotic activity of IGF-I and/or IGF-II (49). The physiological implication of our observation that several members of the IGFBP gene family (IGFBP-1, -2, -5, and -6) are simultaneously regulated by PPARs awaits further investigation. The core function of all family members is the regulation of IGF-I and -II availability in different tissues. Because mammals use for this crucial function a family of six proteins, it is not very surprising that a number of them respond to the same signal, such as PPARs and their ligands and that some functional degeneracy is allowed. In a previous study we have shown that the genes IGFBP-1, -3, and -5 are primary targets of another member of the nuclear receptor superfamily, the VDR (24). Both studies confirm the principle of redundant regulation of important gene families as well as the importance of nuclear receptors in the transcriptional regulation of the genes of this gene family. Interestingly, the genes IGFBP-1 and -5 are both primary PPAR and VDR targets.

PPARs are considered as the sensors of the macronutrients fatty acids and their metabolites, whereas VDR acts as sensor for the metabolites of the micronutrient vitamin D. Both nuclear receptors control critical metabolic reactions as well as cellular growth (50). Moreover, we found that the PPARβ/δ gene is a primary VDR target (51), which suggests that both signaling systems may be even more tightly bound physiologically together. This places the PPAR- and VDR-responsive members of the IGFBP gene family into the position of critical interfaces in the response to these nuclear receptors, and hence through their ligands, dietary components and emphasizes their role as biomarkers in the metabolic syndrome as well as in cancer.

In conclusion, our study provides insight into the regulation of the IGFBP gene family by PPARs. We used an improved in silico screening approach for PPREs and demonstrated that IGFBP-1, -2, -5, and -6 are primary PPAR target genes.

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