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Glycogen synthase 2 is a novel target gene of peroxisome proliferator-activated receptors

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Abstract. Glycogen synthase 2 (Gys-2) is the rate-limiting enzyme in the storage of glycogen in liver and adipose tissue, yet little is known about regulation of Gys-2 transcription. The peroxisome proliferator-activated receptors (PPARs) are transcription factors involved in the regulation of lipid and glucose metabolism and might be hypothesized to govern glycogen synthesis as well. Here, we show that Gys-2 is a direct target gene of PPARα, PPARβ/δ and PPARγ. Expression of Gys-2 is significantly reduced in adipose tissue of PPARα–/–, PPARβ/δ–/– and PPARγ+/– mice. Furthermore, synthetic PPARβ/δ and γ agonists markedly up-regulate Gys-2 mRNA and protein expression in mouse 3T3-L1 adipocytes. In liver, PPARα deletion leads to decreased glycogen levels in the refed state, which is paralleled by decreased expression of Gys-2 in fasted and refed state. Two putative PPAR response elements (PPREs) were identified in the mouse Gys-2 gene: one in the upstream promoter (DR-1prom) and one in intron 1 (DR-1int). It is shown that DR-1int is the response element for PPARs, while DR-1prom is the response element for Hepatic Nuclear Factor 4 alpha (HNF4α). In adipose tissue, which does not express HNF4α, DR-1prom is occupied by PPARβ/δ and PPARγ, yet binding does not translate into transcriptional activation of Gys-2. Overall, we conclude that mouse Gys-2 is a novel PPAR target gene and that transactivation by PPARs and HNF4α is mediated by two distinct response elements.

Keywords. PPAR, HNF4α, liver, adipose tissue, microarray, glycogen synthase 2, gene transcription, PPRE.

Introduction

Metabolic syndrome describes a combination of metabolic abnormalities that include central obesity, dyslipidemia, hypertension, insulin resistance, and a pro-inflammatory and pro-thrombotic state. An important group of pharmacological targets for the treatment of metabolic syndrome are the peroxisome proliferator activated receptors (PPARs). PPARs are ligand-activated transcription factors belonging to the superfamily of nuclear receptors, which include numerous cellular receptors for nutrients and steroids. So far, three PPAR isotypes (α, β/δ, γ) have been identified in a wide range of species, each displaying a
different tissue distribution and ligand specificity [1]. PPARs share a similar structure and a common molecular mechanism of action by forming an obligate heterodimer with the 9-cis retinoic acid receptor RXR. PPAR-RXR heterodimers selectively bind genomic sequences consisting of a direct repeat of the hexameric nucleotide sequence AGGTCA separated by 1 nucleotide (Direct Repeat-1). These so-called peroxisome proliferator response elements (PPRE) are located in the promoter of PPAR target genes or in intronic regions [2–5].

The PPARα isotype (NR1C1) is highly expressed in liver, and governs the adaptive response to fasting [6–8]. PPARα is an extremely important regulator of hepatic nutrient metabolism including fatty acid oxidation (peroxisomal and mitochondrial), fatty acid uptake, amino acid metabolism, glycerol metabolism, and lipoprotein assembly and transport [9–11]. In addition, PPARα potently suppresses the hepatic inflammatory response [12, 13], an effect that is also observed in extra-hepatic tissues such as the vascular wall [14]. Much less is known about the role of PPARα in other tissues, although evidence is accumulating that PPARα induces cardiac and skeletal muscle fatty acid oxidation [15, 16]. Importantly, PPARα mediates the effects of hypolipidemic fibrate drugs, which decrease plasma triglycerides and increase plasma HDL concentrations. In contrast to PPARα, PPARγ (NR1C3) is highly expressed in white adipose tissue (WAT), where it promotes lipid storage. PPARγ is a key transcription factor in the adipogenesis program and is essential for adipocyte survival [17, 18]. It also serves as the molecular target for the thiazolidinedione (TZD) class of insulin-sensitizing drugs that are widely used in the treatment of type 2 diabetes. PPARγ promotes whole body glucose utilization; however, it has been difficult to identify the molecular mechanisms behind this effect. Much of the attention has been focused on possible cross-talk between adipose tissue and skeletal muscle, as muscle is responsible for the major share of whole body glucose utilization. However, adipose tissue is a large organ, especially in the obese, and accordingly it can also be envisioned that the insulin-sensitizing effect of TZDs on glucose uptake is partially exerted at the adipose tissue level.

While PPARα and PPARγ have been extensively studied over many years, much less is known about the function of the PPARβ/δ isotype (NR1C2). Studies with genetically modified PPARβ/δ mice have illustrated the importance of this nuclear receptor in WAT and skeletal muscle, two organs that have a key role in glucose homeostasis [19–22]. It was shown that activation of PPARβ/δ in adipose tissue protects against adiposity and hyperlipidemia by inducing fatty acid catabolism [21]. Moreover, pharmacological activation as well as specific constitutive over-expression of PPARβ/δ leads to a shift in muscle fiber composition towards type I muscle fibers, resulting in increased muscle oxidative capacity [20, 22]. PPARβ/δ has also been shown to stimulate hepatic VLDL production, influence wound healing, and affect colon carcinogenesis [23–25]. However, whether PPARβ/δ has a functional role in glucose homeostasis, in analogy with other PPAR isotypes, remains to be firmly established.

Here we show that glycogen synthase 2 (Gys-2), the rate-limiting enzyme for glycogen synthesis in liver and adipose tissue, is a target gene of PPARα, PPARβ/δ and PPARγ. Transcriptional regulation is achieved via a PPAR response element present in the first intron. We show that an additional direct repeat response element identified in the Gys-2 promoter mediates transactivation by hepatic nuclear factor 4α (HNF4α).

Materials and methods

Chemicals. W14643 was obtained from Eagle Picher Technologies laboratories (Lenexa, KS, USA). Rosiglitazone was from Alexis (Breda, The Netherlands). SYBR Green was from Eurogentec (Seraing, Belgium). Dulbecco’s modified Eagles medium (DMEM), fetal calf serum (FCS), calf serum and penicillin/streptomycin/fungizone were from Cambrex Bioscience (Seraing). Otherwise, chemicals were from Sigma (Zwijndrecht, The Netherlands).

Animal experiments. PPARβ/δ mutant null mice (PPARβ/δ−/−) and PPARγ heterozygous mice (PPARγ+/−) were on a mixed background (Sw129/C57BL/6) and have been described previously [26, 27]. Wild-type littermates served as control animals. PPARα−/− mice and corresponding wild-type mice on an Sw129 background were purchased at Jackson Laboratories (Bar Harbor, ME, USA). Liver-specific hepatocyte nuclear factor 4α (HNF4α)-null mice were generated as described previously. Livers were collected from 45-day-old HNF4α−/− X albumin-Cre− (KO) and HNF4α−/− X albumin-Cre+ (FLOX) mice [28]. Mice were maintained at 20°C with a 12:12 light-dark cycle. All mice were between 3 and 6 months of age. For the fasting experiment, 3-month-old male mice were fasted for different periods of time starting at the onset of the light cycle. For the refeeding experiment, mice were fasted for 24 h after which they were put back on chow for 7 h before sacrifice. After sacrificing the animals, tissues were immediately frozen in liquid nitrogen. The animal experiments were approved by the animal experimentation committee of Wageningen University or the Etat de Vaud (Switzerland).

Oligonucleotide micro-array. Total RNA was prepared from epididymal WAT of wild-type and PPARβ/δ−/− mice (five animals of each genotype) using TRIZol reagent (Invitrogen, Breda, The Netherlands) and subsequently pooled per group. Pooled RNA was further purified using Qiagen RNeasy columns, and the quality was verified using Bioanalyzer 2100 (Agilent, Amsterdam). For one cycle eRNA synthesis (Affymetrix, Santa Clara, USA) 10 μg of RNA was used. Hybridization, washing and scanning of Affymetrix GeneChip mouse genome 430 2.0 arrays was according to standard Affymetrix protocols. Fluorimetric data were processed by Affymetrix GeneChip operating software and the gene chips were globally scaled to all the probe sets with an identical target intensity value. Further analysis was performed by Data Mining Tool (Affymetrix).
3T3-L1 adipogenesis assay. 3T3-L1 fibroblasts were grown in DMEM plus 10% calf serum and plated for final differentiation in DMEM plus 10% FCS. At 2 days after reaching confluence, the medium was changed and the following compounds were added: isobutyl 2-methylxanthine (0.5 mM), dexamethasone (1 μg/mL). After 3 days, the medium was changed to DMEM plus 10% FCS and insulin (5 μg/mL). 3T3-L1 cells were harvested by trypsinization. The cells were plated in 6-well plates (Serva Feinbiochemica, Heidelberg, Germany) coated with a density of 0.5–1.0×10⁶ cells/well. After 24 h of incubation, the medium was removed and replaced with fresh DMEM plus 10% FCS and insulin (5 μg/mL). After 48 h of incubation, the medium was removed and replaced with fresh DMEM plus 10% FCS and insulin (5 μg/mL). After 6 days, the medium was changed to DMEM plus 10% FCS, which was changed every 3 days.

Primary mouse or rat hepatocyte isolation. Mouse primary and rat hepatocytes were isolated as described previously [29]. Briefly, rat liver was isolated according to the protocol described previously [29]. The liver was perfused with a collagenase solution until swelling and degradation of the internal liver structure was observed. The hepatocytes were released, filtered and washed several times using Krebs buffer. The viability was assessed by trypan blue staining and was at least 80%. Cells were cultured in William’s Medium E supplemented with 10% FCS, penicillin-streptomycin, and dexamethasone. Cells were plated in collagen (Serva Feinbiochemica, Heidelberg, Germany) coated wells with a density of 0.5–1.0×10⁶ cells/well. After 4 h of incubation, the medium was removed and replaced with fresh DMEM plus 10% FCS and insulin (5 μg/mL). The next day, hepatocytes were used for experiments.

RNA isolation, reverse-transcription, and real-time quantitative PCR. Total RNA was extracted from tissues with TRIzol reagent (Invitrogen); 1 μg total RNA was then reverse-transcribed with iScript (Bio-Rad, Hercules, CA, USA). The primer sets used for PCR were designed to span the putative promoter/enhancer region (ChIP) on 3T3-L1 cells and mouse liver was carried out as described previously [11]. Sequences of primers used for PCR amplification were deposited in GenBank (accession no. DQ392012 and DQ392013). Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification.

DNA bisulfite conversion. DNA was bisulfite converted using a modified method of purified DNA by incubating with sodium bisulfite (pH 5.0) for 16 h at 50°C. The DNA was then purified with a Wizard PCR Prep DNA Purification System (Promega). Bisulfite-converted DNA was subject to PCR amplification using primers designed specifically for the promoter regions of the genes of interest.

Luciferase assay. Cells were plated in collagen (Serva Feinbiochemica, Heidelberg, Germany) -coated wells with a density of 0.5–1.0×10⁶ cells/well. After 4 h of incubation, the medium was removed and replaced with fresh DMEM plus 10% FCS and insulin (5 μg/mL). 3T3-L1 cells were harvested by trypsinization. The cells were plated in collagen (Serva Feinbiochemica, Heidelberg, Germany) -coated wells with a density of 0.5–1.0×10⁶ cells/well. After 4 h of incubation, the medium was removed and replaced with fresh DMEM plus 10% FCS and insulin (5 μg/mL). After 48 h of incubation, the medium was changed to DMEM plus 10% FCS, which was changed every 3 days.

Transfection efficiency. Luciferase activity was measured 24 h post transfection using the Promega luciferase assay kit (Promega) on a Fluoroskan Ascent Fl apparatus (Thermo labystems, Breda, The Netherlands). β-Galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl-[β]-d-galactopyranoside as a substrate. To disable the mouse Gys-2 promoter, two 150 bp internal overlapping PCR fragments were generated using the wild-type mGys-2 promoter as a template. Primers sets used to generate part B of the mutated mGys-2 promoter fragment were: 5′-TTTGGTCTAAAGGCCTTGGCAGCAAAAGG-3′ and 5′-CCCTTGCCTCTTACAGAGAGGCG-3′. AG3- Primers sets used to generate part B of the mutated mGys-2 promoter fragment were: 5′-CCCTTGGCCTACCTTACAGAGGCG-3′ and 5′-CCCTTGGCCTCTTACAGAGAGGCG-3′. The luciferase activity was subsequently cloned into the pGL3 basic vector reporter (Promega) and sequenced.

RNA isolation, reverse-transcription, and real-time quantitative PCR. Total RNA was extracted from tissues with TRIzol reagent (Invitrogen); 1 μg total RNA was then reverse-transcribed with iScript (Bio-Rad, Hercules, CA, USA). The primer sets used for PCR were designed to span the putative promoter/enhancer region (ChIP) on 3T3-L1 cells and mouse liver was carried out as described previously [11]. Sequences of primers used for PCR amplification were deposited in GenBank (accession no. DQ392012 and DQ392013). Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification.

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from frozen liver were made and analyzed for glycogen accumulation using the periodic acid–Schiff (PAS) reaction. Hematoxylin and eosin staining of liver cryosections was done using standard protocols.

**Western blot.** A mouse anti-glycogen synthase monoclonal antibody was used (clone GS-7H5 MAB3106) (Chemicon International, Hampshire, UK). Western blotting was carried out as previously described [4]. The primary antibody was used at a dilution of 1:1000 and the secondary antibody (anti-mouse IgG, Dako, Glostrup, Denmark) was used at a dilution of 1:8000.

**Results**

**Expression of Gys-2 in WAT is regulated by PPARs.** Our initial aim was to identify novel putative target genes of PPARβ/δ in WAT. Accordingly, we compared gene expression in WAT of wild-type versus PPARβ/δ-/- mice using Affymetrix micro-array analysis. The expression of several genes involved in glucose and lipid metabolism was down-regulated in PPARβ/δ-/- mice, including PPARγ, PGC-1α, and GLUT4, which was confirmed for several genes by real-time quantitative PCR (qPCR) (Table 1). Expression of Gys-2 was most significantly down-regulated in PPARβ/δ-/- mice, and therefore Gys-2 was selected for more detailed investigation. Q-PCR confirmed the marked down-regulation of Gys-2 mRNA in WAT of PPARβ/δ-/- mice (Fig. 1a). Furthermore, expression of Gys-2 also appeared to be down-regulated in WAT of PPARγ+/-- and PPARα-/- mice, although the former result did not achieve statistical significance. In PPARβ/δ-/- mice, the decrease in Gys-2 mRNA was paralleled by a significant down-regulation of PPARα and PPARγ expression, while in PPARα-/- mice expression of PPARβ/δ was significantly down-regulated (Fig. 1b). These data show that PPARs are crucial for maintaining Gys-2 expression in fat, although it is difficult to ascertain which PPAR isotype is the main regulator of Gys-2 expression in WAT.

To investigate whether expression of Gys-2 in adipocytes is under direct control of PPARs, the effect of
PPAR ligands on Gys-2 mRNA was studied in differentiated mouse 3T3-L1 adipocytes. It was observed that the PPARβ/δ agonist L165041, and the PPARγ agonists ciglitazone and rosiglitazone significantly induced Gys-2 mRNA levels (Fig. 1c). This effect was confirmed at the protein level (Fig. 1d). Thus, Gys-2 may represent a direct target gene of PPARγ and PPARβ/δ in adipocytes.

**PPARα governs Gys-2 hepatic expression.** A link between PPARs and glycogen has been previously made. It was observed that liver glycogen levels were reduced in refed PPARα−/− mice compared to refed wild-type mice [30, 31], which we confirmed using histochemical staining (Fig. 2). Furthermore, hepatic expression of Gys-2 was decreased in PPARα−/− mice [32]. Expression of Gys-2 is highest in liver, followed by WAT (our unpublished data). We confirm that hepatic Gys-2 mRNA is markedly reduced in PPARα−/− mice; however, only in the 24-h fasted and refed state (Fig. 3a). To further examine the role of PPARα in Gys-2 expression, primary hepatocytes from wild-type and PPARα−/− mice were treated with the synthetic PPARα agonist Wy14643, allowing for a...
Figure 3. PPARα governs hepatic expression of Gys-2. (a) Relative expression of Gys-2 in fed, fasted and refeed wild-type and PPARα−/− mice, as determined by qPCR. Mice were fasted for 0, 6, 12 or 24 h, with refeeding for 7 h following 24 h fasting. Significant differences between wild-type and PPARα−/− mice were observed in the 24-h fasted and the refeed state. (b) Relative expression of Gys-2 in freshly isolated wild-type and PPARα−/− hepatocytes treated for 24 h with vehicle (DMSO) or Wy14643 (10 μM), as determined by qPCR. Significant effects were observed by two-way ANOVA for genotype (p < 0.001), and for the interaction between the genotype and Wy14643 (p < 0.05). (c) Relative expression of Gys-2 in freshly isolated rat hepatocytes which were treated for 24 h with either vehicle (DMSO), Wy14643 (50 μM) or fenofibrate (50 μM). The effects of Wy14643 and fenofibrate were statistically significant (Student’s t-test: p < 0.01). Error bars represent SEM.

Identification of a putative PPRE in the proximal promoter of the mouse Gys-2 gene. To determine what genomic region could be responsible for the PPAR-induced up-regulation of Gys-2 mRNA, the mouse Gys-2 gene was scanned for potential PPREs (NUBIScan algorithm and Hidden Markov Model framework) [33, 34]. A Direct Repeat-1 motif (DR-1prom) was localized to the proximal Gys-2 gene promoter, about 169 bp upstream from the transcription start site. With the exception of two nucleotides, DR-1prom is identical to the consensus sequence, suggesting that this sequence could serve as a functional PPRE (Fig. 4a). DR-1prom was conserved between mouse and rat.

To examine whether the promoter region containing the putative PPRE is responsible for PPAR-dependent up-regulation of Gys-2 expression, a 553-nucleotide fragment of the mouse Gys-2 promoter gene was cloned in front of a luciferase reporter gene and transactivation studies were carried out in HepG2 cells. Surprisingly, co-transfection of PPARα or PPARγ1 expression vectors in combination with PPAR agonists slightly decreased luciferase activity, while PPARβ/δ activation had little effect (Fig. 4b). Transactivation assays performed with a small genomic fragment surrounding DR-1prom cloned in front of SV40-luciferase led to a similar overall PPAR-mediated repression for PPARα and PPARγ1, while PPARβ/δ had little effect (Fig. 4c). Co-transfection of RXR or of different co-activators such as CBP and PGC1α did not change this pattern (data not shown).

Thus, the PPRE identified in the Gys-2 promoter probably does not mediate the effect of PPARs on Gys-2 expression. Nevertheless, ChIP experiments carried out in 3T3-L1 cells indicated that (1) PPARγ was bound to DR-1prom in mature adipocytes, but not in pre-adipocytes (Fig. 4d), and (2) PPARβ/δ was bound to DR-1prom in pre-adipocytes and, more strongly, in mature adipocytes (Fig. 4e). Thus, despite DR-1prom behaving poorly as a PPRE in classical transactivation assay, it binds both PPARγ and PPARβ/δ in adipocytes. This suggests that in vivo binding of PPARγ and PPARβ/δ to DR-1prom does not translate into transcriptional activation of the Gys-2 gene, and accordingly that activation of Gys-2 expression by PPARs may be mediated by another genomic region. It should be mentioned that ChIP did not reveal any binding of PPARα to DR-1prom in hepatocytes (data not shown).

Interestingly, using the same strategy as described above, a putative PPRE that is homologous to the consensus DR-1 sequence was identified in intron 1 of the mouse Gys-2 gene (Fig. 4a). To assess whether DR-1int was able to mediate PPAR-dependent transactivation, a 314-nucleotide genomic fragment surrounding DR-1int was cloned in front of the SV40 promoter followed by a luciferase reporter gene. In HepG2 cells treatment with the synthetic PPARα agonist Wy14643 induced reporter activity and this activation was further enhanced upon co-transfection of mPPARα (Fig. 5a). Similar inductions of reporter
activity were observed for PPARβ/δ and PPARγ and their respective agonists (Fig. 5a). Thus, DR-1int is able to mediate PPAR-dependent transactivation, irrespective of the PPAR isotype, suggesting that it may at least be partially responsible for PPAR-dependent regulation of Gys-2 expression.

In agreement with the transactivation data, PPARα, PPARβ/δ and PPARγ proteins were able to specifically bind DR-1int in gel shift experiments. A retarded heterodimeric complex was observed only in the presence of both PPAR and obligate binding partner RXRα (Fig. 5b). The complex disappeared in the presence of an excess of cold specific oligonucleotide, but not nonspecific oligonucleotide.

Examination of in vivo PPAR binding to DR-1int by ChIP yielded very similar results as for DR-1prom: PPARγ was bound to DR-1int in mature 3T3-L1 adipocytes, but not in pre-adipocytes (Fig. 6a), whereas PPARβ/δ was bound to DR-1int in both pre- and mature adipocytes (Fig. 6b). In liver, ChIP analysis demonstrated binding of PPARα to DR-1int in wild-type but not PPARα−/− mice, and binding was enhanced by fasting and Wy14643 (Fig. 6c). Together, these data indicate that mouse Gys-2 is a direct PPAR target gene and that regulation by PPARs is at least partially mediated by a PPRE present in intron 1.

Gys-2 is a novel direct target of the liver enriched factor HNF4α. As explained above, PPARα caused a reduction in Gys-2 promoter activity via DR-1prom (Fig. 4b, c). A similar decrease of promoter activity in response to PPARα despite the presence of a putative PPRE has been reported for other genes. Indeed, it was found that PPARα decreases expression of the

| Gene | Product | Fold decrease Micro-array | Q-PCR |
|------|---------|--------------------------|-------|
| Gys2 | glycogen synthase 2 | 13.27 | 9.28 |
| Pik3r1 | phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) | 2.99 | |
| Pik3r1 | phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 1 (p85 alpha) | 2.64 | |
| Gys3 | glycogen synthase 3, brain | 2.25 | |
| Ppilr1r3c | protein phosphatase 1, regulatory subunit 3C, protein targeting to glycogen | 2.19 | |
| Slc2a4 | solute carrier family 2 (facilitated glucose transporter), member 4 | 2.16 | 1.56 |
| Slc2a4 | solute carrier family 2 (facilitated glucose transporter), member 4 | 2.04 | 1.56 |
| PFK-2 | 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase | 2.01 | |
| Gdc1 | glycerol phosphate dehydrogenase 1, cytoplasmic adult | 1.93 | |
| Ppilr1r3c | protein phosphatase 1, regulatory subunit 3C, protein targeting to glycogen | 1.91 | |
| Gdc1 | glycerol phosphate dehydrogenase 1, cytoplasmic adult | 1.84 | |
| Pygl | liver glycogen phosphorylase | 1.71 | |

Lipid metabolism

| Pparg | peroxisome proliferator activator receptor gamma | 2.99 | 3.63 |
| Csd | sterol-C5-desaturase | 2.85 | |
| Lrpl | low density lipoprotein receptor-related protein 1 | 2.22 | |
| Cadh6 | CD36 antigen | 2.06 | 3.09 |
| Fads3 | fatty acid desaturase 3 | 2.06 | |
| Lipe | lipase, hormone sensitive | 1.96 | 1.66 |
| Lrpl2 | low density lipoprotein receptor-related protein 2 | 1.91 | |
| Fabp5 | fatty acid binding protein 5, epidermal | 1.87 | |
| Dgat1 | diacylglycerol acyltransferase | 1.87 | |
| Decz | 2,4-dienoyl CoA reductase 1, mitochondrial | 1.85 | |
| Slc27a1 | solute carrier family 27 (fatty acid transporter), member 1 | 1.85 | |
| Phyh | phytanol-CoA hydroxylase | 1.77 | |
| Dgat2/1 | diacylglycerol O-acyltransferase 2-like 1 | 1.75 | |
| Ppargc1 | peroxisome proliferative activated receptor, gamma, coactivator 1 | 1.74 | 1.97 |
apoCIII and transferrin genes via competition with the HNF4α. Since HNF4α is known to recognize DR-1 sequences as well, we examined whether HNF4α might control the expression of Gys-2 in liver, possibly via DR-1prom. Gys-2 mRNA levels were markedly decreased in liver-specific HNF4α-null mice, thus supporting a role for HNF4α in regulating Gys-2 expression (Fig. 7a). In transactivation assays using the Gys-2 promoter, HNF4α markedly activated reporter activity, suggesting the presence of a HNF4α response element within the 0.55-kb promoter fragment (Fig. 7b). Mutating DR-1prom resulted in an approximately 50% reduction in HNF4α-dependent activation of the Gys-2 promoter (Fig. 7c), which suggests that (a) HNF4α responsiveness is partially mediated by DR-1prom, or (b) the mutations with DR-1prom only partially disabled HNF4α responsiveness. Regardless of these explanations, these data suggest that HNF4α directly regulates the hepatic expression of Gys-2 at least partially via DR-1prom.

Finally, ChIP clearly showed HNF4α binding to DR-1prom but not DR-1int in rat primary hepatocytes (Fig. 7d).

Cross-talk between PPARα and HNF4α in the transcriptional control of Gys-2. Whereas HNF4α activates the Gys-2 promoter via DR-1prom, PPARα does the opposite, suggesting that PPARα may interfere with Gys-2 promoter activation by HNF4α. To examine whether this is the case, the effect of PPARα on HNF4α-mediated transactivation of the 0.55-kb Gys-2 promoter was studied. PPARα activa-
tion significantly reduced HNF4α-dependent transactivation, indicating competition between HNF4α and PPARα in the regulation of the Gys-2 promoter (Fig. 7e). As already mentioned above, we failed to find any evidence for binding of PPARα to DR-1prom in hepatocytes. Thus, the inhibitory effect of PPARα on transcriptional activation of Gys-2 by HNF4α likely does not occur via competition with HNF4α for actual binding to DR-1prom.

**Discussion**

In the present study, we have identified the mouse Gys-2 gene as a direct PPAR and HNF4α target gene. We have shown that the effects of PPARs and HNF4α on Gys-2 expression occur via two distinct response elements. Indeed, while transcriptional activation of the Gys-2 gene by PPARα was found to be mediated by a PPRE present in intron 1 of the mGys-2 gene (DR-1int), the stimulatory effect of HNF4α was mediated by a response element in the immediate upstream promoter (DR-1prom).

Our data are suggestive of the following scenario. In liver, which expresses high amounts of HNF4α, DR-1prom is occupied by HNF4α but not PPARα, while DR-1int is bound by PPARα but not HNF4α. Hence, HNF4α and PPARα activate Gys-2 expression via different response elements. Nevertheless, important negative cross-talk between the two nuclear receptors was observed. In the absence of any mutual binding to the response elements, it can be hypothesized that competition may take place at the level of binding to common co-activator proteins in a mechanism that is often referred to as squelching. In adipose tissue, which does not express HNF4α, DR-1prom is occupied by PPARβ/δ and PPARγ, but this does not result in transcriptional activation. Rather, transactivation occurs via binding of PPARβ/δ and PPARγ to DR-1int.

Our data indicate that HNF4α is an extremely powerful activator of mouse Gys2 transcription, explaining the marked reduction in hepatic Gys-2 expression in liver-specific HNF4α-null mice [35]. As mentioned above, regulation of mGys-2 expression by HNF4α at least partially occurs via DR-1prom. A recent study that combined ChIP with promoter micro-arrays showed that the Gys-2 promoter is bound by HNF4α in human liver [36], thus establishing Gys-2 as a direct target of HNF4α in human as well. It is not very clear why disabling the DR-1prom reduced HNF4α-dependent transactivation by only 50%. It is possible that the 0.55-kb Gys-2 promoter fragment contains an additional HNF4α response element, although *in silico* analysis failed to reveal such an element. Alternatively,
it is possible that mutating the wild-type DR-1prom (AGGCCAAAGGCCA) into a mutated DR-1prom (AGGCCTTTGGCCA) only partially disabled the response element. While functional PPREs are commonly located within regulatory sequences, i.e., proximal promoters, PPREs have also been identified in intronic sequences. Examples are PPREs within intron 3 of human/mouse Angptl4 and rat peroxisomal thiolaese B genes, and within intron 1 of the rat acyl-CoA binding protein gene and human carnitine palmitoyltransferase 1A [2–5]. Our data demonstrate that regulation of Gys-2 expression by PPARs is also mediated by an intronic PPRE.

In the past few years, our understanding of the function of PPARβ/δ in vivo has improved greatly thanks to studies using various transgenic mouse models [19, 37]. At the level of metabolism, PPARβ/δ over-expression promotes skeletal muscle fatty acid oxidation and type I fiber content in mice, resulting in improved endurance exercise performance [22]. Conversely, deletion of PPARβ/δ in cardiomyocytes is associated with impaired fatty acid oxidation and expression of fatty acid oxidative genes, whereas glucose uptake is increased [38]. In WAT, PPARβ/δ stimulates fatty acid oxidation and uncoupling, thereby diminishing adiposity [21]. It is thus clear that PPARβ/δ plays a pivotal role in governing fatty acid oxidation in a variety of tissues. In contrast, data linking PPARβ/δ to regulation of glucose homeostasis remain scarce [20, 39, 40]. Our data reveal that PPARβ/δ is a critical regulator of the adipose expression of the Gys-2 gene. Furthermore, micro-array and qPCR analysis indicated that expression of numerous other genes involved in lipid and glucose metabolism was markedly down-regulated in PPARβ/δ-null mice, including GLUT4, p85, and CD36. Since PPARγ and PGC-1α were significantly down-regulated as well, it is possible that many of the observed changes are not linked to the absence of PPARβ/δ per se but rather reflect indirect effects mediated via decreased PPARγ and PGC-1α mRNA. Although such an effect may contribute to some extent to the down-regulation of Gys-2 in PPARβ/δ-null mice, the in vitro studies leave no doubt that Gys-2 is a direct target gene of PPARβ/δ, as well as of PPARγ.

Glycogen is stored in many tissues, yet it is particularly abundant in liver, muscle, and adipose tissue. In liver, glycogen serves to maintain blood glucose levels between meals, while skeletal muscle glycogen is used to fuel muscle contractions. In contrast, adipose tissue glycogen serves as a source of glycerol 3-phosphate, which is required for (re)-esterification of fatty acids into triglycerides [41]. Several alternative pathways exist to produce glycerol 3-phosphate, including synthesis from glucose, and conversion of gluconeogenic precursors (glyceroneogenesis). Since expression and activity of glycerol kinase are very low in adipose tissue [42], direct phosphorylation of glycerol is not considered as a major pathway to generate glycerol 3-phosphate. However, recent studies suggest that this may change after treatment with synthetic PPARγ agonists, which markedly up-regulate glycerol kinase expression in human and mouse adipocytes [11, 42]. In fact, it has been hypothesized that stimulation of glycerol kinase expression by
TZDs, resulting in increased fatty acid re-esterification, may at least partially account for the suppressive effect of TZDs on plasma free fatty acid levels. Stimulation of fatty acid esterification is part of a general lipogenic and adipogenic effect of PPARγ in the adipocyte. Since adipose glycogen stores yield glycerol 3-phosphate as a precursor for fatty acid (re-)esterification, up-regulation of Gys-2 expression by PPARγ can be placed in the context of the lipogenic role of PPARγ in the adipocyte, which is aimed at promoting energy storage. Besides contributing to lipogenesis, synthesis of glycogen permits continued uptake of glucose into cells. Accordingly, it can be speculated that up-regulation of adipose Gys-2 by PPARγ might partially account for the stimulation of glucose uptake into adipocytes by PPARγ agonists.

It is currently still ambiguous whether PPARβ/δ serves a general anabolic or catabolic function in the adipocyte. On the one hand, it has been reported that PPARβ/δ promotes fatty acid oxidation in adipocytes [20, 21]. On the other hand, PPARβ/δ also seems to have a facilitative, yet important role in lipo- and adipogenesis [43]. As discussed above for PPARγ, up-regulation of Gys-2 expression by PPARβ/δ may indicate a role for PPARβ/δ in fatty acid (re-)esterification, thus contributing to a lipogenic role for PPARβ/δ.

The highest levels of glycogen are found in liver and fluctuate with nutritional status. The hepatic synthesis of glycogen from glucose is catalyzed by Gys-2 [44]. Remarkably, expression of Gys-2 in liver increases during fasting, at the same time when glycogen stores are actively broken down [32]. The reason behind this seemingly counterintuitive regulation is not very
clear, but it may serve to prime the glucose synthesizing system for when dietary glucose becomes available again. In the absence of PPARα, we observed that the expression of Gys-2 drops markedly during prolonged fasting and refeeding. The reduced Gys-2 expression is likely responsible for the diminished rate of glycogen formation upon refeeding, as observed by us and previously by others. Indeed, the effect of PPARα deletion on liver glycogen is minor except under conditions of refeeding [30–32]. It has been reported that after a short-term fast the gluconeogenic flux in PPARα-null mice is directed more towards glycogen, leading to a decrease in hepatic glucose output. However, it is unclear what happens to the gluconeogenic flux toward glycogen in the fasted-refed state, although our and other data clearly indicate that total glycogen synthesis is decreased in PPARα–/– mice.

Mutation of the GYS2 gene in humans leads to lower hepatic glycogen levels and fasting hypoglycemia [45], biochemical features that are also observed in PPARα–/– animals [31]. However, opposite to that observed in patients with a dysfunctional GYS2 gene, PPARα–/– mice show low plasma ketones, which is explained by the stimulatory effect of PPARα on fatty oxidation and ketogenesis. Overall, our data suggest that the decreased hepatic glycogen levels in PPARα–/– and liver-specific HNF4α-null mice [28, 35] may be due to decreased activation of Gys-2 expression via DR-1int and DR-1prom, respectively. Although PPARα and HNF4α stimulate Gys-2 expression via different response elements, important interplay exist between signaling of the two nuclear receptors.

In conclusion, we show that Gys-2 is a direct target gene of PPARα. Transcriptional regulation is achieved via a PPRE present in the first intron. An additional direct repeat response element identified in the Gys-2 promoter mediates transactivation by HNF4α.

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