THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 39, pp. 26385–26393, September 25, 2009
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Peroxisome Proliferator-activated Receptor γ Regulates Expression of the Anti-lipolytic G-protein-coupled Receptor 81 (*GPR81/Gpr81*)*[S]

Received for publication, July 3, 2009 Published, JBC Papers in Press, July 24, 2009, DOI 10.1074/jbc.M109.040741

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The ligand-inducible nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) plays a key role in the differentiation, maintenance, and function of adipocytes and is the molecular target for the insulin-sensitizing thiazoledinediones (TZDs). Although a number of PPARγ target genes that may contribute to the reduction of circulating free fatty acids after TZD treatment have been identified, the relevant PPARγ target genes that may exert the anti-lipolytic effect of TZDs are unknown. Here we identified the anti-lipolytic human G-protein-coupled receptor 81 (*GPR81*), *GPR109A*, and the (human-specific) *GPR109B* genes as well as the mouse *Gpr81* and *Gpr109A* genes as novel TZD-induced genes in mature adipocytes. *GPR81/Gpr81* is a direct PPARγ target gene, because mRNA expression of *GPR81/Gpr81* (and *GPR109A/Gpr109A*) increased in mature human and murine adipocytes as well as *in vivo* in epididymal fat pads of mice upon rosiglitazone stimulation, whereas small interfering RNA-mediated knockdown of PPARγ in differentiated 3T3-L1 adipocytes showed a significant decrease in Gpr81 protein expression. In addition, chromatin immunoprecipitation sequencing analysis in differentiated 3T3-L1 cells revealed a conserved PPAR:retinoid X receptor-binding site in the proximal promoter of the *Gpr81* gene, which was proven to be functional by electromobility shift assay and reporter assays. Importantly, small interfering RNA-mediated knockdown of Gpr81 partly reversed the inhibitory effect of TZDs on lipolysis in 3T3-L1 adipocytes. The coordinated PPARγ-mediated regulation of the *GPR81/Gpr81* and *GPR109A/Gpr109A* genes (and *GPR109B* in humans) presents a novel mechanism by which TZDs may reduce circulating free fatty acid levels and perhaps ameliorate insulin resistance in obese patients.

Because of a high calorie diet and a sedentary lifestyle, obesity and its associated co-morbidities like hypertension, type II diabetes, and atherosclerosis rapidly increase worldwide (1). Adipose tissue is the major site of lipid storage in the body and plays a pivotal role in the regulation of whole body metabolic homeostasis and therefore in the pathophysiology of obesity (2). After a meal, excess fuel substrates are partitioned to adipose tissue where they are processed and stored as triglycerides (TAG).[2] Conversely, during fasting TAGs are hydrolyzed to free fatty acids (FFA) and glycerol, and the FFA released into the bloodstream can subsequently be used by other organs as energy substrates. The latter process, termed lipolysis, is tightly regulated by hormones and cytokines (3). The three main hormones that regulate lipolysis in humans are insulin, which inhibits lipolysis, and catecholamines (adrenaline and noradrenaline) and glucagon, which stimulate lipolysis. In rodents, inhibition of lipolysis by adenosine presents an additional regulatory pathway. Lipolysis is deregulated in obesity; basal lipolysis rates are increased (4), whereas the stimulation of lipolysis by catecholamines (5) as well as the anti-lipolytic action of insulin (6) are inhibited. The impairment of hormonal control of lipolysis may be due to high levels of tumor necrosis factor-α, which is overproduced by adipose tissue in obese humans and rodents (7). Deregulated lipolysis results in increased circulating FFA levels and lipid accumulation in nonadipose tissues,

* This work was supported in part by a short term fellowship of the Federation of European Biochemical Societies (to E. H. J.), the research programme of The Netherlands Metabolomics Centre, which is part of The Netherlands Genomics Initiative/Netherlands Organization for Scientific Research, the Danish Natural Science Research Council, and the STREP Project X-TRA-NET, as part of European Union FP6.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: TAG, triglyceride; ChIP, chromatin immunoprecipitation; EMSA, electromobility shift assay; FA, fatty acid; FFA, free fatty acid; GPR, G-protein-coupled receptor; LPL, lipoprotein lipase; hMADS, human multipotent adipose-derived stem cells; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-response element; RXR, retinoid X receptor; SGBS, Simpson-Golabi Behmel syndrome; TZD, thiazolidinedione; PPARγ, peroxisome proliferator-activated receptor γ; ChIP-seq, sequencing ChIP; siRNA, small interfering RNA; RT, reverse transcription.

ultimately contributing to insulin resistance and other obesity-related metabolic disorders (8).

One of the key regulators of adipocyte differentiation, maintenance, and function is peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear hormone receptor superfamily of ligand-inducible transcription factors (9). PPARγ exists in two isoforms, PPARγ1 and PPARγ2. PPARγ2 has an additional 30 amino acids at the N terminus, and its expression is restricted to adipose tissue, while PPARγ1 is more widely distributed (*e.g.* adipocytes, lower intestine, monocytes, and macrophages). *In vitro* and *in vivo* studies showed that PPARγ is both necessary and sufficient to induce adipogenesis (9). PPARγ bind as an obligate heterodimer with the retinoic acid X receptors (RXRs) to PPAR-responsive elements (PPREs), which consist of two direct repeats of six nucleotides (AGGTCA) interspaced by one nucleotide (DR-1). Upon binding of ligand these proteins undergo a conformational change, which allows the interaction with so-called coactivators, starting a cascade of protein interactions and modifications that finally results in the induction of specific target genes (10). Although the endogenous ligands for PPARγ have not been firmly established, natural compounds like polyunsaturated fatty acids and eicosanoids have been shown to activate PPARγ. In addition, the antidiabetic drugs, such as thiazolidinediones (TZDs) act as high affinity PPARγ ligands (11). Administration of these TZDs to obese and/or insulin-resistant patients has been shown to reduce circulating FFAs and thereby improve insulin sensitivity. Part of these effects may be explained by the stimulatory effect of TZDs on adipocyte differentiation, thereby increasing lipid storage capacity in adipose tissue. In addition, PPARγ also regulates a number of genes essential for the adipocytic phenotype, such as genes involved in lipid uptake, lipid synthesis, lipid droplet stabilization, glycerol/FA recycling, and FA oxidation (12). Because elevated levels of serum FFAs promote insulin resistance (13), an important potential mechanism for the beneficial effects of TZDs is therefore the net partitioning of lipids in adipose tissue. Consistent with this notion, genes encoding proteins involved in lipid uptake in adipocytes, such as lipoprotein lipase, *CD36*, and the oxidized LDL receptor have been reported to be directly regulated by PPARγ (9). In addition, PPARγ directly regulates the expression of genes directly involved in lipid storage, like the lipid-droplet proteins *perilipin* and *S3-12* (14). PPARγ also regulates genes (potentially) involved in the "futile cycle" (9, 15, 16), the re-esterification of fatty acids and glycerol to triglycerides. Several findings suggest that PPARγ and TZDs may also be implicated in the regulation of lipolysis. First, the TZD troglitazone has been shown to lower basal lipolysis rates in differentiated adipocytes (this study and see Refs. (17–19)) as well as tumor necrosis factor-α-activated lipolysis (20, 21). Second, introduction of a dominant-negative form of PPARγ in mature adipocytes resulted in increased lipolysis, suggesting that PPARγ normally inhibits this process (22). Finally, treatment of diabetic patients with TZDs has been shown to restore insulin-mediated suppression of lipolysis (23–26). However, the relevant PPARγ target genes that may exert the anti-lipolytic effect of TZDs are unknown.

To identify novel target genes that may play a role in the effects of TZDs on lipid metabolism, we performed a transcriptome analysis in human adipocytes treated with the TZD rosiglitazone. In this study we show that TZDs induce the expression of two anti-lipolytic G-protein-coupled receptors, *GPR81/Gpr81* and *GPR109A/Gpr109A*, in human and murine adipocytes. In addition, a third anti-lipolytic GPR, the human-specific *GPR109B*, is also induced by rosiglitazone. This PPARγ-mediated activation may occur through a conserved PPRE located in the *GPR81* promoter. The coordinated PPARγ-mediated regulation of the *GPR81/Gpr81* and *GPR109A/Gpr109A* genes (and *GPR109B* in humans) presents a novel mechanism by which TZDs may reduce circulating FFA levels and perhaps ameliorate insulin resistance in obese patients.

EXPERIMENTAL PROCEDURES

Materials—Rosiglitazone and GW9662 were purchased from Alexis Biochemicals and Cayman Chemical, respectively. Anti-PPARγ antibody (2345S) was from Cell Signaling; anti-GPR81 antibody (NLS2095) was from Novus Biologicals; anti-GPR109A (GTX12610) was from GeneTex, and anti-tubulin (T5168) was from Sigma. Anti-PPARγ (sc-7196) and anti-RXR (sc-774) were used for ChIP assays. Anti-PPARγ (sc-7273), anti-RXRα (sc-553), and anti-Gal4 (sc-510) antibodies used for EMSA were from Santa Cruz Biotechnology.

Plasmids—All recombinant DNA work was performed according to standard procedures (27). The murine *Gpr81* reporter, *Gpr81*(−1059/+28)-luc, was generated by inserting the respective promoter into the XhoI/HindIII site of the PGL3-basic vector (Promega). All mutations were generated by QuickChange mutagenesis (Stratagene) and verified by sequencing. All other plasmids have been described earlier (28).

Cell Culture, Differentiation, and Reporter Assays—Culturing of cells was performed as described (28–30). Differentiation of 3T3-L1 (28), the human multipotent adipose-derived stem cells (hMADs) (30), and the human Simpson-Golabi Behmel syndrome cell line (SGBS) (29) have been described earlier. Reporter assays were performed exactly as described (28).

Microarray Analysis—3T3-L1, SGBS, and hMADs were differentiated as described above and at days 6, 8, and 17, respectively, treated with rosiglitazone or DMSO for 6 h. Micro-array experiments were performed as described before (31). In short, total RNA was isolated using TRIzol reagent. Concentrations and purity were determined on a NanoDrop ND-1000 spectrophotometer (Isogen). RNA integrity was checked on an Agilent 2100 Bioanalyzer (Agilent Technologies) with 6000 Nano-Chips. Part of the RNA samples from four 6-cm dishes was used for quantitative RT-PCR (see under "RNA Isolation and Real Time PCR"). Remaining RNA samples from four 6-cm dishes were pooled and used for microarray analysis. Samples were hybridized on human NUGO arrays from Affymetrix. A detailed description of the analysis method is available on request.

Animal Study—Animal study was performed as described earlier (31). In short, Sv129 male mice were purchased at The Jackson Laboratory (Bar Harbor, ME). At 20 weeks of age, the diet of half of the mice group was supplemented with rosiglita-

zone (0.01% w/w) for a week. At the end of the experiment epididymal white adipose tissue was dissected, weighed, and used for RNA isolation. The animal experiments were approved by the animal experimentation committee of Wageningen University.

RNA Isolation and Real Time PCR—3T3-L1 fibroblasts were differentiated as described above. Three independent samples of total RNA were extracted at different time points using TRIzol reagent (Invitrogen). cDNA was synthesized using the superscript first strand synthesis system (Invitrogen) according to manufacturer's protocol. Gene expression levels were determined by quantitative real time PCR with the MyIq cycler (Bio-Rad) using SYBR-green (Bio-Rad) and normalized to *TFIIb* expression.

The primers used were as follows: murine TFIIb forward primer, 5′-TCCTCCTCAGACCGCTTTT-3′, and reverse primer, 5′-CCTGGTTCATCATCGCTAATC-3′; murine Gpr81 forward primer, 5′-GGTGGCACGATGTCATGTT-3′, and reverse primer 5′-GACCGAGCAGAACAAGATGATT-3′; murine Gpr109A forward primer, 5′-TCCAAGTCTCCAAAGGTGGT-3′, and reverse primer, 5′-TGTTTCTCTCCAGCACTGAGTT-3′; murine Fapb4 forward primer, 5′-GAAAACGAGATGGTGACAAGC-3′, and reverse primer, 5′-TTGTGGAAGTCACGCCTTT-3′; human 36B4 forward primer, 5′-CGGGAAGGCTGTGGTGCTG-3′, and reverse primer 5′-GTGAACACAAAGCCCACATTCC-3′; human GPR109A forward primer, 5′-TTCAGAGAATGCGATTTAGGG-3′, and reverse primer 5′-ACACCTTGCAACCAGTCTCC-3′; human GPR109B forward primer, 5′-TTCTGTGGGGCATCACTGT-3′, and reverse primer, 5′-GCCATTCTGGATCAGCAACT-3′; and human GPR81 forward primer, 5′-AATTTGGCCGTGGCTGATTTC-3′, and reverse primer, 5′-ACCGTAAGGAACACGATGCTC-3′.

Western Blot Analysis—For Western blot analyses, differentiated 3T3-L1 cells were lysed in RIPA lysis buffer (200 mM Tris-HCl, pH 8,0; 0,1% SDS, 1% Triton X-100; 10 mM EDTA; 150 mM NaCl; 1% sodium deoxycholate containing protease inhibitors). Total cell lysate was diluted in 4× Laemmli sample buffer, subjected to SDS-PAGE, and transferred to Immobilon membranes (Millipore). α-PPARγ, α-GPR81, α-GPR109A, α-FABP4, and α-tubulin antibodies were used to probe for the respective proteins. ECL Plus (PerkinElmer Life Sciences) was used for detection, and signals were quantified using a densitometer.

ChIP and Sequencing (ChIP-seq)—ChIP assays were performed exactly as described earlier (32). The primers used for ChIP assays were as follows: murine Gpr81 forward primer, 5′-AGTGCCAGAGAGGGGAGACT-3′, and reverse primer 5′-CGTTTCTCTGCAGACCTTCC-3′; murine β-globin forward primer, 5′-CCTGCCCTCTCTATCCTGTG-3′, and reverse primer 5′-GCAAATGTGTTGCCAAAAAG-3′; human GPR81 forward primer, 5′-CTGGAGAGCACACAAAGCTG-3′, and reverse primer 5′-CCACTCCAGGAAATGTTTGG-3′; and human β-globin forward primer, 5′-TGGTATGGGGCCAAGAGATA-3′, and reverse primer 5′-TAGATGCCTCTGCCCTGACT-3′. ChIP-seq was performed as described earlier (12).

EMSA—EMSAs were performed as described earlier (28). The sequences of the double-stranded DNA oligomers used, containing the wild type or mutant PPRE from the mouse G-protein-coupled receptor 81 promoter (between −128 and −98 of the *Gpr81* gene), were as follows: mGpr81 wild type, 5′-CCGGGGACGGGTAGTCAGGCAAAGGTTAGGGAGGA-3′; mGpr81 mutant A, 5′-CCGGGGACGGCAAGTCACCCAAAGGTTAGGGAGGA-3′; and mGpr81 mutant B, 5′-CCGGGACGGGTAGTCTCGCAAACCTTAGGGAGGA-3′.

siRNA Transfection in Differentiated Adipocytes—3T3-L1 cells were differentiated as described above. At day 6 cells were detached using 5× trypsin/EDTA (Invitrogen), washed in medium containing 4% glycerol. For each reaction 2 million cells were resuspended in buffer L (AMAXA cell line Nucleofector kit L), and control (D-001210-01-20; Dharmacon), murine-specific PPARγ (number 2 J-040712-06 Dharmacon), or custom-made Gpr81 (5′-ACCTGGAAGTCAAGCACTATT; Dharmacon) siRNA oligonucleotides were delivered into adipocytes (500 nM of each siRNA/2 million cells) by electroporation (AMAXA Nucleofector II). Cells were reseeded, and 20 h post-electroporation cells were incubated with 1 μM rosiglitazone for an additional 28 h. Subsequently, cells were harvested for Western blot analysis, and media ($n = 4$) were collected for glycerol measurements. Glycerol levels were determined according to the manufacturer's instructions (Instruchemie).

RESULTS

GPR109A, GPR109B, and GPR81 Are Regulated by Rosiglitazone in Mature Adipocytes—To identify novel TZD-regulated genes in mature human adipocytes, we performed transcriptome analysis in differentiated hMADs (30). Out of 361 genes that were up-regulated after 6 h of treatment with the TZD rosiglitazone (data not shown), we selected the human-specific G-protein-coupled receptor 109B (*GPR109B*) to explore in more detail. Together with *GPR109A* and *GPR81*, *GPR109B* belongs to the class A rhodopsin-like G-protein-coupled receptors. GPR109A (also called puma-g) and the human-specific GPR109B are 96% homologous (33) and expressed in adipose tissue, spleen, and immune cells (34–36), whereas GPR81 expression is almost exclusively restricted to adipose tissue (37, 38). GPR109A has been identified as the receptor for the antilipolytic drug nicotinic acid, and in *GPR109A* knock-out mice it has been shown that GPR109A was the receptor mediating the lipid-lowering effects of nicotinic acid (34–36). Recently, the ketone body β-hydroxybutyrate was reported as an endogenous agonist for GPR109A (39), whereas aromatic D-amino acids can activate GPR109B (40). In addition, very recently two reports (41, 42) showed that GPR81 functions as a receptor for lactate, which reduces lipolysis *in vitro* and *in vivo* (43, 44). Interestingly, the *GPR81*, *GPR109A*, and *GPR109B* genes are colocalized on human chromosome 12 and share synteny with murine *Gpr81* and *Gpr109A* on mouse chromosome 5 (Fig. 1*A*) (45). For this reason, expression of the *GPR109A* and *GPR81* genes, which were not represented on the microarray, was determined together with the *GPR109B* gene in differentiated hMADs cells. Using quantitative RT-PCR, mRNA expression of these three genes was found to increase 4–5-fold after treat-

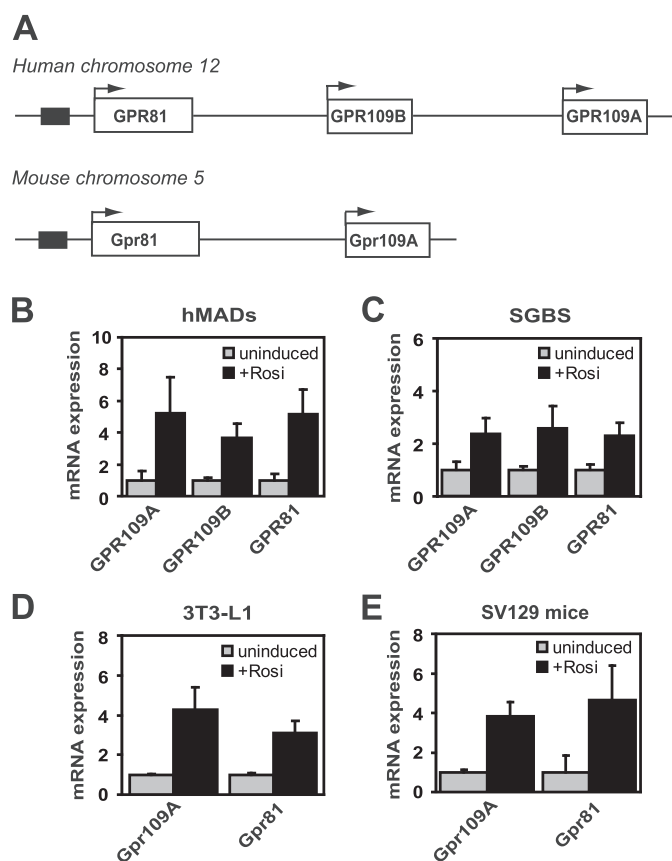

FIGURE 1. ***GPR109A/Gpr109A*, *GPR81/Gpr81*, and *GPR109B* are induced by the PPARγ ligand rosiglitazone in mature adipocytes and *in vivo* in adipose tissue.** *A*, schematic representation of the genomic arrangement of the GPR family in human and mouse. *GPR109A/Gpr109A*, *GPR109B*, and *GPR81/Gpr81* mRNA expression in fully differentiated hMADs (*B*), human SGBS (*C*), and murine 3T3-L1 adipocytes (*D*) after incubation with 1 μM rosiglitazone (*Rosi*) for 6 h. Relative mRNA expression levels were related to untreated cells and normalized for the *36B4* or *TFIIb* reference gene for human and murine adipocytes respectively. *E*, *Gpr109A* and *Gpr81* mRNA expression levels in epididymal fat pads of control or rosiglitazone-treated male SV129 mice. Relative mRNA expression levels were related to control mice and normalized for the *TFIIb* reference gene.

ment with rosiglitazone for 6 h (Fig. 1*B*). The same experiment was performed in another human adipocyte cell line, the SGBS cell line (29). In these cells a similar mRNA expression profile was observed (Fig. 1*C*). To investigate whether conserved mechanisms of regulation exist in mouse adipocytes, we examined the effect of rosiglitazone treatment on *Gpr81* and *Gpr109A* mRNA expression in differentiated 3T3-L1 adipocytes. As was observed for the human adipocytes, treatment of murine adipocytes with rosiglitazone stimulated the mRNA expression levels of *Gpr81* and *Gpr109A* up to 4-fold (Fig. 1*D*). Finally, we examined the effect of rosiglitazone treatment on the mRNA expression of *Gpr81* and *Gpr109A* in mouse adipose tissue *in vivo*. For this Sv129 male mice received a diet supplemented with rosiglitazone for 1 week; RNA was isolated from epididymal fat pads and subjected to quantitative RT-PCR analysis. As shown in Fig. 1*E*, mRNA expression of both *Gpr109A* and *Gpr81* was up-regulated in epididymal fat pads of the mice administered rosiglitazone compared with control mice.

In summary, rosiglitazone treatment induces the mRNA expression of the human *GPR109A*, *GPR109B*, and *GPR81*

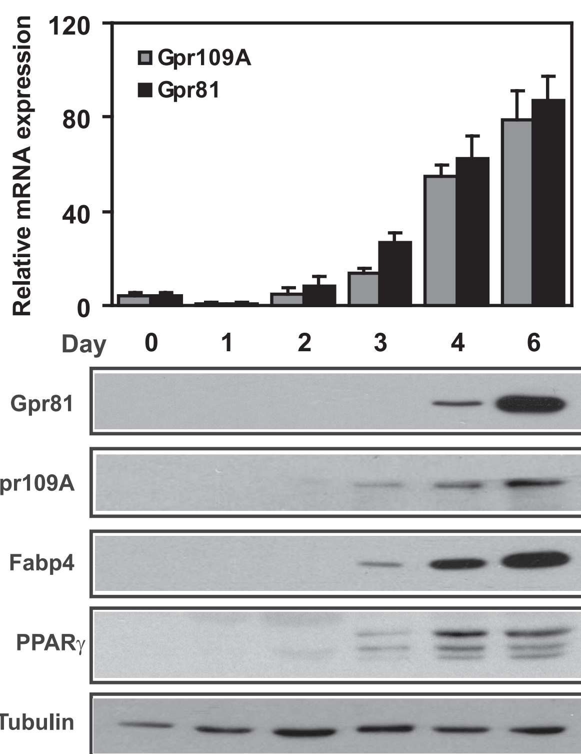

FIGURE 2. **mRNA and protein expression of *Gpr109A* and *Gpr81* increases during differentiation of 3T3-L1 adipocytes.** *Gpr109A*, *Gpr81*, and *Fabp4* mRNA expression (*upper panel*) and protein expression levels (*lower panels*) at days 0, 1, 2, 3, 4, and 6, respectively, of differentiating 3T3-L1 cells. Relative mRNA expression levels were related to undifferentiated cells (day 0) and normalized for the *TFIIb* reference gene. Tubulin protein expression was used as a loading control in Western blot analysis.

genes in differentiated adipocytes. A conserved regulatory mechanism may underlie this induction, because the mouse *Gpr109A* and *Gpr81* genes were also significantly up-regulated, *in vitro* and *in vivo*, upon rosiglitazone treatment.

GPR81 and GPR109A mRNA and Protein Expression Increase during 3T3-L1 Differentiation—Because PPARγ plays an essential role in adipogenesis and the expression of known PPARγ target genes increases during differentiation, we studied the protein and mRNA expression levels of Gpr81 and Gpr109A during differentiation of 3T3-L1 pre-adipocytes into mature adipocytes. At different time points of differentiation, cells were harvested to determine mRNA and protein expression levels. Quantitative RT-PCR showed that mRNA expression of both *Gpr81* and *Gpr109A* steadily increased during adipocyte differentiation, starting at day 2 (Fig. 2). In addition, protein expression levels of GPR81 and GPR109A were determined during adipogenesis. Protein expression of GPR81 could be detected from day 4 onward, whereas expression of the Gpr109A protein was observed slightly earlier (day 3). As a control, protein expression levels of PPARγ and the well established PPARγ target gene Fabp4 were determined and showed a similar increase during adipocyte differentiation (Fig. 2). In conclusion, Gpr81 and Gpr109A mRNA and protein expression clearly increased during differentiation of 3T3-L1 adipocytes.

PPARγ Directly Regulates GPR81 Protein Expression in Mature Adipocytes—To investigate whether the activation of the *Gpr81* and *Gpr109A* genes by rosiglitazone is directly reg-

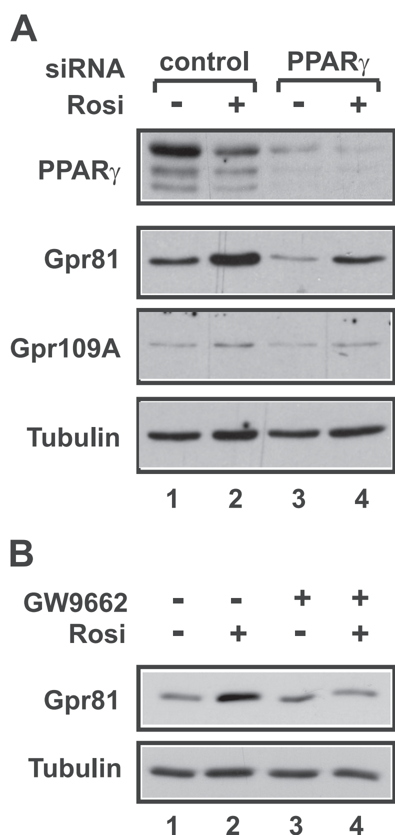

FIGURE 3. **PPARγ directly regulates GPR81 and GPR109A protein expression.** *A,* differentiated 3T3-L1 adipocytes (day 6) were electroporated with control or PPARγ siRNA. Twenty hours after electroporation medium was replaced by medium with or without 1 μM rosiglitazone and incubated for an additional 28 h. Cells were lysed and subjected to Western blot analysis. α-Tubulin was used as a loading control. This blot is a representative of at least three independently performed experiments. *B,* differentiated 3T3-L1 adipocytes (day 6) were treated with or without 1 μM rosiglitazone and with or without 1 μM GW9662 for 24 h. Western blot analysis was performed as described under *A*.

ulated by PPARγ, we reduced expression of the PPARγ protein in mature 3T3-L1 adipocytes by siRNA-mediated knockdown. As described earlier (46), rosiglitazone treatment resulted in reduced PPARγ protein levels (Fig. 3, *lanes 1* and *2*). Electroporation of siRNA oligonucleotides directed against PPARγ significantly reduced, but did not completely abolish, PPARγ protein expression in the absence and presence of rosiglitazone (Fig. 3). In agreement with the mRNA expression data (Fig. 1*B*), protein expression of Gpr81 increased 3-fold in the presence of rosiglitazone in these cells (Fig. 3*A*, *lanes 1* and *2*). Knockdown of PPARγ resulted in a significant reduction (4-fold, as determined by densitometry) of both basal and rosiglitazone-induced Gpr81 protein expression compared with 3T3-L1 adipocytes treated with nontargeting siRNA oligonucleotides. In the case of Gpr109A protein, a 1.5-fold induction was observed upon rosiglitazone treatment, which was reduced 2-fold upon knockdown of the PPARγ protein under both basal and treated conditions. In addition, the ability of rosiglitazone to induce Gpr81 protein expression was examined in the presence of the PPARγ-specific antagonist GW9662. As depicted in Fig. 3*B*, treatment with GW9662 inhibited the induction of Gpr81 by rosiglitazone. Taken together, these results indicate that the PPARγ protein is essential for the activation of the *Gpr81* gene, and to a lesser extent the *Gpr109A* gene, by rosiglitazone.

Endogenous PPARγ and RXR Bind to the Proximal Promoter of GPR81—The rapid activation of the *Gpr81* and *Gpr109A* genes by rosiglitazone (Fig. 1) together with the essential role of the PPARγ protein in this process (Fig. 3) prompted us to examine whether PPARγ and its heterodimeric partner RXR are recruited to the proximal promoter of the *Gpr81* and/or *Gpr109A* genes. Very recently, a genome-wide analysis of PPARγ and RXR binding during 3T3-L1 differentiation by ChIP sequencing technology was reported (12). Detailed analysis of the chromosomal region surrounding the *Gpr81* and *Gpr109A* genes revealed clear PPARγ and RXR binding in the proximal promoter (−294/−55) of *Gpr81*, suggesting a PPARγ:RXR-binding site at this location. Interestingly, no significant peaks in close proximity of the *Gpr109A* gene were observed (Fig. 4*A*). The recruitment of PPARγ and RXR to the proximal promoter of the *Gpr81* gene in mature 3T3-L1 adipocytes (day 6) was confirmed by ChIP-PCR (Fig. 4*B*). In addition, the recruitment in preadipocytes (day 0), in which PPARγ expression is low, was negligible, and neither PPARγ nor RXR was detected on an arbitrary gene region of the β-globin gene on chromosome 7, which served as a negative control (Fig. 4*B*).

Because the proximal promoter region of the *Gpr81* gene is well conserved between human and mouse, we investigated if PPARγ/RXR also binds to the proximal promoter of the human *GPR81* gene in SGBS preadipocytes (day 0) and mature SGBS adipocytes (day 8). Interestingly, both PPARγ and RXR were recruited to the proximal promoter of *GPR81* in differentiated SGBS cells but not in preadipocytes (day 0) (Fig. 4*C*). Also, in this case binding of PPARγ and RXR was absent in the negative control (Fig. 4*C, human beta-globin*). Taken together these results indicate that a PPARγ/RXR heterodimer binds to the proximal promoter of the mouse *Gpr81* gene as well as the human *GPR81* gene.

Identification of a Functional PPRE in the Proximal Promoter of GprR81—To identify the PPRE in the proximal promoter of *Gpr81*, we subjected the sequence underneath the peaks (−294/−55) of the ChIP-seq data (Fig. 4*A*) to an *in silico* promoter analysis (Nuclear Hormone Receptor scan (47)). A potential PPRE was detected, which was conserved between humans and mice (Fig. 5*A*). To assess if PPARγ/RXRα binds to this potential PPRE, we first performed electrophoretic mobility shift assays. A ^{32}P-labeled probe containing the PPRE was incubated with *in vitro* translated PPARγ2 and/or RXRα. As shown in Fig. 5*B*, a specific PPARγ2-RXRα heterodimeric complex was formed on the *Gpr81* PPRE, as formation of this protein-DNA complex could be diminished by addition of an excess of unlabeled wild type PPRE but not by an excess of mutant PPRE (Fig. 5*B*). Specific antibodies against PPARγ and RXRα, but not an antibody directed against an irrelevant protein (Gal4), supershifted the protein-DNA complex, confirming the heterodimeric composition of the complex (Fig. 5*C*).

Next, we fused the 5′-flanking region and start site of the mouse *Gpr81* gene (−1059/+28) to a luciferase gene. The activity of this reporter was determined in human osteosarcoma (U2OS) cells, which express negligible levels of endogenous PPARγ protein but display a robust response upon over-

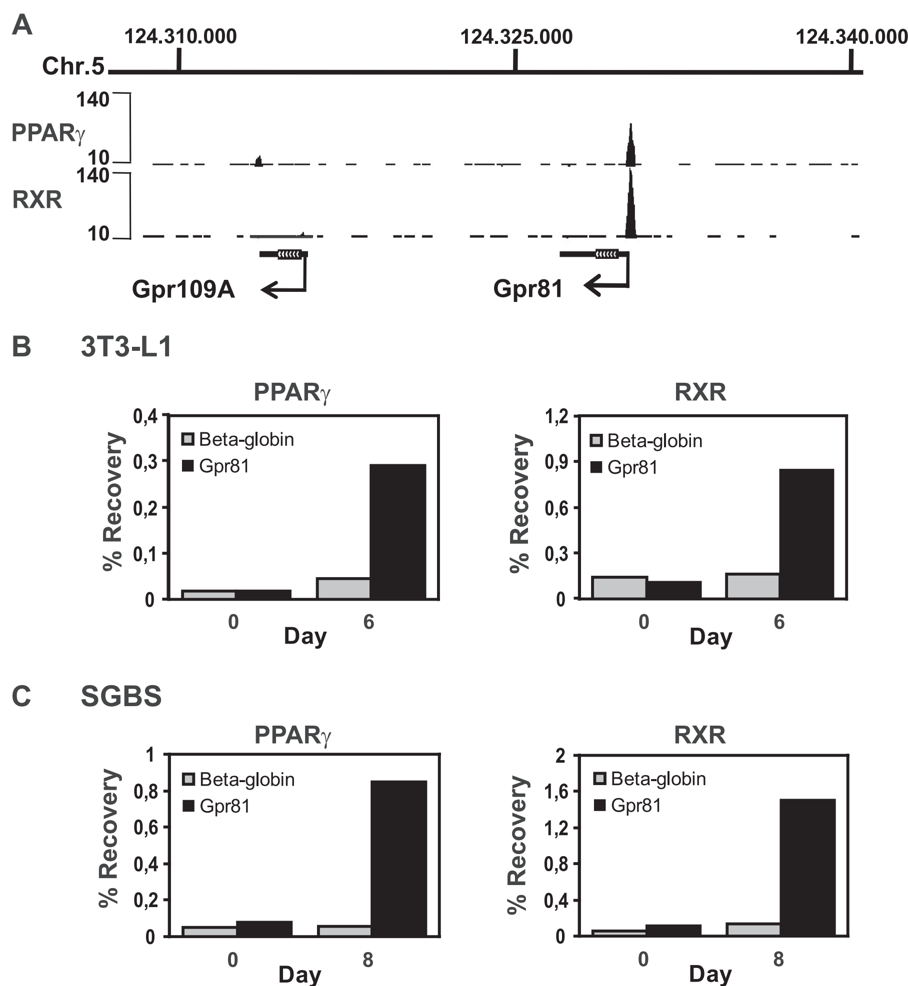

FIGURE 4. **PPARγ and RXR are recruited to the proximal promoter of GPR81.** *A,* ChIP-seq data of differentiated 3T3-L1 adipocytes (day 6) viewed in the University of Southern California (UCSC) browser showing PPARγ- and RXR-binding sites for GPR81. *Chr.*, chromosome. *B,* ChIP-PCR for PPARγ and RXR was performed in 3T3-L1 (*B*) and SGBS (*C*) at the days indicated. As a negative control the arbitrary gene β-globin was used. Results are indicated as percentage of immunoprecipitated chromatin compared with the input.

expression of the protein (28). As shown in Fig. 6*A*, transfection of cells with an expression vector encoding PPARγ2 markedly activated the reporter gene compared with empty vector control (pCDNA). Activation of PPARγ by rosiglitazone further enhanced the PPARγ-mediated activation of the reporter. Mutation of the potential PPRE completely abolished the PPARγ-mediated activation of the reporter, both in the absence and presence of rosiglitazone (Fig. 6*A*).

To examine the regulation of the *Gpr81* promoter in more detail, we tested the ability of the PPARγ1 isoform, as well as three PPARγ2 mutants to activate this reporter. As shown in Fig. 6*B*, PPARγ1 activated the reporter to a comparable level as PPARγ2, suggesting there is no isoform specificity for this PPRE. The two natural PPARγ2 mutants R425C and P495L displayed reduced and negligible activity, respectively, in agreement with their activity on other promoters (28, 48). The heterodimerization defective mutant L464R failed to activate the reporter (Fig. 6*B*), confirming that dimerization of PPARγ and RXRα is a prerequisite for binding to the *Gpr81* PPRE (Fig. 5, *B* and *C*). Taken together, these results indicate that PPARγ activates transcription of the *Gpr81* gene by binding of a PPARγ/RXR heterodimer to a conserved PPRE located in the proximal promoter (−141/−129) of the gene.

siRNA-mediated Knockdown of Both Gpr81 and PPARγ in Mature Adipocytes Increased Lipolysis—Next, we wished to establish the relevance of the PPARγ-mediated up-regulation of the *Gpr81* gene in the anti-lipolytic action of TZDs. For this, we reduced Gpr81 or PPARγ protein expression by siRNA-mediated knockdown in mature 3T3-L1 adipocytes and determined glycerol levels as a measure for lipolysis. In agreement with previous studies (17–19), TZD treatment decreased glycerol levels by 2-fold (Fig. 7*B*). TZD treatment also inhibited glycerol release when lipolysis was stimulated with the β-adrenergic agonist isoprotenerol (supplemental Fig. 1). Partial knockdown of PPARγ increased lipolysis and reduced the effect of rosiglitazone treatment (35% reduction; Fig. 7*B*, *right panel*). Similarly, knockdown of Gpr81, which was also partial (Fig. 7*A*), resulted in a slight increase in glycerol levels and a 35% reduction of the rosiglitazone-mediated inhibition (Fig. 7*B*, *right panel*). Taken together, these data suggest that the anti-lipolytic action of rosiglitazone is partly mediated through the PPARγ-mediated regulation of the *Gpr81* gene in mature adipocytes.

DISCUSSION

PPARγ plays a key role in (pre)adipocyte biology by regulating their differentiation, maintenance, and lipid metabolism. The insulin-sensitizing TZDs have been shown to be high affinity ligands for PPARγ and are administered to patients with insulin resistance. This class of antidiabetic drugs increases systemic insulin sensitivity in diabetic animal models and humans (49). The number of target genes that help to explain the beneficial effects of these ligands is limited, however. Here we present compelling evidence that the anti-lipolytic G-protein-coupled receptor 81 (*GPR81/Gpr81*) is a novel direct PPARγ target gene in human and murine adipocytes. Interestingly, in addition to the *Gpr81/GPR81* gene, expression of the anti-lipolytic *Gpr109A/GPR109A* gene (and the *GPR109B* gene in human adipocytes) was also stimulated by TZD-activated PPARγ, but a functional PPRE could only be identified in the proximal promoter of the *Gpr81* gene (Fig. 1*A* and Fig. 4*A*). It is possible that this site also controls the *Gpr109A/GPR109A* promoter, which is 16 kb further downstream. Of note, the genome-wide profiles

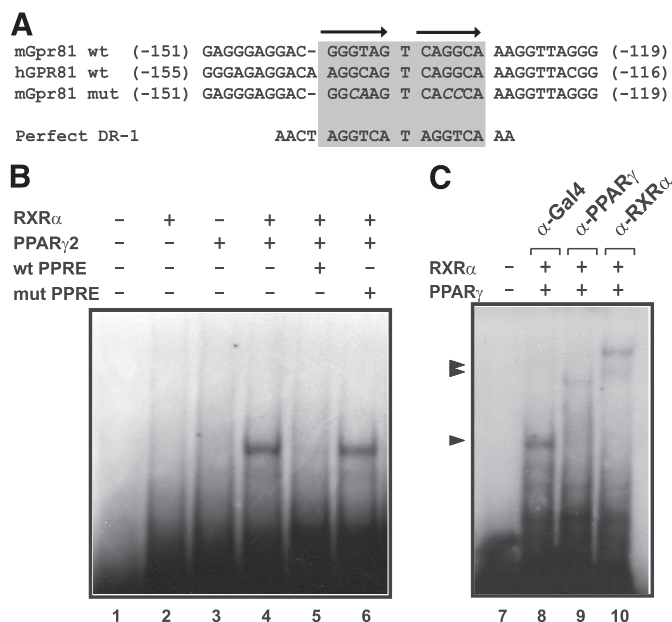

FIGURE 5. **Identification of the PPRE in the proximal promoter of *GPR81/Gpr81*.** *A*, alignment of the murine, human *GPR81/Gpr81*, and perfect PPRE. Electrophoretic mobility shift assay using a ^{32}P-labeled fragment from the proximal *Gpr81* promoter containing the PPRE incubated with *in vitro* translated PPAR-γ2 and/or RXRα proteins in the presence or absence of an excess of unlabeled wild type (*wt*) or mutant (*mut*) fragment (*B*) or α-Gal4, α-PPARγ, or α-RXRα antibody, respectively (*C*). Protein-DNA complexes were separated from unbound DNA on nondenaturing polyacrylamide gels and visualized by autoradiography of dried gels.

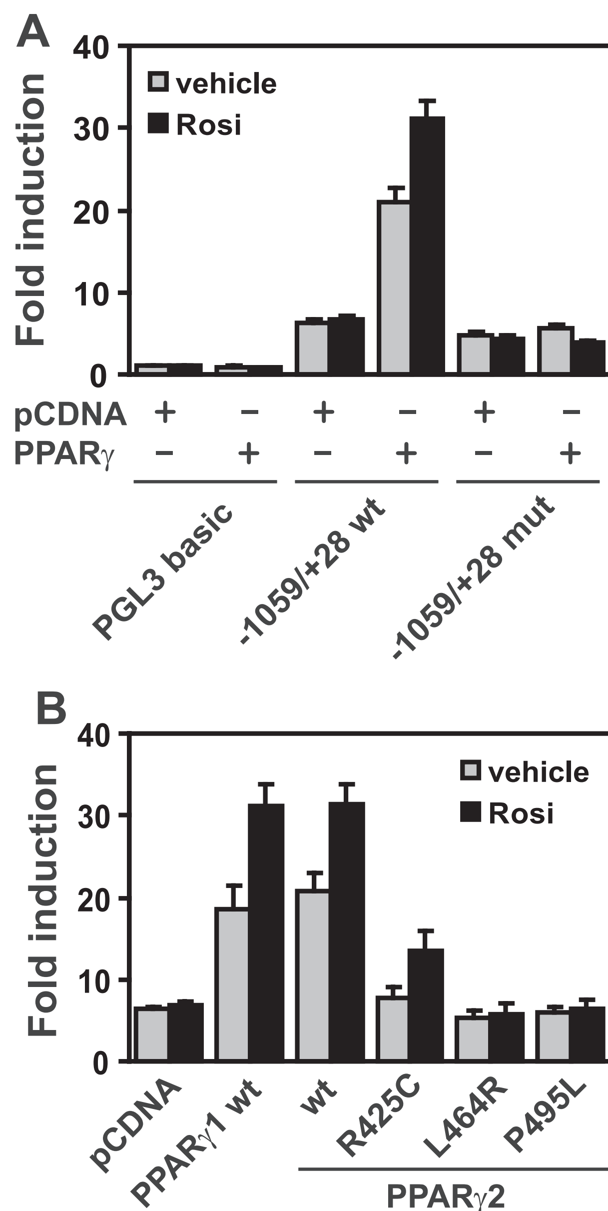

FIGURE 6. **Identified PPRE in the proximal promoter of *Gpr81* is functional in cells.** *A*, U2OS cells were cotransfected with a reporter construct (pGL3-*Gpr81*(−1059/+28)) containing wild type (*wt*) or mutant (*mut*) PPRE together with empty (*pCDNA*) or PPARγ-encoding expression vectors. Activation of the luciferase reporter in the absence or presence of 1 μM rosiglitazone (*Rosi*) is expressed as fold induction over that with empty reporter cotransfected with pCDNA in the absence of rosiglitazone after normalization for *Renilla* luciferase activity. *B*, U2OS cells were transfected with the *Gpr81*(−1059/+28) reporter and expression vectors encoding wild type PPARγ1, PPARγ2, or mutant PPARγ2. Activation of the luciferase reporter is expressed as described above. Results are averages of at least three independently performed experiments assayed in duplicate means ± S.E.

of PPARγ:RXR in 3T3-L1 have unequivocally shown that only a very small percentage of PPARγ:RXR target sites lie in the proximal promoters (12, 50). Distal gene regulation has been proposed to occur via a mechanism by which a transcription factor bound to a distal site directs looping, thereby bringing coactivators and chromatin remodelers at the distal sites in proximity of transcription start site of target genes and facilitating recruitment of RNA polymerase II (51). The rapid increase (within 6 h) and synchronous induction of *GPR81/Gpr81* and *GPR109A/Gpr109A* in mature adipocytes observed in our studies support the direct regulation of both genes by PPARγ. Alternatively, PPARγ could regulate the *GPR109A/Gpr109A* gene (and the *GPR109B* gene in humans) in an indirect manner. Future studies are therefore required to establish the exact molecular mechanisms underlying the regulation of the *GPR109A/Gpr109A* gene.

Based on the data presented here showing that PPARγ directly regulates the anti-lipolytic *GPR81*, *GPR109A*, *GPR109B* (this study), and *GPR43* genes (52, 53) in adipocytes together with previous reports showing that TZDs reduce lipolysis in these cells (17–21), we propose the following model for the anti-lipolytic effect of TZDs in adipocytes. Administration of TZDs to mature adipocytes activates PPARγ leading to increased transcription of *GPR81* and *GPR109A* (and *GPR109B* in humans) and subsequent increase in protein expression of both receptors. GPR109A and GPR81 couple to members of the G_i family of G-proteins (38, 45). In adipocytes activation of G_i preferentially results in the inhibition of adenylyl cyclases, which counteracts the activity of pro-lipolytic receptors (*e.g.* β-adrenergic and glucagon receptors). As a result, intracellular cAMP levels will be lowered and protein kinase A will be less active. Protein kinase A phosphorylates a number of proteins, most notably hormone-sensitive lipase and perilipin, which are required for hydrolysis of TAGs. Phosphorylation of perilipin allows access to the TAG-containing droplets by the now activated hormone-sensitive lipase and a second lipase, adipose triglyceride lipase, which hydrolyzes the TAG in FFA and glycerol. Our data in mature 3T3-L1 adipocytes suggest that the PPARγ-mediated up-regulation of Gpr81 and Gpr109A con-

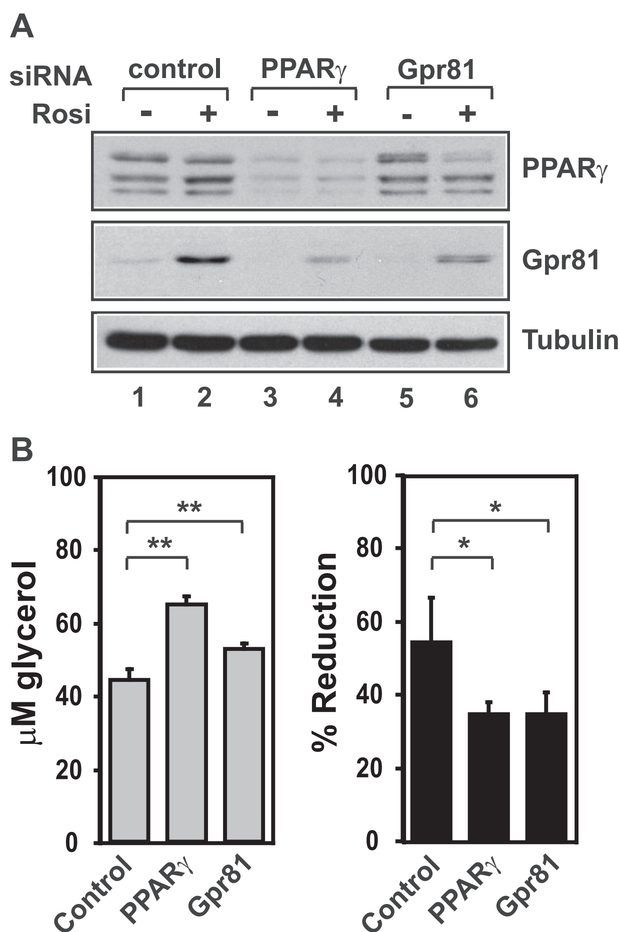

FIGURE 7. ***Gpr81* contributes to the anti-lipolytic action of rosiglitazone.** siRNA-mediated knockdown of PPARγ or Gpr81 in differentiated 3T3-L1 adipocytes was performed as described in Fig. 3. *A,* cells were lysed and subjected to Western blot analysis, using α-tubulin as a loading control. *B,* media were collected ($n = 4$), and glycerol levels were determined, as a measure for lipolysis. Presented is the glycerol concentration and the percentage reduction upon rosiglitazone treatment over vehicle-treated cells. Indicated are the mean values ($n = 4$) ± S.D. *Asterisks* indicate significant differences (Student's *t* test: *, $p < 0.05$; **, $p < 0.01$).

tributes to the anti-lipolytic action of TZDs *in vitro*. It is therefore tantalizing to speculate that also *in vivo* the insulin-sensitizing, antidiabetic and hypolipidemic actions of TZDs are partly mediated via PPARγ-mediated up-regulation of the *Gpr81* and *Gpr109A* genes in rodents and the *GPR81*, *GPR109A*, and *GPR109B* genes in humans. It should be noted, however, that adipocytes also express at least one additional anti-lipolytic GPR, *Gpr43*, which is also transcriptionally controlled by PPARγ (53). The presence of numerous anti-lipolytic GPRs in adipocytes may explain the relatively modest effect of Gpr81 knockdown on rosiglitazone-mediated inhibition of lipolysis observed in our experiments (Fig. 7). Furthermore, inhibition of lipolysis is clearly not the only mechanism by which TZDs reduce circulating FFA, as these drugs also stimulate adipogenesis and increase lipid uptake, lipid synthesis, lipid droplet stabilization, glycerol/FA recycling, and FA oxidation in adipose tissue (12). Investigations on the effects of TZDs in *Gpr81* knock-out mice, *Gpr109A* knock-out mice, and double knock-out animals will help to establish the relative importance of these GPRs in mediating the lipid-lowering effects of these drugs.

Acknowledgments—We thank Shohreh Keshtkar and Dr. Guido Hooiveld (Nutrition, Metabolism and Genomics Group, Wageningen University, Wageningen, The Netherlands) for their help with microarray analysis and Dr. Thomas Åskov Pedersen (Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark) for help in analyzing the ChIP sequence data.

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