Novel Genes Regulated by the Insulin Sensitizer Rosiglitazone During Adipocyte Differentiation

Tatjana Albrektsen,[1,2] Klaus Stensgaard Frederiksen,[1] William E. Holmes,[1] Esper Boel,[1] Karen Taylor,[3] and Jan Fleckner[1]

Thiazolidinediones (TZDs) are a new class of compounds that improve insulin sensitivity in type 2 diabetic patients as well as in rodent models of this disease. These compounds act as ligands for a member of the nuclear hormone receptor superfamily, peroxisome proliferator–activated receptor-γ (PPAR-γ), which is highly expressed in adipose tissue and, moreover, has been shown to play an important role in adipocyte differentiation. The strong correlation between the antidiabetic activity of TZDs and their ability to activate PPAR-γ suggests that PPAR-γ, through downstream-regulated genes, mediates the effects of TZDs. In this report, we present the isolation and characterization of 81 genes, encoding proteins of known function, differentially expressed during TZD-stimulated differentiation of 3T3-L1 cells. By the use of different reverse–Northern blot techniques, the differential expression of 50 of these genes could be verified, and 21 genes were specifically regulated by a potent TZD during the course of adipocyte differentiation, whereas no effect of a PPAR-γ antagonist could be observed in mature adipocytes. The differential expression of a large fraction of the isolated genes was also shown to occur in white adipose tissue of *ob/ob* mice treated with rosiglitazone; combined, our results suggest that an important effect of rosiglitazone in adipose tissue is based on activation of PPAR-γ in preexisting preadipocytes found among the mature adipocytes, resulting in subsequent adipocyte differentiation.
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Thiazolidinediones (TZDs) are a new class of compounds that improve the insulin sensitivity in type 2 diabetic patients as well as in rodent models of this condition. Repeated administration of TZDs to type 2 diabetic patients and insulin-resistant *KK* and *ob/ob* mice, as well as Zucker fatty rats, has been shown to ameliorate hyperglycemia, hyperinsulinemia, and hyperlipidemia, thereby reversing, at least partially, the diabetic state (1,2).

TZDs are high-affinity ligands for peroxisome proliferator activated–receptor-γ (PPAR-γ), which is a member of the nuclear hormone receptor superfamily (3). PPAR-γ forms heterodimers with members of the retinoid X receptor (RXR) family and regulates gene expression by binding to PPAR response elements in the promoter region of a variety of genes (4–8). Analyses of the PPAR-γ expression pattern have revealed that the receptor is expressed abundantly in adipose tissue, and several observations suggest that PPAR-γ is essential for adipocyte differentiation. First, forced expression of PPAR-γ in fibroblasts leads to the development of an adipocyte phenotype, an event that is enhanced by the presence of ligands for this receptor (9). Second, full or partial PPAR-γ antagonists have recently been shown to prevent adipocyte differentiation in vitro (10–13). Third, a homozygous PPAR-γ knockout mouse (PPAR-$\gamma^{-/-}$ mouse) is completely devoid of adipose tissue (14), heterozygotes (PPAR-$\gamma^{+/-}$ mice) are characterized by decreased adipose tissue mass (15), and adipose tissue of mice chimeric for wild-type and PPAR-γ–null cells is composed exclusively of PPAR-$\gamma^{+/+}$ cells (16).

PPAR-γ also seems to play a role in the development/treatment of diabetes. Barroso et al. (17) recently identified a loss-of-function mutation in the human PPAR-γ gene that is associated with severe insulin resistance. Moreover, a correlation between TZD potencies as antidiabetic agents in animal models and binding affinities to recombinant PPAR-γ (18,19) as well as binding affinities to differentiated 3T3-L1 cells and rat and human adipocytes has been observed (19,20). In addition, synthetic RXR agonists increase insulin sensitivity in obese mice and work in combination with TZDs to enhance antidiabetic activities (21).

White adipose tissue (WAT) seems to play an essential role for glucose homeostasis, because transgenic mice lacking WAT are severely diabetic (22,23) and WAT is required for the antidiabetic effect of TZDs (24). In concert, these observations have led to the suggestion that the antidiabetic actions of the TZDs result from their ability to bind to and activate PPAR-γ in adipose tissue. In contrast to these findings, it has been observed that heterozygous PPAR-γ–deficient mice are less susceptible to high-fat diet– or aging-induced insulin resistance than wild-type mice (15). In addition, the Pro12Ala polymorphism in human PPAR-γ2, which moderately reduces the transcriptional activity of PPAR-γ, has been shown to confer

From the [1]Department of Molecular Genetics, Novo Nordisk, Bagsvaerd, Denmark; the [2]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark; and [3]Research & Development, Amersham Pharmacia Biotech, Bucks, U.K.

W.E.H. is currently affiliated with Millennium Pharmaceuticals, Cambridge, Massachusetts.

Address correspondence and reprint requests to Jan Fleckner, Novo Nordisk A/S, Novo Allé 6B2.83, DK-2880 Bagsvaerd, Denmark. Email: jafl@novo.dk.

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Dex, dexamethasone; DTT, dithiothreitol; EST, express sequence tag; PPAR, peroxisome proliferator–activated receptor; RXR, retinoid X receptor; SSC, sodium chloride–sodium citrate; TGF, transforming growth factor; TZD, thiazolidinedione; WAT, white adipose tissue.

resistance to type 2 diabetes (25). This raises at least one unresolved issue: How can both PPAR-γ activation and moderate PPAR-γ reduction result in improved insulin resistance? Recent studies suggest that both states prevent adipocyte hypertrophy of existent adipocytes; heterozygous PPAR-γ deficiency prevents it by decreasing lipogenesis, whereas TZDs stimulate adipocyte differentiation and apoptosis (26). In line with these findings are several studies indicating that TZDs result in a relative increase of the number of smaller adipocytes (27,28).

To gain a detailed molecular understanding of the mechanism of action of TZDs, we have performed mRNA differential display analysis on differentiating 3T3-L1 cells and identified genes exhibiting altered expression after exposure to the potent TZD rosiglitazone. Furthermore, we have studied the importance of ligand-activated PPAR-γ during the differentiation process as well as the influence of ligand-inactivated PPAR-γ in mature adipocytes. Finally, we have analyzed the effect of rosiglitazone on the expression levels of the genes isolated by differential display in WAT, liver, and muscle of *ob/ob* mice.

RESEARCH DESIGN AND METHODS

Cell culture and differentiation assays. Murine 3T3-L1 cells were obtained from the American *Type Culture* Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Taastrup, Denmark), 100 units/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies). Cells were grown to confluence and induced to differentiate 2 days postconfluence by incubation with media containing either 1 μmol/l dexamethasone (Dex; Sigma, St. Louis, MO) and 10 μg/ml human insulin (Novo Nordisk, Bagsvaerd, Denmark), or 1 μmol/l Dex, 10 μg/ml human insulin, and 1 μmol/l rosiglitazone (Novo Nordisk). For antagonist experiments, cells were differentiated for 7 days with 1 μmol/l Dex, 10 μg/ml human insulin, and 1 μmol/l rosiglitazone, after which rosiglitazone was exchanged with 10 μmol/l LG100641 (Novo Nordisk) for an additional 2 days.

mRNA differential display. mRNA differential display was performed essentially as described (29) and (30) by the use of three different 1-bp-anchored 3′ oligo(dT) primers and 35 arbitrarily designed 5′ oligos.

Reverse Northern blot analysis

Conventional method. [α-^{32}P]dATP-labeled first-strand cDNAs were prepared by reverse transcription of RNA isolated from 3T3-L1 cells as well as adipose tissue, liver, and muscle tissue from *ob/ob* mice. Each reverse transcription reaction was performed by incubating 40 μg total RNA and 1.5 μg oligo-$dT_{12–18}$ at 70°C for 10 min in a volume of 20 μl, followed by quick cooling on ice and the addition of a reaction mixture containing 50 mmol/l Tris-HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol (DTT), 500 μmol/l dNTP (-dATP), 10 μl [α-^{32}P]dATP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and 800 units Superscript II reverse transcriptase (Life Technologies) in a final volume of 50 μl. The reaction was incubated for 2 h and 30 min at 37°C and finally chased with 500 μmol/l dATP and 200 units Superscript II reverse transcriptase for 1 h. A Nick-column (Pharmacia Biotech, Uppsala, Sweden) was used to separate incorporated from unincorporated radioactive nucleotides. Hybond-N^+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) were prepared for blotting, and DNA was fixed according to the manufacturer's instructions. Briefly, the membranes were cut to the size of the slot blot manifold (Schleicher & Schull, Keene, NH), wet with 5 × SSC, placed in the manifold, and clamped. Differentially regulated gene fragments were amplified by PCR using T7 and M13 reverse primers, and ~300 ng DNA was denatured for 15 min at room temperature in 0.4 N NaOH in a total volume of 200 μl, loaded into the manifold, and allowed to sit for 30 min before suction was applied. The wells were washed with 300 μl 5 × SSC, and the membranes were removed from the manifold and dried in air. The membranes were subsequently prehybridized at 65°C for 3 h in a solution containing 5 × SSC, 1% SDS, 10 × Denhardt's solution, 150 μg/ml poly(A) (Sigma), 50 μg/ml yeast RNA (Sigma), and 10 mmol/l EDTA, followed by overnight hybridization in the same solution with the addition of [α-^{32}P]dATP-labeled heat-denatured first-strand cDNA prepared from treated and untreated 3T3-L1 cells and from adipose tissue, liver, and muscle from treated and untreated *ob/ob* mice. The results were quantitated by a PhosphoImager (Molecular Dynamics), and signals resulting from hybridization to a vector-specific PCR product were subtracted.

DNA microarray analysis. All cDNA inserts from the differential display experiments were PCR-amplified using T7 and M13 reverse primers. PCR (100 μl each) were purified using a Qiagen 96-well PCR purification system. The purified PCR products were mixed 1:1 in DMSO (Sigma) and spotted in duplicate onto silanized glass slides (Amersham Pharmacia Biotech) using an Amersham Pharmacia Biotech Generation III microarray spotter. All slides were UV cross-linked (50 mJ) in a Stratagene UV cross-linker. Cy-3– and Cy-5–labeled first-strand cDNA probes were prepared from either 15 μg total RNA or 0.5 μg mRNA from treated and untreated 3T3-L1 cells. The RNA was incubated with 1 μg anchored oligo-dT primer at 70°C for 10 min and subsequently chilled on ice for 30 s. The primer-annealed RNA was incubated with reaction mixture (1 × Superscript II buffer; Life Technologies), 10 mmol/l DTT, 200 μmol/l dGAT(TP), 100 μmol/l dCTP, 100 μmol/l Cy-3/Cy-5–dCTP (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and 200 units Superscript II reverse transcriptase (Life Technologies) at 42°C for 2 h and 30 min in a final volume of 20 μl. The RNA template was removed by alkaline denaturation (incubation with 2 μl of 2.5 mol/l NaOH at 37°C for 15 min.). Labeled probes were purified on GFX spin columns (Amersham Pharmacia Biotech, Uppsala, Sweden). The Cy-3– and Cy-5–labeled cDNAs were mixed 1:1 in hybridization buffer (version 2; Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and subsequently 1:1 in formamide (Sigma). The mixed probes were injected into an automated slide processor (Amersham Parmacia Biotech) holding the spotted slide and hybridized at 42°C for 16 h. After hybridization, the slide was washed in 1 × sodium chloride–sodium citrate (SSC) and 0.2% SDS at 55°C for 10 min and then in 0.1 × SSC and 0.2% SDS at 55°C for 10 min. The slide was scanned in a Generation III microarray laser scanner (Molecular Dynamics). Image analyses were performed with ArrayVision (Imaging Research). All ratios were calculated using background-subtracted, artifact-removed median densities. The Cy-3– and Cy-5–signals were normalized to the ribosomal phosphoprotein 36B4.

Northern blot Analysis. Total RNA was isolated from 3T3-L1 cells using RNA-Zol following the vendor's instructions. 20 μg RNA was size-fractionated in a denaturing gel containing 1% agarose, 20 mmol/l MOPS, 5 mmol/l NaOAc, 6% formaldehyde, and 1 mmol/l EDTA, transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech) by capillary blotting, and immobilized by UV cross-linking. cDNAs from differential display–isolated fragments were labeled with a Prime It kit (Stratagene, Aarhus, Denmark) using [α-^{32}P]dATP (3,000 Ci/mmol; Amersham Pharmacia Biotech) and hybridized using Express Hyb (Clontech, Stockholm, Sweden) following the manufacturer's instructions, and results were visualized by autoradiography.

Quantitative PCR. Total RNA isolated from 3T3-L1 cells were DNase treated, and three reverse transcription reactions were performed using Superscript II reverse transcriptase (Gibco-BRL) following the manufacturer's instructions. mRNA expression levels were analyzed by real-time fluorescent detection using a Lightcycler (Roche) and the following primer combinations: 5′-CAGTCCCTCTTAACATGTAAGAC-3′ and 5′-TACAGGATATTCCTTTAGTGGTGG-3′ for the transducer of erbB-2; 5′-CCTGCCTCGCCCTTATCCAAAGC-3′ and 5′-TGGCTAGCTAGTTCAGACATATAC-3′ for drm; 5′-GACAGTCAGTGGTAGGCATGTGAC-3′ and 5′-CAATTCAAATAGTCCTTACCTCCC-3′ for novel mRNA from the rennin-expressing kidney tumor cell line; 5′-CGGTAGTGACCAGCAAGAGG-3′ and 5′-CAGTACAGAAGGAAAACCACC-3′ for the TIS11 primary response gene; and 5′-TGTCCCAAGGTGTACCCCAGC-3′ and 5′-GGAAGATAAAGGTAGCACTTT-3′ for Tax binding protein (TXBP181). Expression levels were normalized to the expression levels of 18S as described by the vendor (Applied Biosystems).

In vivo studies. *ob/ob* mice at the age of 10 weeks were divided into groups of six animals and dosed once daily for 16 days with rosiglitazone (100 mg · kg^{-1} · $dose^{-1}$) or vehicle (0.5% carboxymethylcellulose) by oral gavage. At the end of the dosing period, animals were killed by decapitation, and then liver, muscle, and WAT was removed and frozen in liquid nitrogen, and RNA was isolated by RNAZol (BioSite, Täby, Sweden) according to the manufacturer's instructions. RNA from all animals in each group was pooled. All animal experiments were conducted in accordance with Danish law.

RESULTS

Isolation of genes regulated by TZDs during differentiation of 3T3-L1 cells. To identify genes regulated by TZDs in developing adipocytes, we treated 3T3-L1 cells with Dex, insulin, and rosiglitazone and used mRNA differential display analysis to isolate differentially regulated cDNAs. The differential display analysis was performed using total RNA isolated from 3T3-L1 cells at three

TABLE 1
Differential display analysis of differentiating 3T3-L1 cells

Unique differentially expressed clones	132
With known function	81
With EST entries	34
Without EST entries	17

Data are *n*.

different time points: *1*) from cells 2 days postconfluence (referred to as day 0), *2*) from cells treated for 1 day, and *3*) from cells treated to differentiate for 7 days. As shown in Table 1, the differential display analysis resulted in the identification of 132 unique differentially expressed cDNAs. Of these 132 cDNAs, 81 were identical to sequences in public databases encoding proteins with known function, and 34 were homologous to express sequence tags (ESTs), whereas 17 had no significant homology to any known gene or EST available in the databases (Tables 1 and 2).

As shown in Table 2, the genes encoding proteins of known function could be divided into a variety of groups, including: *1*) proteins involved in synthesis, degradation, and transport of lipids; *2*) secreted proteins; *3*) proteins involved in cytoskeleton and extracellular structure; *4*) transcription factors and transcription repressors; *5*) proteins involved in cell growth control; *6*) RNA-interacting proteins; *7*) proteins involved in oxidative phosphorylation; *8*) proteins important for binding and metabolization of drugs and radicals; and *9*) proteins involved in amino acid metabolism. Several of these genes were expected to be regulated because the cells differentiate from fibroblasts to mature adipocytes, and several genes have previously been identified as differentially expressed during the adipocyte differentiation process (Table 2) (31–34), confirming the validity of our methods. Among the differentially regulated genes involved in lipid metabolism, we found genes whose products are required for the pyruvate/malate cycle (including tricarboxylate carrier and malate dehydrogenase), genes encoding proteins involved in the TCA cycle (e.g. citrate synthase and NAD^+-dependent isocitrate dehydrogenase subunits 3 and 4), and genes encoding proteins involved in conversion of fatty acids (e.g., steaoryl-CoA desaturase 1 and peroxisomal acyl-CoA oxidase as well as inorganic pyrophosphatase, involved in the hydrolysis of PP_i (inorganic pyrophosphate) formed by acyl-CoA synthetase). Furthermore, several genes that have been suggested to be involved in the control of the proliferative state of cells were downregulated, such as metastatic cell protein, drm (downregulated in mos-transformed cells), a 21-kDa peptide that is under translational control in tumor cells, hydrophobic nucleolar protein (HNP36), a large subunit of transforming growth factor (TGF)-β masking protein, gas 1 (growth-arrest specific gene 1), and a polypeptide having biological activity relating to proliferation and propagation of cells. This is, in most cases, well in agreement with previous findings indicating that differentiation of 3T3-L1 cells involves growth arrest (35) and that ligand-activated PPAR-γ is sufficient to induce growth arrest (36). However, the downregulation of the large subunit of TGF-β masking protein, which neutralizes the activity of TGF-β1, is surprising because TGF-β1 has previously been shown to inhibit adipocyte differentiation (37). Similarly, the downregulation of gas 1 is unexpected because this gene has previously been reported to be downregulated during the clonal expansion phase of 3T3-L1 cells (38). Interestingly, several of the genes encoded mitochondrial and peroxisomal proteins (see Table 2), organelles that among other things are responsible for fatty acid catabolism, suggesting that the differentiated 3T3-L1 cells have increased metabolic levels.

Taken together, these changes in gene expression suggest that differentiation of 3T3-L1 cells from fibroblast to mature adipocytes (as induced by treatment with Dex, insulin, and rosiglitazone) involves cessation of cell proliferation; cytoskeleton and extracellular structure changes; changes in the secondary structure of RNA; the capability to metabolize drugs and radicals; and an increased uptake, storage, and conversion of fatty acids.

Rosiglitazone regulates genes during adipocyte differentiation, whereas a PPAR-γ antagonist has no effect in fully differentiated adipocytes. Previous studies have shown that a considerable fraction of 3T3-L1 cells can differentiate into adipocytes in the presence of Dex and insulin alone (39). However, a remarkably more pronounced differentiation of the 3T3-L1 cells was obtained by the use of a differentiation mixture containing a combination of Dex, insulin, and rosiglitazone (data not shown). Being interested in TZD-regulated gene expression patterns, we sought to identify genes regulated specifically by rosiglitazone during the course of adipocyte differentiation. To do this, we omitted rosiglitazone from the mixture of differentiation reagents (day 7−), and using cDNA microarray technology, we compared the pattern of gene expression with that of cells treated with the full complement of compounds (day 7+). The cDNA microarray technique was based on immobilization of the isolated cDNAs on glass plates followed by hybridization with fluorescently labeled first-strand cDNAs synthesized from day 7− and day 7+ total RNA. In parallel experiments, we performed cDNA microarray analyses in which we analyzed the changes in gene expression in undifferentiated cells (day 0) and cells differentiated for 7 days with all compounds (day 7+). As shown in Tables 2 and 3, the differential expression of 50 of the 81 isolated cDNAs encoding proteins of known function could be verified to be regulated ≥2.5-fold during the differentiation process. For ~50% (21 of 50) of these cDNAs, the differential expression seemed to be caused or accelerated by the presence of rosiglitazone, whereas the up- or downregulation of the remaining cDNAs could be attributed to the treatment with Dex and insulin alone (Tables 2 and 3).

Another way of studying the importance of TZDs in adipocytes is the use of antagonists of their receptors. Therefore, we analyzed the effects of a full PPAR-γ antagonist, LG100641 (12), in mature adipocytes. 3T3-L1 cells were differentiated for 7 days with Dex, insulin, and rosiglitazone, after which rosiglitazone was exchanged with the PPAR-γ antagonist, and the cells were treated for an additional 2 days. RNA from these cells was isolated, and the pattern of gene expression was compared with that of cells treated for 9 days with Dex, insulin, and rosiglitazone. However, according to cDNA microarray analyses, no genes were induced or reduced more than 2.5-fold, indicating that the PPAR-γ antagonist had no

TABLE 2
Known genes isolated by differential display and corresponding reverse Northern blot analysis in vitro and in vivo

	3T3-L1 (d7+/d0)	3T3-L1 (d7+/d7−)	WAT (rosi+/rosi−)	Liver (rosi+/rosi−)	Muscle (rosi+/rosi−)	Putative function
aP2*†	++++	++	+	+	‡	Synthesis, degradation, and transport of lipids
Fatty acid transporter (FAT)*†	+ +	+	+	‡	‡	
Stearoyl-CoA desaturase 1 *†	++++	+	+	‡	‡	
Peroxisomal acyl-CoA oxidase*§	++++	+	+	‡	‡	
Lipoprotein lipase*†	+++	‡	+	‡	‡	
Malate dehydrogenase*§	+	‡	‡	‡	‡	
Medium-chain acyl-CoA dehydrogenase*§	‡	‡	+++	‡	‡	
FSP27*	+++	++	+	‡	++	Adipose tissue-related proteins of unknown function
adipoQ*	+++	‡	+	‡	‡	Secreted
PK-120 precursor (itih-4)	‡	‡	++	‡	‡	
Insulin-like growth factor binding protein*	++	‡	‡	‡	‡	
Microtubule-associated proteins 1A and 1B light-chain 3 subunit*	‡	‡	‡	‡	‡	Cytoskeleton and extracellular structure
Type VI collagen α3 chain*	+	‡	‡	‡	‡	
Integrin a6 subunit*	++	‡	‡	‡	‡	
β-actin*	‡	‡	‡	‡	‡	
α1 type 1 procollagen*	−−−−	−−	++	‡	+++	
PPARγ*	+	‡	‡	‡	‡	Transcription factors/transcription repressors
FOS-related antigen I (Fra-I)	‡	+	+	‡	+	
NF-E2 related transcription factor (NRFI)	+	‡	‡	‡	‡	
Transcriptional repressor MPc	‡	‡	‡	‡	‡	
Metastasis associated protein mta-1	‡	‡	‡	−	‡	Cell growth control
mts1 (metastatic cell protein)	−−−	−	+	‡	‡	
drm (down-regulated in mos-transformed cells)	‡	‡	‡	‡	‡	
21-kDa peptide that is under translational control in tumor cells	‡	‡	‡	‡	‡	
Hydrophobic nucleolar protein (HNP36)	‡	‡	‡	‡	+	
Large subunit of TFG-β masking protein	−	‡	+	‡	‡	
gas1*	−−	−	‡	‡	‡	
Polypeptide having biological activity relating to proliferation and propagation of cells	−−	‡	‡	‡	‡	
Ribosomal protein 23	‡	‡	‡	‡	‡	Ribosomal proteins
60S ribosomal protein L37	‡	‡	‡	‡	‡	
TNZ p68 RNA helicase*	‡	‡	‡	‡	‡	RNA interaction
G-rich sequence factor-1 (GRSF1)	+ +	‡	+	‡	‡	
NADH-ubiquinone oxidoreductase 9-kDa subunit precursor*§	‡	+	‡	‡	‡	Oxidative phosphorylation
NADH-ubiquinone oxidoreductase chain 4§	+	‡	+	‡	‡	
Cytochrome C oxidase polypeptide II§	+	‡	‡	‡	‡	
NADH-ubiquinone oxidoreductase chain 1§	+	‡	+	‡	‡	
Cytochrome C oxidase polypeptide VIB§	+	‡	+	‡	‡	
Cytochrome C oxidase polypeptide I*§	+	‡	‡	‡	‡	
Ubiquinol cytochrome C reductase§	+ +	‡	‡	‡	‡	
Cytochrome b§	+	‡	+−	‡	‡	
Cu-Zn-superoxide dismutase*	+	+	‡	‡	‡	Binding and metabolization of drugs and radicals
Phenol/aryl form of sulfotransferase*	‡	‡	‡	‡	‡	
Immunophilin FKBP51*	++	‡	+	‡	+	
Branched chain α-ketoacid dehydrogenase*†§	+	‡	‡	‡	+	Amino acid metabolism
Fumarylacetoacetate hydrolase*†	+	+	‡	‡	‡	
Adenine nucleotide translocase 2§	+ +	‡	+	‡	‡	
Tricarboxylate carrier*†§	‡	‡	+	‡	‡	
Heme-oxygenase-2	+++	‡	‡	‡	‡	
Protein phosphatase 2C	+	+	‡	‡	‡	
Citrate synthase†§	++	‡	‡	‡	‡	
Pyruvate dehydrogenase*§	+++	‡	+	‡	‡	

Continued on following page

TABLE 2
Continued

	3T3-L1 (d7+/d0)	3T3-L1 (d7+/d7−)	WAT (rosi+/rosi−)	Liver (rosi+/rosi−)	Muscle (rosi+/rosi−)	Putative function
Huntingtin interacting protein	+	‡	‡	‡	‡	
Ras like Rap 1B	‡	‡	+	‡	‡	
Inorganic pyrophosphatase*§	++++	++	‡	‡	‡	
Virus-like (VL30) retroelement	++++	++	+	‡	‡	
Glutamine synthetase*	++++	‡	‡	‡	‡	
Paraneoplastic cerebellar degeneration antigen cdr2	+	‡	+	‡	‡	
Serine-rich RNA polymerase suppressor protein-1	‡	‡	+	‡	‡	
Phosphomannomutase	++	+	++	‡	‡	
NAD+ dependent isocitrate dehydrogenase subunit 3 and 4§	++	+	+	‡	‡	
Mitochondrial encoded hydrophobic protein§	+	‡	++	‡	‡	
AMP deaminase isoform C	‡	‡	‡	‡	‡	
Mitochondrial E1B 19K/Bcl-2-binding protein homolog (Nip3)§	‡	‡	‡	‡	‡	
Protein-tyrosine kinase substrate p36	‡	‡	‡	‡	‡	
β-Catenin*	‡	‡	‡	‡	‡	
Transducer of ErbB-2 (*tob*)*	‡	‡	‡	‡	+	
Mitochondrial genes encoding three transfer RNAs§	+	+	+	‡	‡	
Aldose reductase	‡	‡	‡	‡	‡	
COP9 complex subunit4	‡	‡	‡	‡	‡	
ATP syntase oligomycin sensitive conferral protein precursor*§	+	+	‡	‡	+	
Adjoining tRNA spec for Pro and Phe mitochondrial D-loop region§	++	++	+	‡	‡	
Mitochondrial D-loop region§	+++	+	++	‡	‡	
PAT-12 product	‡	‡	−	‡	‡	
Tissue inhibitor (type2) of metalloproteinases	−−−−	‡	‡	‡	‡	
Osteoblast-specific factor 2*	‡	‡	+	‡	‡	
tax 1 binding protein TXBP181	−−	‡	+	‡	‡	
Novel mRNA from renin-expressing kidney tumor cell line (clone 4.9)	‡	‡	+	‡	‡	
Pentylenetetrazol-related cDNA PTZ-17*	−	‡	+	‡	+	
Putative steroid membrane binding protein	‡	‡	‡	‡	‡	
Heterochromatin protein M31	‡	‡	‡	‡	‡	
TIS11 primary response gene	‡	‡	‡	‡	‡	

Genes found by differential display analysis in 3T3-L1 cells to be upregulated and downregulated are indicated in red and blue, respectively. (+)(−), induced or reduced 2.5- to 5-fold; (++) (−−), induced or reduced 5- to 10-fold; (+++) (−−−), induced or reduced 10- to 20-fold; (++++) (−−−−), induced or reduced >20-fold in reverse Northern blot analysis, d7+/d0, 3T3-L1 cells differentiated 7 days with Dex, insulin, and rosiglitazone compared with cells 2 days postconfluence; d7+/d7−, 3T3-L1 cells differentiated 7 days with Dex, insulin, and rosiglitazone compared with cells differentiated for 7 days with Dex and insulin. *Genes previously shown to be differentially regulated during adipocyte differentiation in vitro; †genes previously shown to be differentially regulated by TZDs in vivo; and ‡genes not regulated or below detection level in reverse Northern blot analysis; §mitochondrial or peroxisomal genes.

effect on the gene expression profiles in fully differentiated 3T3-L1 cells. Northern blot analyses of selected genes that were specifically regulated by rosiglitazone (such as *fos*-related antigen 1, inorganic pyrophosphatase, and VL30) showed that *fos*-related antigen 1 was unaffected by LG100641, whereas inorganic pyrophosphatase and VL30 were only very weakly downregulated, by 1.5-fold each (data not shown).

Verification of differential display and reverse Northern analyses. As described above, we used cDNA microarray technology to verify the results obtained by differential display as well as to examine the changes in gene expression caused either by rosiglitazone during the differentiation process or by a PPAR-γ antagonist in fully differentiated 3T3-L1 cells. To further verify the differential expression of the transcripts, we also used another technique, the so-called conventional reverse Northern blot technique. As described in RESEARCH DESIGN AND METHODS, this technique was based on immobilization of all cDNAs isolated by differential display on nylon membranes. Subsequently, [α-^{32}P]dATP-labeled first-strand cDNAs from 3T3-L1 cells 2 days postconfluence (day 0) and from cells differentiated for 7 days with Dex, insulin, and rosiglitazone (day 7+) were synthesized and used as probes in a reverse Northern blot hybridization. Comparison of the conventional reverse Northern blot technique and the cDNA microarray technique revealed that the two methods resulted in verification of differential regulation

TABLE 3
Regulation of differential display isolated transcripts encoding proteins of known function in vitro and in vivo

	Fold regulation								
	2.5–5 ×		5–10 ×		10–20 ×		>20 ×		
	Up	Down	Up	Down	Up	Down	Up	Down	Total
3T3-L1 cells									
7+/0	19	2	11	3	6	1	6	2	50
7+/7−	13	2	5	1	—	—	—	—	21
AT	29	1	6	—	1	—	—	—	37
Liver	1	1	—	—	—	—	—	—	2
Muscle	6	—	2	—	1	—	—	—	9

of almost identical cDNAs, suggesting that these methods can be used equally well. Figure 1*A* and *B* shows the differential regulation of selected genes such as aP2, adenine nucleotide translocase, metastatic cell protein, and FSP27 according to the conventional reverse Northern blot analysis and DNA microarray analysis, respectively.

To further confirm the results obtained by differential display, reverse Northern blot, and microarray analyses, we performed Northern blot analyses of 13 selected clones (Fig. 2*A*). Genes such as *fos*-related antigen 1, inorganic pyrophosphatase, VL30, and Cu-Zn-superoxide dismutase, in agreement with reverse Northern analyses, required the presence of all three components of the differentiation cocktail to be upregulated, whereas G-rich sequence factor 1, cytochrome C oxidase polypeptide II, adenine nucleotide translocase 2, immunophilin FKBP51, and NADH-ubiquinone oxidoreductase chain 4 were upregulated by Dex and insulin alone (Table 2, column 2). Among the downregulated genes, metastatic cell protein, in agreement with the results obtained by reverse Northern blot, was downregulated by Dex and insulin alone, whereas an increased downregulation was observed in the presence of all components. Two other downregulated genes,

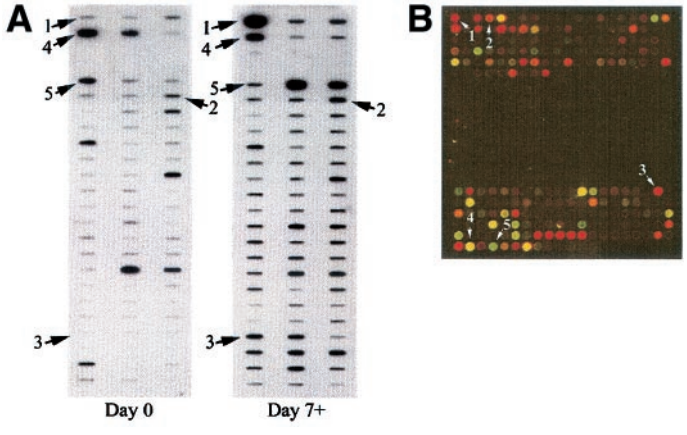

FIG. 1. Reverse Northern blot analysis of selected genes isolated by differential display analysis. *A*: ^{32}P-labeled first-strand cDNAs were prepared by reverse transcription of RNA isolated from 3T3-L1 cells 2 days postconfluence (day 0) and from cells treated for 7 days with Dex, insulin, and rosiglitazone (day 7+) and hybridized to slot-blotted cDNAs of genes isolated by differential display. *B*: RNA from day 0 and day 7+ cells were used to prepare cDNA labeled with Cy3-dCTP and Cy-5-dCTP, respectively. The two cDNA probes were mixed and simultaneously hybridized to the DNA microarray containing spotted cDNAs of genes isolated by differential display. The image of the subsequent scan shows upregulated genes as red spots, downregulated genes as green spots, and nondifferentially expressed genes as yellow spots. The arrows indicate bands and spots representing the following genes: 1, aP2; 2, adenine nucleotide translocase; 3, FSP27; 4, 36B4; and 5, metastatic cell protein.

pentylenetetrazole-related cDNA PTZ-17 and a polypeptide having biological activity relating to the proliferation and propagation of cells, were correctly found to be downregulated by treatment with Dex, insulin, and rosiglitazone. However, in contrast to the results obtained by reverse Northern blot, suggesting no effect of rosiglitazone, Northern blot analyses showed that Dex and insulin alone did reduce the expression of these genes, but that maximal downregulation was only observed by the presence of rosiglitazone. Besides the Northern blot analyses of these 13 genes, we performed real-time PCR analyses to quantify the expression of five genes: transducer of Erb-2, novel mRNA from rennin-expressing kidney tumor cell line, drm (downregulated in mos-transformed cells), TIS11 primary response gene, and tax binding protein (TXBP181), which were all found not to be regulated or to be below the detection levels of reverse Northern blot and microarray analyses (Fig. 2*B*). All of these selected genes were up- or downregulated in agreement with the data obtained by differential display analyses. When combined, the Northern blot and real-time PCR analyses of 18 genes show that all genes were up- or downregulated as predicted by differential display, indicating that our differential display analyses resulted in the identification of genes that are truly differentially expressed during adipocyte differentiation.

TZDs regulate gene expression in vivo. A variety of experiments have shown that differentiation of 3T3-L1 preadipocytes faithfully mimics the process of adipocyte differentiation in vivo, resulting in cells that possess many biochemical and morphologic characteristics of adipocytes (40,41). However, the 3T3-L1 model system is limited by the fact that it lacks the endocrine and paracrine interactions between the different cell types found in animals. Therefore, we decided to study the effect of rosiglitazone on adipose tissue in vivo. To test whether cDNAs isolated by differential display from 3T3-L1 cells were also differentially regulated in vivo, we used the conventional reverse Northern blot technique to compare the gene expression of adipose tissue from vehicle-fed *ob/ob* mice with that of *ob/ob* mice treated with rosiglitazone. Tables 2 and 3 show that many of the cloned cDNAs that were regulated in 3T3-L1 cells were also regulated in adipose tissue of rosiglitazone-treated animals. In total, 37 of the 81 isolated genes encoding proteins of known function were confirmed by reverse Northern blot analysis to be regulated in adipose tissue of animals treated with rosiglitazone. Among the genes of known function that were verified to be differentially regulated by reverse Northern blot were genes encoding proteins involved in

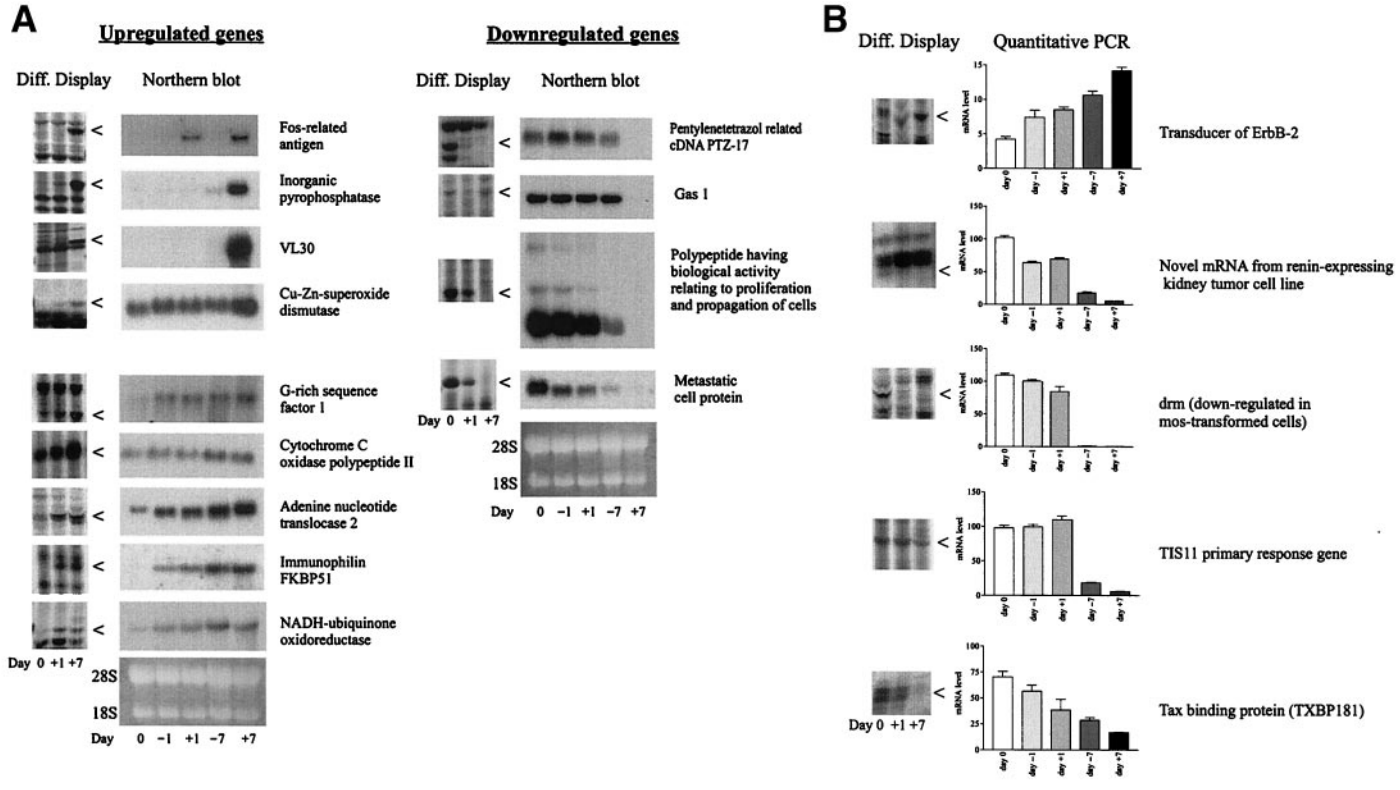

FIG. 2. Differential expression profiles of selected differential display clones. *A*: Differential display (Diff. Display) analysis of 3T3-L1 cells induced to differentiate with Dex, insulin, and rosiglitazone and corresponding Northern blot analysis of 3T3-L1 cells treated with a combination of Dex and insulin (−) or treated with a combination of Dex, insulin, and rosiglitazone (+). *B*: Differential display analysis of 3T3-L1 cells induced to differentiated as described in *A* and corresponding quantitative PCR analyses of 3T3-L1 cells differentiated as described in *A*. Data are arbitrary numbers from quantitative PCR using real-time fluorescent detection of mRNA levels and are presented as the means ± SE ($n = 3$, $P = 0.05$).

the uptake and transport of fatty acids, such as fatty acid transporter, lipoprotein lipase, and aP2 as well as several genes encoding proteins involved in the conversion of fatty acids and metabolites within the adipocytes, such as stearoyl-CoA desaturase 1, peroxisomal acyl-CoA oxidase, and medium-chain acyl-CoA dehydrogenase. Interestingly, several of the regulated proteins were mitochondrial and peroxisomal (Table 2), which is well in accordance with a recent report illustrating rosiglitazone-induced numbers of mitochondria per cell in WAT of Sprague Dawley rats (42).

Furthermore, we tested whether any of these genes were regulated by the rosiglitazone treatment in the liver and muscle tissues. In contrast to the results obtained from adipose tissue, only very few genes were regulated in the liver, whereas slightly more genes were regulated in the muscle tissues of the animals treated with the insulin sensitizer (Tables 2 and 3). Taken together, these findings show that rosiglitazone induces many common genes in the adipose tissue of *ob/ob* mice and 3T3-L1 cells induced to differentiate with Dex, insulin, and rosiglitazone, whereas only minor effects of rosiglitazone were detected on these genes in muscle and liver.

DISCUSSION

To gain a detailed molecular understanding of the mechanism of action of the TZD class of insulin sensitizers in the process of adipocyte differentiation, we identified genes exhibiting altered expression during the differentiation process driven by the potent TZD rosiglitazone. The model system used for these studies was 3T3-L1 cells, which are mouse embryo fibroblasts that by treatment with Dex, insulin, and TZDs can differentiate into adipocytes. To detect and identify genes that were regulated during differentiation, we chose to use mRNA differential display as a relatively unbiased random approach that does not depend on existing genetic data. In total, we isolated 132 unique cDNAs, of which 81 were identical to genes encoding proteins of known function. Recent studies have shown that an abundance of >1,200 genes is changed during the differentiation process of 3T3-L1 cells (31,32,34). Comparison of our list of genes encoding proteins of known function (81 genes) with previously identified genes revealed an overlap of 33 genes, strongly suggesting that additional studies have to be conducted to obtain a comprehensive list of genes that are differentially expressed during adipocyte differentiation.

Many novel differentially expressed genes have been isolated in our experiments, most of which were confirmed by other technologies to be truly differentially regulated transcripts. These conclusions were based on the results obtained by two different methods of reverse Northern blot, conventional analysis using ^{32}P-labeled cDNAs as probes, and the more recently developed method of DNA microarray analysis. By the use of these techniques, 50 of 81 clones encoding proteins of known function were verified to be differentially regulated during the differentiation of 3T3-L1 fibroblasts into adipocytes. Although the remaining 31 clones may potentially be false positives, this is not considered likely when taking the relative sensitivity of the applied methods into account.

Much of a cell's mRNA mass consists of many copies of a small number of genes, and 96% of transcripts are estimated to be expressed at <50 copies per cell (43). Consistent with this, five clones for which the differential regulation could not be confirmed by reverse Northern blot techniques turned out to be truly regulated clones when analyzed by real-time PCR analyses. Several of the remaining 26 clones are therefore likely low-abundance messages that are true positives but simply not detectable above background in the cDNA microarray and reverse Northern blot assays. Because mRNA differential display analysis is inherently a very sensitive technique allowing for the detection of genes expressed at highly varying levels, it is expected (and desired) to identify some transcripts that cannot be found or analyzed for using Northern technologies. Therefore, this study is likely to have uncovered novel regulated transcripts that would be hard to find using, for example, DNA chip/microarray approaches.

The differentiation protocol involving the use of a combination of Dex, insulin, and rosiglitazone resulted in the identification of genes specifically regulated or enhanced/reduced by TZDs, but it also identified genes regulated by Dex and insulin alone. To identify genes exhibiting altered expression specifically caused by rosiglitazone, we compared gene expression levels in 3T3-L1 cells differentiated using a combination of Dex and insulin alone with cells differentiated using all three components. Microarray analyses revealed that the differential expression of ~50% of the genes of known function (21 of 50 reverse Northern–verified genes) was caused by or accelerated by rosiglitazone. In contrast to these findings, during the process of differentiation, the PPAR-γ antagonist LG100641 (12) had no or very weak effects on the pattern of gene expression in fully differentiated 3T3-L1 cells. These observations are in good agreement with a recent report using another PPAR-γ antagonist, which had no effect on the morphology and basal lipolysis status in mature adipocytes (11). Although it has been observed that TZDs can downregulate PPAR-γ itself (44), we did observe a high expression of PPAR-γ in fully differentiated 3T3-L1 cells (data not shown), suggesting a potential for effects of a full antagonist. To our knowledge, the data presented here are the first illustrating the lack of effect of a PPAR-γ antagonist as detected in a large number of genes in fully differentiated adipocytes. Another study has demonstrated a reduced expression of selected proteins such as fatty acid translocase in WAT of high fat–fed KKA_y mice in response to other PPAR-γ antagonists (26), and these antagonists were suggested to prevent adipocyte hypertrophy. The reasons for differences between our results and those of Yamauchi et al. (26) are at present not clear, although it is tempting to speculate that endocrine and paracrine factors present in the in vivo settings (e.g., leptin) are playing an important role.

Interestingly, the differential regulation of the genes found by differential display analysis of differentiating 3T3-L1 cells was shown in many cases to also occur in WAT of *ob/ob* mice treated with rosiglitazone (37 of 81 genes encoding proteins of known function). This is well in agreement with recent reports demonstrating TZD-induced differential regulation of a variety of genes in WAT of other diabetic animal models, such as Zucker diabetic fatty rats and *db/db* mice (28,45–47). However, comparison of our 37 reverse Northern–verified genes with these genes revealed an overlap of only 7 genes. Our results therefore add a significant number of novel genes, which are transcriptionally regulated by TZDs in WAT. The differential expressions of genes in the adipose tissue of *ob/ob* mice is likely to stem from differentiation of preexisting preadipocytes within the adipose tissue or occur in mature adipocytes or both. The pronounced overlap of genes differentially regulated in vivo and in vitro suggests that rosiglitazone induces in vivo adipocyte differentiation in *ob/ob* mice. This is consistent with previous reports demonstrating that other insulin sensitizers, such as pioglitazone and troglitazone, increase the number of small-sized adipocytes and stimulate the expression of genes involved in the lipid metabolism of adipocytes in obese Zucker rats (27,28). Combined with the lacking effect of the PPAR-γ antagonist in fully differentiated adipocytes in vitro, our data therefore suggest that the beneficial effect of TZDs in vivo may be caused by an increased differentiation of preadipocytes within the adipose tissue. It is important to note, though, that the present analyses are based on a fraction of genes, and the existence of other genes (such as CAP) (48) specifically regulated by TZDs in mature adipocytes cannot be excluded to play important roles.

A few discrepancies were observed between the results obtained by the use of 3T3-L1 cells and the use of adipose tissue from *ob/ob* mice. First, some genes were verified as differentially regulated by reverse Northern blot analysis in vivo but not in vitro (e.g., medium-chain acyl-CoA dehydrogenase, *fos*-related antigen 1, tricarboxylate carrier, *ras* like *rap* 1B, serine-rich RNA polymerase suppressor protein-1, PK-120 precursor [itih-4], and osteoblast-specific factor 2). One explanation for these findings could be a combination of different expression levels in vitro and in vivo and the limitations of the reverse Northern blot technique. Previous studies have shown that some genes are expressed at high levels in vivo and are absent or expressed at lower levels in vitro (31). Second, a few genes seemed to be regulated in one direction in 3T3-L1 cells and regulated in the opposite direction in the adipose tissue of rosiglitazone-treated *ob/ob* mice. α1 Type I procollagen, metastatic cell protein, a large subunit of TGF-β masking protein, osteoblast-specific factor 2, tax binding protein TXB181, novel mRNA from the rennin-expressing kidney tumor cell line, and pentylenetetrazole-related cDNA PTZ-17 were all found to be downregulated in 3T3-L1 cells during the differentiation process but upregulated in the adipose tissue of rosiglitazone-treated animals. There is no evident explanation for these differences, but, speculatively, different requirements may exist for the formation of cytoskeleton and extracellular structure during the process of adipocyte differentiation in vitro compared with the in vivo situation, explaining the opposite regulation of some genes such as the α1 type I procollagen. The increased expression in vivo of the large subunit of TGF-b masking protein, which neutralizes the activity of TGF-β1, is well in agreement with the expected reduced proliferation of cells undergoing differentiation. The significance of the surprisingly reduced expression of the large subunit

of TGF-β masking protein in vitro remains to be elucidated.

Moreover, our results show that rosiglitazone has considerably larger effects on the expression of the selected genes in adipocytes compared with liver and muscle tissues. This is well in agreement with the low expression in these tissues of PPAR-γ, which is considered to be the receptor for the TZDs. It should be noted, though, that our analysis is biased toward adipose-specific genes and as such may not reflect the full picture of PPAR-γ agonist action. In the liver, we only detected upregulation of two transcripts encoding proteins of known function, one of which was identified as aP2, perhaps caused by a minor contamination of the liver samples with macrophages. We do not expect interference with PPAR-α because no induced expression of fatty acid transporter was seen in the liver, which has previously been demonstrated to be induced by PPAR-α activators (6). In the muscle tissue, we identified a few extra differentially expressed genes. A previous nonbiased analysis has shown that another TZD, GW1929, in muscle tissue of Zucker diabetic fatty rats decreases the expression of pyruvate dehydrogenase 4 as well as other genes involved in fatty acid transport (45). Combining these results, it is therefore tempting to speculate that the observed effects of TZDs might be a pleiotropic response. First, it is plausible that it involves differentiation of novel active adipocytes in the adipose tissue, resulting in increased uptake and conversion of fatty acids by the adipose tissue with a concomitant reduction of the level of free fatty acids in the plasma, thereby—in accordance with the Randle cycle (49)—leading to increased insulin sensitivity in other peripheral tissues, such as liver and muscle. Second, it is possible that TZDs induce/reduce specific responsive genes in all tissues involved in glucose metabolism.

In summary, the differential display analysis of 3T3-L1 cells differentiating to adipocytes by the treatment with Dex, insulin, and the potent insulin sensitizer rosiglitazone has resulted in the identification of a variety of novel genes that are differentially regulated during the course of TZD-induced differentiation. The expression levels of several genes were specifically regulated or enhanced/reduced by rosiglitazone during the course of differentiation, whereas a specific PPAR-γ antagonist had no effect in terminally differentiated adipocytes in vitro. Furthermore, most of the identified genes were confirmed to be regulated in adipocytes of *ob/ob* mice treated with rosiglitazone, and our results therefore support the notion, on the molecular level, that TZDs activate PPAR-γ in the adipose tissue, resulting in differentiation of preexisting preadipocytes found among the mature adipocytes in adipose tissue.

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REFERENCES

1. Fujiwara T, Yoshioka S, Yoshioka T, Ushiyama I, Horikoshi H: Characterization of new oral antidiabetic agent CS-045: studies in KK and *ob/ob* mice and Zucker fatty rats. *Diabetes* 37:1549–1558, 1988
2. Kumar S, Boulton AJM, Beck-Nielsen H, Berthezene F, Muggeo M, Persson B, Spinas GA, Donoghue S, Lettis S, Stewartlong P: Troglitazone, an insulin action enhancer, improves metabolic control in NIDDM patients. *Diabetologia* 39:701–709, 1996
3. Lehmann JM, Moore LB, Smitholiver TA, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high-affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR-gamma). *J Biol Chem* 270:12953–12956, 1995
4. Schoonjans K, Peinado-Onsurbe J, Lefebvre AM, Heyman RA, Briggs M, Deeb S, Staels B, Auwerx J: PPARalpha and PPARgamma activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *Embo J* 15:5336–5348, 1996
5. Tontonoz P, Hu ED, Devine J, Beale EG, Spiegelman BM: PPAR-gamma-2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol Cell B* 15:351–357, 1995
6. Motojima K, Passilly P, Peters JM, Gonzalez FJ, Latruffe N: Expression of putative fatty-acid transporter genes are regulated by peroxisome proliferator-activated receptor-alpha and receptor-gamma activators in a tissue- and inducer-specific manner. *J Biol Chem* 273:16710–16714, 1998
7. Sears IB, MacGinnitie MA, Kovacs LG, Graves RA: Differentiation-dependent expression of the brown adipocyte uncoupling protein gene: regulation by peroxisome proliferator-activated receptor gamma. *Mol Cell B* 16:3410–3419, 1996
8. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM: mPPAR-gamma-2- tissue-specific regulator of an adipocyte enhancer. *Genes Dev* 8:1224–1234, 1994
9. Tontonoz P, Hu ED, Spiegelman BM: Stimulation of adipogenesis in fibroblasts by PPAR-gamma-2, a lipid-activated transcription factor. *Cell* 79:1147–1156, 1994
10. Oberfield JL, Collins JL, Holmes CP, Goreham DM, Cooper JP, Cobb JE, Lenhard JM, Hull-Ryde EA, Mohr CP, Blanchard SG, Parks DJ, Moore LB, Lehmann JM, Plunket K, Miller AB, Milburn MV, Kliewer SA, Willson TM: A peroxisome proliferator-activated receptor gamma ligand inhibits adipocyte differentiation. *Proc Natl Acad Sci U S A* 96:6102–6106, 1999
11. Camp HS, Chaudhry A, Leff T: A novel potent antagonist of peroxisome proliferator-activated receptor gamma blocks adipocyte differentiation but does not revert the phenotype of terminally differentiated adipocytes. *Endocrinology* 142:3207–3213, 2001
12. Mukherjee R, Hoener PA, Jow L, Bilakovics J, Klausing K, Mais DE, Faulkner A, Croston GE, Paterniti JR: A selective peroxisome proliferator-activated receptor-gamma (PPAR gamma) modulator blocks adipocyte differentiation but stimulates glucose uptake in 3T3–L1 adipocytes. *Mol Endocri* 14:1425–1433, 2000
13. Wright HM, Clish CB, Mikami T, Hauser S, Yanagi K, Hiramatsu R, Serhan CN, Spiegelman BM: A synthetic antagonist for the peroxisome proliferator-activated receptor gamma inhibits adipocyte differentiation. *J Biol Chem* 275:1873–1877, 2000
14. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P, Chien KR, Koder A, Evans RM: PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell* 4:585–595, 1999
15. Kubota N, Terauchi Y, Miki H, Tamemoto H, Yamauchi T, Komeda K, Satoh S, Nakano R, Ishii C, Sugiyama T, Eto K, Tsubamoto Y, Okuno A, Murakami K, Sekihara H, Hasegawa G, Naito M, Toyoshima Y, Tanaka S, Shiota K, Kitamura T, Fujita T, Ezaki O, Aizawa S, Kadowaki T: PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* 4:597–609, 1999
16. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM: PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* 4:611–617, 1999
17. Barroso I, Gurnell M, Crowley VE, Agostini M, Schwabe JW, Soos MA, Maslen GL, Williams TD, Lewis H, Schafer AJ, Chatterjee VK, O'Rahilly S: Dominant negative mutations in human PPARgamma associated with severe insulin resistance, diabetes mellitus and hypertension. *Nature* 402:880–883, 1999
18. Berger J, Bailey P, Biswas C, Cullinan CA, Doebber TW, Hayes NS, Saperstein R, Smith RG, Leibowitz MD: Thiazolidinediones produce a conformational change in peroxisomal proliferator-activated receptor-gamma: binding and activation correlate with antidiabetic actions in *db/db* mice. *Endocrinology* 137:4189–4195, 1996
19. Willson TM, Cobb JE, Cowan DJ, Wiethe RW, Correa ID, Prakash SR, Beck KD, Moore LB, Kliewer SA, Lehmann JM: The structure-activity relationship between peroxisome proliferator-activated receptor-gamma agonism

and the antihyperglycemic activity of thiazolidinediones. *J Med Chem* 39:665–668, 1996
20. Young PW, Buckle DR, Cantello BC, Chapman H, Clapham JC, Coyle PJ, Haigh D, Hindley RM, Holder JC, Kallender H, Latter AJ, Lawrie KWM, Mossakowska D, Murphy GJ, Roxbee-Cox L, Smith SA: Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radioiodinated ligand for peroxisomal proliferator-activated receptor gamma. *J Pharmacol Exp Ther* 284:751–759, 1998
21. Mukherjee R, Davies PJA, Crombie DL, Bischoff ED, Cesario RM, Jow L, Hamann LG, Boehm MF, Mondon CE, Nadzan AM, Paterniti JR, Heyman RA: Sensitization of diabetic and obese mice to insulin by retinoid-x-receptor agonists. *Nature* 386:407–410, 1997
22. Moitra J, Mason MM, Olive M, Krylov D, Gavrilova O, Marcus-Samuels B, Feigenbaum L, Lee E, Aoyama T, Eckhaus M, Reitman ML, Vinson C: Life without white fat: a transgenic mouse. *Genes Dev* 12:3168–3181, 1998
23. Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL, Brown MS: Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes Dev* 12:3182–3194, 1998
24. Chao L, Marcus-Samuels B, Mason MM, Moitra J, Vinson C, Arioglu E, Gavrilova O, Reitman ML: Adipose tissue is required for the antidiabetic, but not for the hypolipidemic, effect of thiazolidinediones. *J Clin Invest* 106:1221–1228, 2000
25. Deeb SS, Fajas L, Nemoto M, Pihlajamaki J, Mykkanen L, Kuusisto J, Laakso M, Fujimoto W, Auwerx J: A Pro12Ala substitution in PPAR gamma 2 associated with decreased receptor activity, lower body mass index and improved insulin sensitivity. *Nat Genet* 20:284–287, 1998
26. Yamauchi T, Waki H, Kamon J, Murakami K, Motojima K, Komeda K, Miki H, Kubota N, Terauchi Y, Tsuchida A, Tsuboyama-Kasaoka N, Yamauchi N, Ide T, Hori W, Kato S, Fukayama M, Akanuma Y, Ezaki O, Itai A, Nagai R, Kimura S, Tobe K, Kagechika H, Shudo K, Kadowaki T: Inhibition of RXR and PPAR gamma ameliorates diet-induced obesity and type 2 diabetes. *J Clin Invest* 108:1001–1013, 2001
27. Hallakou S, Doare L, Foufelle F, Kergoat M, Guerre-Millo M, Berthault MF, Dugail I, Morin J, Auwerx J, Ferre P: Pioglitazone induces in vivo adipocyte differentiation in the obese Zucker fa/fa rat. *Diabetes* 46:1393–1399, 1997
28. de Souza CJ, Eckhardt M, Gagen K, Dong M, Chen W, Laurent D, Burkey BF: Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. *Diabetes* 50:1863–1871, 2001
29. Liang P, Pardee AB: Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257:967–971, 1992
30. Albrektsen T, Richter HE, Clausen JT, Fleckner J: Identification of a novel integral plasma membrane protein induced during adipocyte differentiation. *Biochem J* 359:393–402, 2001
31. Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM: Distinct transcriptional profiles of adipogenesis *in vivo* and *in vitro*. *J Biol Chem* 276:34167–34174, 2001
32. Guo X, Liao K: Analysis of gene expression profile during 3T3–L1 preadipocyte differentiation. *Gene* 251:45–53, 2000
33. Zhou LB, Halvorsen YD, Cryan EV, Pelton PD, Burris TP, Demarest KT: Analysis of the pattern of gene expression during human adipogenesis by DNA microarray. *Biotechnol Tech* 13:513–517, 1999
34. Imagawa M, Tsuchiya T, Nishihara T: Identification of inducible genes at the early stage of adipocyte differentiation of 3T3–L1 cells. *Biochem Biophys Res Commun* 254:299–305, 1999
35. Scott RE, Hoerl BJ, Wille JJ, Florine DL, Krawisz R, Yun K: Coupling of proadipocyte growth arrest and differentiation. II. A cell cycle model for the physiological control of cell proliferation. *J Cell Biol* 94:400–405, 1982
36. Altiok S, Xu M, Spiegelman BM: PPARgamma induces cell cycle withdrawal: inhibition of E2F/DP DNA-binding activity via down-regulation of PP2A. *Genes Dev* 11:1987–1998, 1997
37. Sparks RL, Allen BJ, Strauss EE: TGF-beta blocks early but not late differentiation-specific gene expression and morphologic differentiation of 3T3 T proadipocytes. *J Cell Physiol* 150:568–577, 1992
38. Shugart EC, Levenson AS, Constance CM, Umek RM: Differential expression of gas and gadd genes at distinct growth arrest points during adipocyte development. *Cell Growth* 6:1541–1547, 1995
39. Mandrup S, Sorensen RV, Helledie T, Nohr J, Baldursson T, Gram C, Knudsen J, Kristiansen K: Inhibition of 3T3–L1 adipocyte differentiation by expression of acyl-coa-binding protein antisense RNA. *J Biol Chem* 273:23897–23903, 1998
40. Green H, Kehinde O: Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J Cell Physiol* 101:169–171, 1979
41. Novikoff AB, Novikoff PM, Rosen OM, Rubin CS: Organelle relationships in cultured 3T3–L1 preadipocytes. *J Cell Biol* 87:180–196, 1980
42. Toseland CDN, Campbell S, Francis I, Bugelski PJ, Mehdi N: Comparison of adipose tissue changes following administration of rosiglitazone in the dog and rat. *Diabetes Obes Metab* 3:163–170, 2001
43. Lillie J: Probing the genome for new drugs and targets with DNA arrays. *Drug Dev R* 41:160–172, 1997
44. Camp HS, Whitton AL, Tafuri SR: PPAR gamma activators down-regulate the expression of PPAR gamma in 3T3–L1 adipocytes. *FEBS Lett* 447:186–190, 1999
45. Way JM, Harrington WW, Brown KK, Gottschalk WK, Sundseth SS, Mansfield TA, Ramachandran RK, Willson TM, Kliewer SA: Comprehensive messenger ribonucleic acid profiling reveals that peroxisome proliferator-activated receptor gamma activation has coordinate effects on gene expression in multiple insulin-sensitive tissues. *Endocrinology* 142:1269–1277, 2001
46. Ahuja HS, Liu S, Crombie DL, Boehm M, Leibowitz MD, Heyman RA, Depre C, Nagy L, Tontonoz P, Davies PJA: Differential effects of rexinoids and thiazolidinediones on metabolic gene expression in diabetic rodents. *Mol Pharmacol* 59:765–773, 2001
47. Suzuki A, Yasuno T, Kojo H, Hirosumi J, Mutoh S, Notsu Y: Alteration in expression profiles of a series of diabetes-related genes in *db/db* mice following treatment with thiazolidinediones. *Jpn J Pharmacol* 84:113–123, 2000
48. Ribon V, Printen JA, Hoffman NG, Kay BK, Saltiel AR: A novel, multifunctional c-cbl binding-protein in insulin-receptor signaling in 3T3–L1 adipocytes. *Mol Cell B* 18:872–879, 1998
49. Randle PJ: Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14:263–283, 1998