

Differential Gene Regulation by PPAR γ Agonist and Constitutively Active PPAR γ 2

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The PPAR γ is a key adipogenic determination factor. Ligands for PPAR γ such as antidiabetic thiazolidinedione (TZD) compounds are adipogenic, and many adipocyte genes that are activated by TZDs contain binding sites for PPAR γ . Like ligands for other nuclear receptors, TZDs can regulate genes positively or negatively. Here, we sought to understand the importance of positive regulation of gene expression by PPAR γ in adipogenesis. Fusion of the potent viral transcriptional activator VP16 to PPAR γ 2 (VP16-PPAR γ) created a transcription factor that constitutively and dramatically activated transcription of PPAR γ -responsive genes in the absence of ligand. Forced expression of VP16-PPAR γ in 3T3-L1 preadipocytes using retroviral vectors led to adipogenesis in the absence of standard differentiating medium or any exogenous PPAR γ ligand. Gene

microarray analysis revealed that VP16-PPAR γ induced many of the genes associated with adipogenesis and adipocyte function. Thus, direct up-regulation of gene expression by PPAR γ is sufficient for adipogenesis. TZD-induced adipogenesis up-regulated many of the same genes, although some were divergently regulated, including resistin, whose gene expression was reduced in VP16-PPAR γ adipocytes treated with TZDs. These results show that, although activation of PPAR γ by a heterologous activation domain is sufficient for adipogenesis, it is not equivalent to TZD treatment. This conclusion has important implications for understanding biological effects of the TZDs on adipogenesis and insulin sensitization. (*Molecular Endocrinology* 16: 1040–1048, 2002)

THE NUCLEAR RECEPTOR PPAR γ is expressed at highest level in adipose tissue and is a key transcription factor involved in adipocyte differentiation (1, 2). Thiazolidinedione (TZD) ligands that have been shown to bind and activate PPAR γ (3) are currently used in the treatment of type 2 diabetes in humans (4). Like other PPAR γ ligands, TZDs are adipogenic (5–8). Many adipocyte genes that are activated by TZDs, such as the adipocyte-specific aP2 gene, contain binding sites for PPAR γ (9). PPAR γ is required for adipose development *in vivo* and in cultured cells (10–12). Interestingly, mice with heterozygous loss of the PPAR γ gene showed improved insulin sensitivity (11, 13).

Positive regulation by nuclear receptors, including PPAR γ , involves a fairly well understood exchange of corepressors and coactivators leading to induction of receptor-bound genes (14). TZDs, like other nuclear receptor ligands, can regulate genes negatively as well as positively. Negative regulation is much less well understood and may involve positive regulation of a repressor protein (15), direct DNA binding (16), binding to other transcription factors (17), as well as competition for coactivators in solution off DNA (18).

TZDs function as potent insulin sensitizing agents *in vivo* despite the fact that they increase body weight

(19), and increased adiposity is associated with insulin resistance (20). This suggests that some adipocyte genes whose expression is altered during adipogenesis might be differentially regulated by TZDs. In adipocytes, the secreted hormone resistin is an example of a gene that is up-regulated during adipogenesis but down-regulated by TZDs (21, 22). Like other examples of negative regulation by TZDs, the mechanism of resistin down-regulation is not well understood. Clearly, however, it cannot be identical to the marked up-regulation of the resistin gene that is observed during adipogenesis (21, 23).

Here we sought to understand the importance of positive and negative regulation of gene expression by PPAR γ and its ligands in adipogenesis. We found that the fusion of the potent viral transcriptional activator VP16 to PPAR γ 2 (VP16-PPAR γ) created a transcription factor that constitutively and dramatically activated transcription of PPAR γ -responsive genes in the absence of ligand. Like nuclear receptor coactivators, VP16 positively regulates transcription by increasing histone acetylation and remodeling the chromatin environment of genes to which it is recruited (24). VP16-PPAR γ is thus a model for positive regulation of target genes that are bound directly by PPAR γ . Forced expression of VP16-PPAR γ in 3T3-L1 preadipocytes using retroviral vectors led to adipogenesis in the absence of standard differentiating medium (DM) or any exogenous PPAR γ ligand. Gene microarray analysis

Abbreviations: DM, Differentiation medium; LXSN, 3T3-L1 cells infected with control retrovirus; TZD, thiazolidinedione; VP16, potent viral transcriptional activator.

revealed that VP16-PPAR γ induced many adipocyte genes. However, despite the presence of activated PPAR γ in the cells, TZD treatment markedly down-regulated resistin gene expression in VP16-PPAR γ adipocytes. These results show that constitutively active PPAR γ is sufficient for adipogenesis and that TZD treatment is not equivalent to activation of PPAR γ in adipocytes.

RESULTS

VP16-PPAR γ Constitutively Activates Transcription of PPAR γ -Responsive Genes in the Absence of Ligand

To create a transcription factor that constitutively activates PPAR γ -responsive genes in the absence of ligand, the potent viral transcriptional activator VP16 was fused to PPAR γ 2 (VP16-PPAR γ). To compare the transcriptional activity of VP16-PPAR γ and ligand-activated PPAR γ , the plasmid VP16-PPAR γ and a PPAR response reporter were transiently transfected into 293T cells and studied in the presence or absence of a potent TZD, rosiglitazone. As expected, rosiglitazone markedly activated transcription (3- to 4- fold) in the presence of cotransfected PPAR γ (Fig. 1). Transcriptional activation by VP16-PPAR γ was dramatically greater (over 100-fold), even in the absence of rosiglitazone. Moreover, the PPAR γ ligand had no fur-

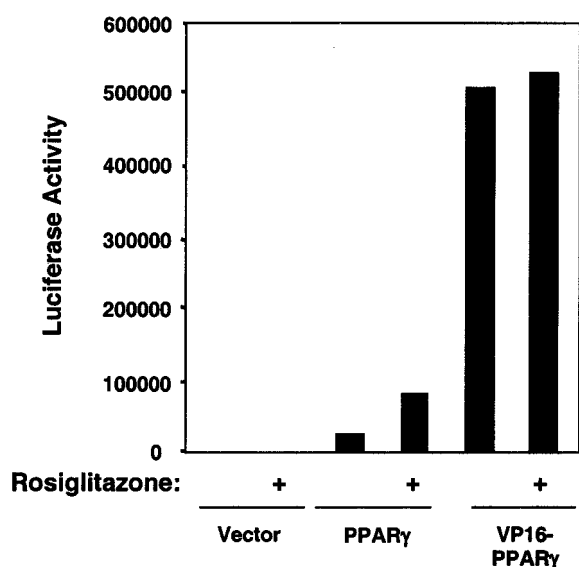


Fig. 1. VP16-PPAR γ Is a Potent Activator of PPAR-Responsive Genes

Transfection of 293T cells with indicated expression vectors using (acyl-coenzyme A PPAREx3)-TK-luciferase reporter plasmid in the presence or absence of PPAR γ ligand, rosiglitazone (1 μ M). The isoform of PPAR γ used in this and all experiments is PPAR γ 2. The relative luciferase activity has been normalized to β -galactosidase activity.

ther effect on the transcriptional activity of VP16-PPAR γ . These results indicate that VP16-PPAR γ is a potent activator of positively regulated PPAR γ -responsive genes.

Expression of VP16-PPAR γ in 3T3-L1 Cells Induces Adipocyte Differentiation in the Absence PPAR γ Ligand

Rosiglitazone induces adipocyte differentiation of preadipocytic 3T3-L1 cells, most likely by activating low levels of PPAR γ , which is autoinduced during adipogenesis (25). By contrast, forced expression of wild-type PPAR γ is only weakly adipogenic in the absence of ligand (7, 26). To determine the effect of constitutive activation of positively regulated PPAR γ target genes, VP16-PPAR γ was expressed from a retroviral vector in 3T3-L1 preadipocytes as we have previously described for wild-type PPAR γ , as well as C/EBP α and C/EBP β (25, 27, 28). Notably, expression of VP16-PPAR γ led to adipogenesis in the absence of standard DM or any exogenous PPAR γ ligand, as indicated by Oil Red O staining (Fig. 2a). The extent of differentiation was similar to that observed with rosiglitazone treatment. As expected (25, 27, 28), both DM and rosiglitazone also induced the adipogenic bZip transcription factor C/EBP α (Fig. 2b). VP16-PPAR γ induced the expression of C/EBP α to a similar extent, but in a manner that was independent of external adipogenic stimulus (Fig. 2b). Note that both the larger and smaller forms of C/EBP α , respectively known as LAP and LIP, were induced by VP16-PPAR γ . As in differentiation by DM or TZD treatment, the induction of C/EBP α is likely to contribute to the adipogenic effects of VP16-PPAR γ expression.

We also performed microarray analysis to compare gene expression in 3T3-L1 cells infected with control retrovirus (LXSN) and with the VP16-PPAR γ retrovirus. The arrays contained probe sets for about 6,000 known genes, representing about 10–20% of the total number in the genome. Seventy-eight genes were increased greater than 3-fold in the VP16-PPAR γ 3T3-L1 cells, whereas only 28 were down-regulated more than 3-fold. The high proportion of induced genes is consistent with the strong activational nature of the VP16-PPAR γ protein. Negatively regulated genes are likely to be regulated indirectly, either as a consequence of primary gene activation or due to squelching of positive cofactors. The list of induced genes includes many that are characteristic of adipogenesis and the adipocyte phenotype, including adipogenic transcription factors (PPAR γ , C/EBP α), adipose-specific secreted proteins (resistin, adipisin, adiponectin/Acrp30), and adipocyte metabolic genes (glycerol phosphate dehydrogenase, pyruvate carboxylase, hormone sensitive lipase, steroyl coenzyme A desaturase I, fatty acid coenzyme A synthase, fatty acid synthase) (Table 1). Thus, the 3T3-L1 cells expressing VP16-PPAR γ are *bona fide* adipocytes by many criteria.

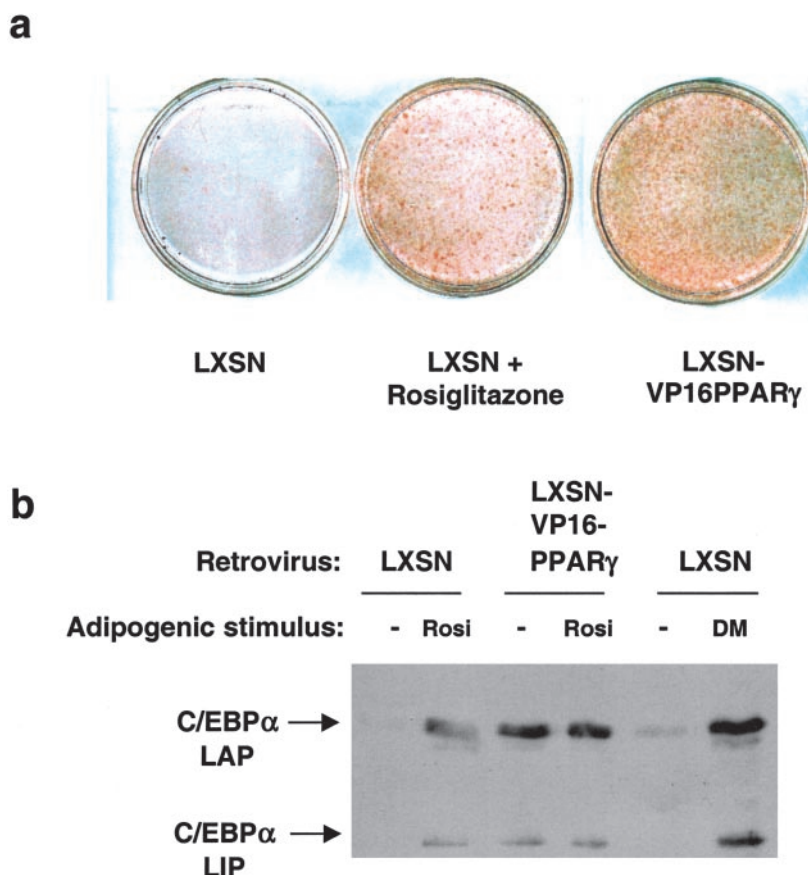


Fig. 2. VP16-PPAR γ Is Adipogenic in the Absence of DM or PPAR γ Ligand

3T3-L1 cells were infected with LXSN retrovirus alone or with VP16-PPAR γ . a, Oil Red O staining of cells were grown in growth media or 1 μ M rosiglitazone on d 7 after confluency. b, Western analysis of C/EBP α .

Differential Gene Regulation in Adipogenesis Induced by VP16-PPAR γ and PPAR γ Ligand

The above results show that many genes induced by VP16-PPAR γ were previously known to be adipocyte genes, consistent with the phenotype of the VP16-PPAR γ cells. TZDs also induce adipocyte differentiation but might regulate a different set of genes because TZDs can negatively regulate gene expression via PPAR γ (29) and may have effects that are independent of PPAR γ (30). 3T3-L1 cells were infected with control LXSN retrovirus, then cultured in the presence of rosiglitazone. This experiment was performed at the same time as the VP16-PPAR γ infection, and the cells underwent adipogenesis to similar extents (see Fig. 2). Microarray analysis of the same gene set as described above was performed, and many of the same adipocyte genes induced by VP16-PPAR γ were also induced by treatment with rosiglitazone, although not always to the same extent (see Table 1). Northern analysis for aP2 and PPAR γ , two adipocyte-predominant genes found to be induced by microarray analysis of VP16-PPAR γ cells, showed that both are induced by VP16-PPAR expression to levels comparable to that due to treat-

ment with rosiglitazone and standard differentiating conditions (Fig. 3, compare lane 4 with lanes 2 and 3).

The VP16-PPAR γ cells were compared with the rosiglitazone-treated, LXSN control cells. Overall, many fewer genes were differentially regulated more than 3-fold by rosiglitazone-induced differentiation as compared with differentiation due to constitutively activated PPAR γ . Moreover, only eight genes were induced at least 3-fold greater by rosiglitazone than in the VP16-PPAR γ cells. The most differentially regulated gene in this category was the mammalian homolog of the yeast secretory pathway protein Sec53p, whose expression was 11.3-fold greater in the microarray analysis of cells induced by rosiglitazone. Northern analysis confirmed that this gene is induced by rosiglitazone, but not by VP16-PPAR γ , suggesting that the TZD may activate this gene independent of PPAR γ (Fig. 4, compare lanes 2 and 5 with each other and with lanes 1 and 4, respectively). By contrast, a much greater number of genes were found to be down-regulated at least 3-fold in the rosiglitazone-differentiated LXSN cells relative to the cells differentiated by VP16-PPAR γ in the absence of ligand. The most differentially regulated gene in this category was the IGF-II gene, whose expression was 30-fold lower

Table 1. Adipocyte Genes Induced by VP16-PPAR γ 2 and PPAR γ Ligand

Adipocyte Gene (Reference)	Fold Induction vs. Control (LXSN)	
	VP16-PPAR γ	Rosiglitazone
Resistin (21)	147	40
Adipsin (55)	131	45
Glycerol phosphate dehydrogenase (56, 57)	119	183
β -3 adrenergic receptor (58)	67	23
Pyruvate carboxylase (59)	44	61
Hormone-sensitive lipase (60)	42	39
Acrp30/Adiponectin/AdipoQ (61, 62)	41	47
SCD1 (63)	32	15
Long chain fatty acid CoA synthase (64)	29	38
aP2/422 (65, 66)	25	26
Angiotensinogen (67)	13.3	3.7
LXR (68)	10.3	8.1
Diacylglyceride acyltransferase (69)	8.8	9.7
Amine oxidase (70)	6.1	2.9
PPAR γ (9, 54)	5.6	3.8
C/EBP α (63)	5.6	4.1
Fatty acid synthase (71)	4.0	4.0

Microarray analysis was performed as described in *Materials and Methods* on 3T3-L1 cells infected with control LXSN retrovirus and cultured with or without rosiglitazone (1 μ M), and 3T3-L1 cells infected with VP16-PPAR γ retrovirus. Fold-induction is relative to LXSN control. Seventeen genes induced more than 3-fold and known to be induced during adipogenesis are shown (due to space limitations, only one to two citations could be shown for each). Overall, 78 genes present on the chip were induced more than 3-fold by VP16-PPAR γ .

in microarray comparison of the rosiglitazone-induced cells and the VP16-PPAR γ cells. Northern analysis confirmed the lower expression in rosiglitazone-treated cells (Fig. 4, lanes 2 and 5). Interestingly, IGF-II was also expressed at similar levels in the preadipocytes as in the VP16-PPAR γ expressing cells (lanes 1 and 4), suggesting that activated PPAR γ is not responsible for the high (but comparable) expression. Consistent with this, rosiglitazone down-regulated IGF-II gene expression in the VP16-PPAR γ 2 cells as well as in the undifferentiated cells.

Resistin Is Down-Regulated by Rosiglitazone in VP16-PPAR γ Adipocytes

Another gene whose expression was lower in rosiglitazone-differentiated adipocytes than in VP16-PPAR γ adipocytes was resistin, whose expression was 3.6-fold higher in the VP16-PPAR γ adipocytes. Resistin is an adipocyte-secreted hormone that is known to be up-regulated during adipogenesis (21, 23). Resistin was the most highly induced gene in our microarray profile of VP16-PPAR γ adipocytes (144-fold; see Table 1). Resistin is also known to be down-regulated by TZDs in 3T3-L1 cells (21), although the molecular mechanism by which TZDs down-regulate resistin is not known. Multiple TZDs down-regulate resistin expression in adipocytes, with a dose-response profiles that parallel their ability to bind PPAR γ (Refs. 21 and 23; and data not shown). The dramatic induction of resistin in the VP16-PPAR γ cell adipogenesis was confirmed by Northern analysis, and comparable to the induction of resistin during adipocytic differentia-

tion of control cells with DM (Fig. 4). These data strongly suggest that transcriptional activation of PPAR γ activates resistin expression, either directly or indirectly, the latter by initiating the adipogenic cascade. Consistent with the microarray analysis, resistin gene induction was much less during rosiglitazone-induced adipogenesis (Fig. 4, compare lanes 2 and 3). Importantly, the combination of rosiglitazone treatment and forced expression of VP16-PPAR γ led to resistin expression at approximately the level of the control cells differentiated with rosiglitazone, and much lower than that in the VP16-PPAR γ cells (compare lanes 4 and 5). This contrasts with the effect on other adipose genes including PPAR γ and aP2, which were not decreased by the combination of VP16-PPAR γ expression and rosiglitazone (see Fig. 3).

The preceding experiments compared the effects of rosiglitazone and VP16-PPAR γ expression in the context of conversion of preadipocytes to adipocytes. We next investigated the effects of rosiglitazone on resistin expression in the differentiated VP16-PPAR γ adipocytes. If the effect of TZDs was solely to activate PPAR γ , and if the induction of resistin in the VP16-PPAR γ cells was due to direct activation of the resistin promoter, then TZD treatment should not decrease resistin expression. By contrast, rosiglitazone markedly reduced resistin gene expression in the VP16-PPAR γ adipocytes (Fig. 5). This suggests that this effect of rosiglitazone was not due to activation of PPAR γ and dramatically illustrates a molecular distinction between the effect of rosiglitazone and acti-

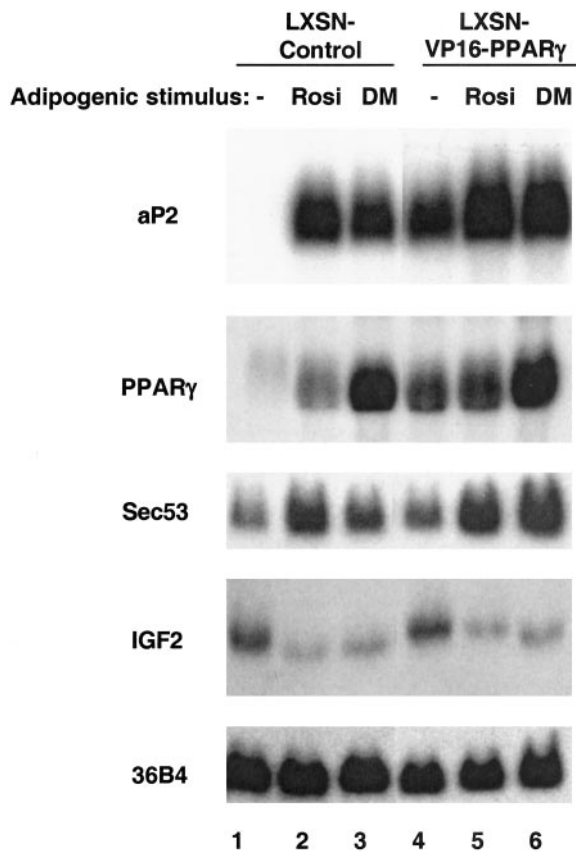


Fig. 3. Differential Regulation of Sec53 and IGF-II by VP16-PPAR γ and PPAR γ Ligand

Northern analysis for aP2, PPAR γ , Sec53, IGF-II, and 36B4 at d 7 after control medium, differentiation medium, or rosiglitazone treatment (1 μ M) of LXSN and LXSN-VP16-PPAR γ cells.

vation of PPAR γ by a heterologous transcriptional activation domain.

DISCUSSION

We have shown that a constitutively active form of PPAR γ is sufficient for adipogenesis. The similarities between 3T3-L1 adipocytes treated with DM or rosiglitazone and those ectopically expressing constitutively activation of PPAR γ are striking. Adipocyte differentiation was induced to similar extents, and many of the same adipocyte genes were induced in all manipulations. In VP16-PPAR γ , the viral coactivator functions by recruiting coactivator complexes including histone acetyltransferases to the vicinity of PPAR γ -bound genes (31). This strongly supports the notion that gene induction and adipogenesis due to TZDs are directly related to that the role of ligand in coactivator recruitment to PPAR γ binding sites. This also indicates that activation of gene transcription by PPAR γ is sufficient for adipogenesis.

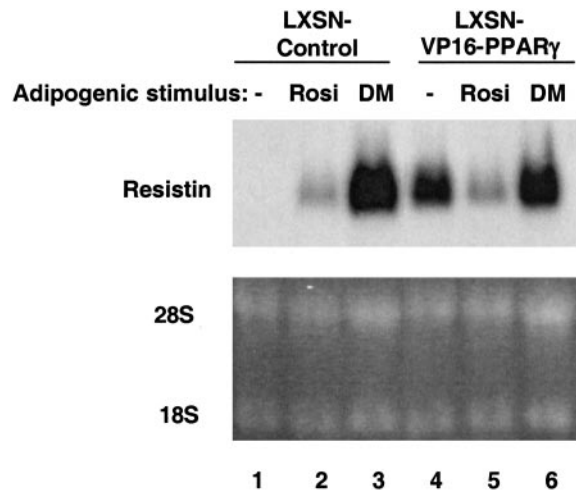


Fig. 4. Resistin Is Induced during VP16-PPAR γ -Mediated Adipogenesis, but Down-Regulated by TZDs

Cells were treated as in Fig. 3, and probed for resistin gene expression. Ethidium bromide staining of rRNA is shown as evidence of equal loading.

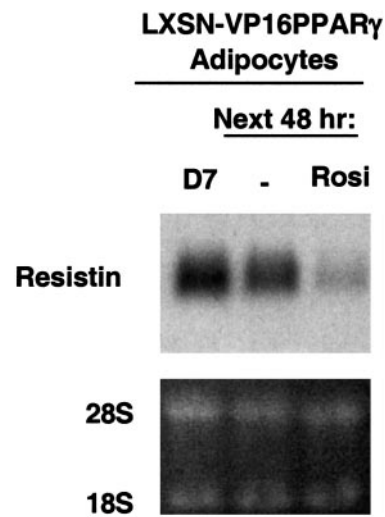


Fig. 5. Down-Regulation of Resistin Expression in VP16-PPAR γ Adipocytes by TZDs

3T3-L1 cells were differentiated using VP16-PPAR γ , then d 7 adipocytes (D7) were treated with 1 μ M rosiglitazone or vehicle for 48 h. Ethidium bromide staining of rRNA is shown as evidence of equal loading.

A number of genes were noted to be down-regulated during adipogenesis due to VP16-PPAR γ . There are a number of potential explanations for this. Given the role of VP16 as an activator, we favor the possibility such effects involve induction of one or more repressor proteins. VP16-PPAR γ could also down-regulate the expression certain genes by transrepression due to squelching of coactivators such as p300/CBP (18). It is also possible that the mechanism of negative regulation by VP16-PPAR γ would be identical to that of transrepression by TZD binding to

PPAR γ . This, however, seems unlikely because the latter requires the charge clamp that mediates ligand-dependent interactions with proteins containing LXXLL motifs. (29).

Although VP16-PPAR γ expression and TZD treatment both triggered adipogenesis, TZDs clearly regulated some genes differently than transcriptional activation of PPAR γ -RXR, as accomplished by VP16-PPAR γ . A number of mechanisms are likely to contribute to the difference between constitutive and ligand activation of PPAR γ . First, although both VP16-PPAR γ and rosiglitazone-bound PPAR γ recruit many of the same coactivators containing histone acetyltransferase activity, such as CBP/p300 (32) and the activated-recruited cofactor/VDR interacting protein/TR-associated protein complex (33–35), coactivators may be recruited with different affinities and potencies in terms of gene activation. This point is emphasized by the striking ability of VP16-PPAR γ to activate a PPRE-containing reporter nearly 100-fold better than rosiglitazone-bound PPAR γ (Fig. 1). Yet, although rosiglitazone did not further activate the PPRE-containing reporter, it did enhance activation of the endogenous aP2 gene in adipocytes expressing VP16-PPAR γ (Fig. 3). This is consistent with previous observations that the magnitude of activation of the aP2 promoter varies among coactivators of different classes (36). The p160 class of coactivators are relatively specific for the nuclear receptor superfamily (37). Even among nuclear receptors, different ligand-bound receptors may prefer one class of coactivators over another. PPAR γ prefers CBP to the p160 coactivator, whereas its heterodimer partner RXR prefers the p160 coactivator in the same complex (38, 39). PPAR γ also binds corepressors nuclear receptor corepressor/silencing mediator for RA and TRs, and it is likely that the binding is different for VP16-PPAR γ than for PPAR γ bound to rosiglitazone (40, 41). It is also possible that rosiglitazone functions as a partial agonist of PPAR γ on a subset of target genes, as has been described for other TZDs including troglitazone (42) and MCC-555 (43) as well as non-TZD PPAR γ ligands (44). By contrast, VP16-PPAR γ would be expected to be a pure transcriptional activator. In addition, TZD binding to PPAR γ transrepresses certain genes in a manner that would not be expected for VP16-PPAR γ because, as noted before, this function requires ligand and the charge clamp that mediates interactions with LXXLL containing proteins such as the p160 coactivators (29).

Relatively few genes are established as being differentially regulated by TZD treatment and by adipogenesis *per se*. One gene that is strongly induced in adipocytes by TZDs encodes Cbl-associated protein (45), which plays important roles in insulin-stimulated glucose transport (46). The present study shows that the Sec53 protein is also induced in adipocytes by rosiglitazone. Conversely, IGF-II gene expression is altered significantly by adipocyte differentiation but is down-regulated by TZD treatment. The functions of

Sec53 and IGF-II in TZD actions apart from adipogenesis remain to be elucidated.

Resistin induction during adipogenesis occurs with all inducers, but it is dramatically greater with DM or VP16-PPAR γ than with rosiglitazone. Negative regulation of resistin gene expression by TZD could be mediated via a PPAR γ binding site on the resistin gene, such that constitutively activated PPAR γ would paradoxically induce resistin expression. Arguing against this, TZD treatment of VP16-PPAR γ cells either during adipogenesis or as mature adipocytes led to reduction of resistin gene expression. It is also unlikely that activated PPAR γ induces a repressor of resistin gene expression because resistin was dramatically up-regulated by VP16-PPAR γ . Down-regulation of resistin gene expression by TZD could be by an off DNA mechanism involving PPAR γ , such as squelching of limiting coactivators (18), or could even be independent of PPAR γ . TZDs have been suggested to have PPAR γ -independent effects on muscle (47), and recent studies of PPAR γ null cells differentiated into macrophages suggests that negative regulation of cytokine gene expression by TZDs in that system may be independent of PPAR γ (30). Unlike macrophages, PPAR γ is required for adipocyte differentiation, making it impossible for us to study the effects of TZDs on resistin expression in PPAR γ null adipocytes at this time (48, 49). Thus, we cannot draw any conclusion about whether PPAR γ mediates the transrepressive effects of TZD that are not mimicked by VP16-PPAR γ in adipocytes.

The induction of resistin may well be an indirect consequence of adipogenesis. Indeed, resistin is induced during TZD-induced adipogenesis, albeit markedly (3.6-fold) less than with VP16-PPAR γ , which is comparable to DM (Fig. 4). Thus, resistin might be increased by TZD *in vivo* under circumstances where *de novo* adipogenesis is dominant over metabolic effects on mature adipocytes, where resistin expression is reduced. These characteristics of resistin gene expression could explain why some studies have found that rosiglitazone treatment of mice down-regulates resistin gene expression (21, 50), whereas another noted up-regulation of the resistin gene with PPAR γ ligands (51).

Thus, our work shows that constitutively active PPAR γ is sufficient for adipogenesis, but points out that gene regulation during this process is not identical to that during adipocyte differentiation stimulated by PPAR γ ligand. Intriguingly, a PPAR γ antagonist that blocks TZD-induced adipocyte differentiation does not inhibit adipogenesis by DM (52). This suggests that, as with VP16-PPAR γ , the adipogenic stimulus of DM can be distinguished from the effects of TZDs. It remains to be determined whether genes regulated by TZD but not by adipogenesis *per se* or by VP16-PPAR γ are mechanistically related to the insulin-sensitizing effects of these compounds.

MATERIALS AND METHODS

Cell Culture and Differentiation

3T3-L1 preadipocytes were maintained in growth media containing 10% FBS and differentiated as described previously (25). Briefly, for standard adipocyte differentiation, 2 d after cells reached confluency (referred as d 0), cells were exposed to DM containing 10% FBS for 48 h, then were maintained in postdifferentiation medium containing 10% FBS. For BRL 49653-induced adipogenesis, 1 μ M BRL 49653 was added to post-confluent cells for 7–10 d until fat cells were seen. The cDNA of VP16 fused with full-length PPAR γ was ligated into retroviral vector LXS. Ectopically expressing cells were made as described before (25).

Transient Transfection Transcription Assay

Cell Culture and Transient Transfection. 293T cells were maintained in DMEM with high glucose supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD) and grown at 37 C in 5% CO₂. Cells were transiently transfected using lipofectamine (Life Technologies, Inc.) according to the manufacturer's directions. Ligands were added 16 h after transfection, and cells were harvested 24 h later. The results were normalized to transfection efficiency as determined by β -galactosidase expression, as described previously (53).

Oil Red O Staining

3T3-L1 cells were washed three times with PBS, fixed by 10% formalin in phosphate buffer for 1 h, and washed again with PBS. The cells were then stained with a filtered Oil Red O stock solution [0.5 g of Oil Red O (Sigma, St. Louis, MO) in 100 ml of isopropyl alcohol] for 15 min at room temperature, then washed twice with water for 15 min each and visualized.

Western Analysis

3T3-L1 cells were washed in PBS and lysed with NET-N buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl at pH 8.0, 0.5% Nonidet P-40, and 10% glycerol) containing protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) on ice for 30 min, followed by centrifugation at 17,000 rpm at 4 C for 30 min. Supernatant was collected, and protein concentration was determined by Bio-Rad Laboratories, Inc. (Hercules, CA) protein assay. Sixty micrograms of protein were subjected to 10% polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane. The membrane was incubated first with primary antibody [anti-PPAR, 1:1500 (23); anti-C/EBP, 1:300 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA)] for 2 h, followed by secondary antibody (horseradish peroxidase conjugated) for 1 h. Blots were developed using ECL chemiluminescence detection reagent (Amersham Pharmacia Biotech, Inc., Arlington Heights, IL) and visualized by exposure to autoradiography film.

Northern Blotting and Microarray Analysis

Total RNA was extracted from 3T3-L1 cells with Trizol reagent (Life Technologies, Inc.) and Northern analysis were performed as described previously (54). Biotin-labeled cRNA was prepared according to the Affymetrix (Santa Clara, CA) expression analysis technical manual. Hybridization to Murine Genome U74A arrays was performed at the Research Genetics, Inc. (Huntsville, AL).

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