Role of Peroxisome Proliferator–Activated Receptor-γ in Maintenance of the Characteristics of Mature 3T3-L1 Adipocytes

Yoshikazu Tamori, Jiro Masugi, Naonobu Nishino, and Masato Kasuga

Peroxisome proliferator–activated receptor (PPAR)-γ plays an important role in adipogenesis. However, the functions of PPAR-γ in differentiated adipocytes have remained unclear. The role of PPAR-γ in mature 3T3-L1 adipocytes was therefore investigated by overexpression of a dominant negative mutant of this protein (PPAR-γΔC) that lacks the 16 COOH-terminal amino acids and that has been shown to prevent the thiazolidinedione-induced differentiation of 3T3-L1 cells into adipocytes. Overexpression of PPAR-γΔC in mature 3T3-L1 adipocytes by adenovirus gene transfer resulted in a decrease in both cell size and intracellular triglyceride content, an increase in the extent of lipolysis, and a reduction in the rate of free fatty acid uptake. Furthermore, overexpression of this mutant reduced the abundance of mRNAs for several key enzymes that contribute to triglyceride and free fatty acid metabolism as well as the amounts of GLUT4, insulin receptor, insulin receptor substrate (IRS), and C/EBPα mRNAs. It also reduced both the concentration of IRS2 and the insulin-stimulated glucose uptake. These results suggest that PPAR-γ plays an important role in mature 3T3-L1 adipocytes at least in part by maintaining the expression of genes that confer the characteristics of mature adipocytes. Diabetes 51:2045–2055, 2002

Peroxisome proliferator-activated receptors (PPARs) constitute a subfamily of nuclear hormone receptors, the transcriptional activities of which are regulated by interaction with the corresponding ligand (1). Among PPARs, the expression of PPAR-γ exhibits the greatest specificity for adipose tissue (2). The role of this isoform in adipogenesis has been studied extensively in vitro with the use of cultured preadipocytic cell lines that undergo differentiation in the presence of PPAR-γ ligands. The expression of PPAR-γ is induced early in adipogenesis, subsequent to induction of the transcription factors C/EBPβ and C/EBPδ (3), and it in turn leads to an increase in the abundance of C/EBPα. C/EBPα, together with PPAR-γ, promotes development of the differentiated phenotype (4). PPAR-γ thus plays an important role in adipogenesis. Moreover, the observations that PPAR-γ–deficient cells fail to differentiate into adipocytes demonstrated that PPAR-γ is absolutely required for adipocyte differentiation (5–7).

PPAR-γ has also been identified as the receptor for thiazolidinediones (TZDs) (8), a class of oral antidiabetic agents that improve glycemic control by reducing the level of peripheral insulin resistance. The relative ability of different TZDs to bind to and to activate PPAR-γ in vitro correlates with the antidiabetic action of these drugs in vivo (9,10). The antidiabetic effects of TZDs are thus thought to be mediated through PPAR-γ. The predominant expression of PPAR-γ in differentiated adipocytes (11,12) suggests that adipose tissue is one of the main target tissues of TZDs. However, the mechanism by which and the tissues in which TZDs act to increase insulin sensitivity remain unclear (13,14).

Several mutations in the PPAR-γ gene have been detected in human subjects. A Pro115 → Gln mutation that inhibits the phosphorylation of Ser112 and increases the transcriptional activity of PPAR-γ was identified in four markedly obese individuals (15). A Pro12 → Ala mutation that reduces the transcriptional activity of PPAR-γ is associated with lower BMI and improved insulin sensitivity (16,17). Furthermore, two dominant negative mutations of PPAR-γ, Pro467 → Leu and Val290 → Met, have been identified in individuals with severe insulin resistance (18). These genetic studies suggest that PPAR-γ also contributes to lipid and glucose metabolism in the body as a whole.

The observation that the expression of PPAR-γ not only is induced early during adipocyte differentiation (19) but also continues at a high level in mature adipocytes (11,12) suggests that PPAR-γ may also have important functions in fully differentiated cells. We have previously shown that expression of a PPAR-γ mutant (PPAR-γΔC) that lacks the 16 COOH-terminal amino acids of the full-length protein completely inhibited TZD-induced adipogenesis in 3T3-L1 cells (20); this mutant retains DNA-binding activity but has lost the ability to bind ligand. With the use of this dominant negative mutant, we investigated the role of PPAR-γ in mature 3T3-L1 adipocytes.

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ACS, acyl-CoA synthase; CAP, c-Cl−-associated protein; C/EBPα, CCAAT/enhancer binding protein-α; DMEM, Dulbecco’s modified Eagle’s medium; FFA, free fatty acid; HSL, hormone-sensitive lipase; IR, insulin receptor; IRS, insulin receptor substrate; KREB, Krebs-Ringer-HEPES; LPL, lipoprotein lipase; MOI, multiplicity of infection; PEPCK, phosphoenolpyruvate carboxykinase; PPU, plaque-forming units; PPAR, peroxisome proliferator–activated receptor; TNP-α, tumor necrosis factor-α; TZD, thiazolidinedione.
RESEARCH DESIGN AND METHODS

Materials. Polyclonal antibodies to PPAR-γ2 were generated by injection of rabbits with 5′-triphosphate γ-glutamate of mouse PPAR-γ2. Rabbit polyclonal antibodies to insulin receptor substrate 1 (IRS1) and to IRS2 were obtained from Upstate Biotechnology (Lake Placid, NY). A mouse monoclonal antibody to phosphotyrosine (PY20) was from Transduction Laboratories (Lexington, KY). Recombinant murine tumor necrosis factor-α (TNF-α) was obtained from Wako (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were from Sigma (St. Louis, MO) and JRH Biosciences (Lenexa, KA), respectively. The pAdex1CAwt vector and BRL49653 were provided by I. Saito (University of Tokyo, Japan) and SmithKline Beecham, U.K., respectively. Mouse PPAR-γ2 cDNA was generated by RT-PCR with mRNA from 3T3-L1 adipocytes (20).

Cell culture. 3T3-L1 fibroblasts were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% fetal bovine serum. Adipogenesis was induced by treatment of the cells with insulin, dexamethasone, and isobutylmethylxanthine as described previously (21).

Preparation of and infection with adenovirus vectors. Adenovirus expression vectors encoding PPAR-γ-WT or PPAR-γ-ΔC were constructed as described (20). In brief, cDNAs encoding hemagglutinin epitope-tagged wild-type and COOH-terminally truncated PPAR-γ2 proteins were subcloned separately into the pAdex1CAwt adenovirus vector (22), which contains the CAP promoter (23). The resulting constructs, together with DNA-terminal protein complex, were introduced by transfection into 293 cells with the use of CellPhect (Pharmacia). A single clone of each recombinant adenovirus (Adex-PPAR-γ-WT and Adex-PPAR-γ-ΔC) was isolated by serial dilution with a plaque assay. Viral titer was determined by plaque assay. Fifteen days after induction of differentiation, 3T3-L1 cells were infected with adenovirus vectors for 2 h.

Measurement of size and triglyceride content of 3T3-L1 cells. 3T3-L1 adipocytes were fixed in 10% formaldehyde/phosphate-buffered saline, stained with Giemsa’s stain solution, and then examined by light microscopy. The sizes of adipocytes were determined by tracing the diameters of 100 3T3-L1 adipocytes manually and analyzing with Mac Scope (Mitani, Chiba, Japan). The triglyceride content of isopropyl alcohol extracts prepared from 3T3-L1 adipocytes grown in six-well plates was quantitated with an acetyl acetone-based colorimetric kit (Wako).

Measurement of lipolysis and free fatty acid uptake. For measurement of lipolysis, 3T3-L1 adipocytes cultured in six-well plates were incubated for 12 h in DMEM containing 1% bovine serum albumin (BSA) in the absence of serum. Measurement of lipolysis and free fatty acid uptake. 3T3-L1 adipocytes cultured in six-well plates were incubated for 12 h in DMEM containing 1% bovine serum albumin (BSA) in the absence of serum.

RESULTS

Effects of overexpression of PPAR-γ-WT and PPAR-γ-ΔC on the morphology of mature 3T3-L1 adipocytes. To investigate the role of PPAR-γ in mature 3T3-L1 adipocytes, with the use of an adenovirus expression system we engineered cells that had been cultured for >15 days after the induction of differentiation to overexpress either the wild-type protein (PPAR-γ-WT) or the dominant negative mutant PPAR-γ-ΔC. Mature 3T3-L1 adipocytes were infected with the control adenovirus (Adex-control), an adenovirus encoding PPAR-γ-WT (Adex-PPAR-γ-WT), or an adenovirus encoding PPAR-γ-ΔC (Adex-PPAR-γ-ΔC) at a multiplicity of infection (MOI) of 20 to 60 plaque-forming units (PFU) per cell. The recombinant adenovirus that we used was shown to be expressed with an efficacy of >95% at an MOI of 30 in 3T3-L1 adipocytes as assessed by β-galactosidase staining (24). Immunoblot analysis of cell lysates with antibodies to PPAR-γ revealed that 48 h after infection with the last two constructs, the cells overexpressed the encoded PPAR-γ proteins in an MOI-dependent manner (Fig. 1A). The expression levels of PPAR-γ-WT or PPAR-γ-ΔC proteins were quantified with NIH image and shown to be 11.9-fold or 10.3-fold higher than that of endogenous PPAR-γ at an MOI of 60, respectively. Furthermore, the amounts of overexpressed proteins were not significantly changed by 72 h after infection (Fig. 1B).

We next investigated the effects of overexpression of PPAR-γ-WT or PPAR-γ-ΔC on the morphology of mature 3T3-L1 adipocytes. Forty-eight hours after infection with Adex-PPAR-γ-WT or Adex-PPAR-γ-ΔC at an MOI of 60 PFU/cell, fully differentiated cells were fixed, stained with oil red O, and examined macroscopically and microscopically. Overexpression of PPAR-γ-WT resulted in an increase both in cell size and in the cytoplasmic deposition of triglyceride droplets, compared with adipocytes infected with Adex-control (Fig. 2A and B). In contrast, overexpression of PPAR-γ-ΔC resulted in a decrease in both cell size and intracellular triglyceride content (Fig. 2A and C). We also investigated the effects of BRL49653, a potent TZD that binds to PPAR-γ with an affinity in the nanomolar range (9), and of TNF-α, which inhibits the
differentiation of 3T3-L1 cells to mature adipocytes (25). Treatment of mature 3T3-L1 adipocytes with 5 μmol/l BRL49653 for 48 h induced increases in both cell size and intracellular triglyceride content (Fig. 2D and E), similar to the effects of overexpression of PPAR-γ-WT. In contrast, exposure of cells to TNF-α (10 ng/ml) for 48 h resulted in decreases in both cell size and triglyceride content (Fig. 2D and F), similar to the effects of overexpression of PPAR-γ-ΔC.

Quantitative analysis revealed that overexpression of PPAR-γ-WT increased the size of mature 3T3-L1 adipocytes by 12% and the intracellular triglyceride content by 34%, compared with cells infected with Adex-control (Fig. 3A and C). In contrast, overexpression of PPAR-γ-ΔC reduced cell size and triglyceride content by 15 and 19%, respectively (Fig. 3A and C). The similar effects were obtained with treatment with BRL49653 or TNF-α as with overexpression of PPAR-γ-WT or PPAR-γ-ΔC, respectively. BRL49653 increased the size of 3T3-L1 adipocytes by 18% and the intracellular triglyceride content by 29%, compared with control cells (Fig. 3B and D). On the contrary, TNF-α treatment decreased them by 21 and 29%, respectively (Fig. 3B and D).

**Effects of overexpression of PPAR-γ-WT or PPAR-γ-ΔC on lipolysis and FFA uptake in mature 3T3-L1 adipocytes.** We next measured lipolysis and FFA uptake in mature 3T3-L1 adipocytes overexpressing PPAR-γ-WT or PPAR-γ-ΔC. Lipolysis was determined by measurement of FFAs released into the medium during incubation of cells for 12 h. Whereas overexpression of PPAR-γ-WT had

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**TABLE 1**

Primer pairs and PCR conditions for semiquantitative RT-PCR analysis

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer pair (5′ → 3′ and 3′ → 5′, respectively)</th>
<th>Annealing temperature (°C)</th>
<th>No. of cycles</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP</td>
<td>Forward TACATCGAAGGGAGAAAGTGG&lt;br&gt;Reverse TCTTTATCATCTGTCGGCGTCC</td>
<td>61</td>
<td>28</td>
<td>509</td>
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<tr>
<td></td>
<td>Forward AGCGGATTAGTGGAAGAAC&lt;br&gt;Reverse GGTCTCCACTCTGGTTC</td>
<td>56</td>
<td>26</td>
<td>465</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward AGTACCCCATTAGAACAG&lt;br&gt;Reverse TGCAGAAAATGGCAGCCTGAC</td>
<td>59</td>
<td>23</td>
<td>732</td>
</tr>
<tr>
<td>aP2</td>
<td>Forward CAAAATGTGTAGCTTGGCTTAC&lt;br&gt;Reverse CTTCCTCTTGCTCAGTGCC</td>
<td>58</td>
<td>24</td>
<td>417</td>
</tr>
<tr>
<td>ACS</td>
<td>Forward ACGTTCGCAATGGCATCG&lt;br&gt;Reverse TTTGATGATGGGTCG</td>
<td>58</td>
<td>25</td>
<td>899</td>
</tr>
<tr>
<td>CD36</td>
<td>Forward AGTCTTTGGATCTTGGTATGC&lt;br&gt;Reverse TTCATAGGTCTGAAACATC</td>
<td>58</td>
<td>25</td>
<td>883</td>
</tr>
<tr>
<td>LPL</td>
<td>Forward ATGGAGAGCAAGCCCTGTC&lt;br&gt;Reverse AGTCTCTCCTGTCAACATC</td>
<td>60</td>
<td>24</td>
<td>770</td>
</tr>
<tr>
<td>HSL</td>
<td>Forward ATGGATTTACGACAGTGACACAG&lt;br&gt;Reverse ACTGAGGCCCTGTC</td>
<td>59</td>
<td>26</td>
<td>609</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Forward AGGGCTCTGGTGAGTGATGCC&lt;br&gt;Reverse TAAAGGATGCAACCGATGGTCC</td>
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<td>25</td>
<td>651</td>
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<tr>
<td>GLUT4</td>
<td>Forward CAACGTGGGCTTGGGTAGGCA&lt;br&gt;Reverse ACACATCGACGCAGCCGGT</td>
<td>62</td>
<td>25</td>
<td>589</td>
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<tr>
<td>IR</td>
<td>Forward ATGGACCATCCGGAAACAACCT&lt;br&gt;Reverse TTGATGACAGTGCGAGGACA</td>
<td>60</td>
<td>27</td>
<td>494</td>
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<tr>
<td>IRS1</td>
<td>Forward CCCACAGCAGCTATTACC&lt;br&gt;Reverse AGAGACGAAGATGCTGAGTGC</td>
<td>65</td>
<td>28</td>
<td>446</td>
</tr>
<tr>
<td>IRS2</td>
<td>Forward GGGCTCTGTTGGAAAATGTCCTC&lt;br&gt;Reverse CTGGGCTTTCCTACAGTGATG</td>
<td>63</td>
<td>31</td>
<td>443</td>
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<tr>
<td>C/EβPα</td>
<td>Forward AGACATCGCGCCCTACATCG&lt;br&gt;Reverse TGTAAGGTGACAGTGCTGTG</td>
<td>67</td>
<td>30</td>
<td>446</td>
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no effect on lipolysis, that of PPAR-γ-ΔC markedly increased the release of FFAs into the medium in an MOI-dependent manner (Fig. 4A). Measurement of oleate uptake during a 2-min incubation revealed no significant difference among cells infected with Adex-control, Adex-PPAR-γ-WT, or Adex-PPAR-γ-ΔC at an MOI of 40 PFU/cell (Fig. 4B). However, at an MOI of 60 PFU/cell, infection with Adex-PPAR-γ-ΔC resulted in a decrease in FFA uptake of 26% compared with that apparent in cells infected with Adex-control; infection with Adex-PPAR-γ-WT at this MOI had no effect on FFA uptake.

Effects of overexpression of PPAR-γ-WT or PPAR-γ-ΔC on the abundance of specific mRNAs in mature 3T3-L1 adipocytes. With the use of semiquantitative RT-PCR analysis, we examined the effects of overexpression of PPAR-γ-WT or PPAR-γ-ΔC on the abundance of mRNAs that encode various proteins associated with fatty acid and triglyceride metabolism, glucose uptake, or insulin signaling. We also evaluated the effects of BRL49653, which promotes adipocyte differentiation and increases insulin sensitivity (26), and of TNF-α, which inhibits both adipocyte differentiation and insulin action (27), on the

FIG. 1. Overexpression of PPAR-γ-WT or PPAR-γ-ΔC in mature 3T3-L1 adipocytes by adenovirus-mediated gene transfer. A: Mature 3T3-L1 adipocytes were infected with the empty adenovirus vector (Adex-control) or adenovirus vectors encoding either PPAR-γ-WT (Adex-PPAR-γ-WT) or PPAR-γ-ΔC (Adex-PPAR-γ-ΔC) at an MOI of 20, 40, or 60 PFU/cell. After 48 h, total cell lysates were prepared and subjected to immunoblot analysis with antibodies to PPAR-γ. B: Mature 3T3-L1 adipocytes were infected with Adex-PPAR-γ-WT or Adex-PPAR-γ-ΔC at an MOI of 60 PFU/cell. After 24, 48, and 72 h, total cell lysates were prepared and subjected to immunoblot analysis with antibodies to PPAR-γ.

FIG. 2. Effects of overexpression of PPAR-γ-WT or PPAR-γ-ΔC or of treatment with BRL49653 or TNF-α on the morphology of mature 3T3-L1 adipocytes. Cells were infected with Adex-control (A), Adex-PPAR-γ-WT (B), or Adex-PPAR-γ-ΔC (C) at an MOI of 60 PFU/cell or were incubated in the absence (D) or presence of 5 μmol/l BRL49653 (E) or TNF-α (10 ng/ml) (F). After 48 h, the cells were fixed, stained with oil red O, and examined macroscopically (left) and microscopically (right). Magnification ×400.
abundance of these mRNAs. To confirm the suitability of our RT-PCR technique, we first measured the abundance of CAP mRNA, the production of which is stimulated by activators of PPAR-γ, such as TZDs, as a result of the direct binding of PPAR-γ to the putative PPAR-γ response element located in the CAP gene promoter (28). Consistent with previous results (29), treatment of mature 3T3-L1 adipocytes with 5 μmol/l BRL49653 for 48 h increased the amount of CAP mRNA in these cells (Fig. 5A). In contrast, the abundance of CAP mRNA was decreased in cells treated with TNF-α (10 ng/ml) for 48 h. Overexpression of PPAR-γ-ΔC (MOI 60 PFU/cell) also resulted in a decrease in the amount of CAP mRNA in mature 3T3-L1 adipocytes, whereas overexpression of PPAR-γ-WT had no effect on this parameter. In addition to CAP, we analyzed the abundance of PEPCK mRNA to further confirm the suitability of our RT-PCR technique, because it is demonstrated that the binding site for the PPAR-γ/RXRα heterodimer in the PEPCK 5′-flanking region is critical for its enhancer activity in adipocytes (30). Overexpression of PPAR-γ-WT or PPAR-γ-ΔC and treatment with BRL49653 or TNF-α also resulted in effects similar to the expression of PEPCK and the expression of CAP gene in 3T3-L1 adipocytes (Fig. 5B).

Analysis of the expression of genes encoding six proteins that contribute to triglyceride and FFA metabolism...
revealed that the amounts of mRNAs for aP2, ACS, CD36, LPL, HSL, and perilipin all were reduced in mature 3T3-L1 adipocytes overexpressing PPAR-γΔC but were unaffected by overexpression of PPAR-γ-WT (Fig. 5C). The abundance of aP2, ACS, CD36, HSL, and perilipin mRNAs was increased by treatment of cells with BRL49653, whereas that of aP2, ACS, CD36, LPL, HSL, and perilipin mRNAs was decreased by exposure of cells to TNF-α.

We also examined expression of the genes encoding GLUT1 and GLUT4, which are the major GLUT isoforms of 3T3-L1 adipocytes. Overexpression of PPAR-γΔC significantly reduced the amount of the mRNA for the insulin-sensitive GLUT4; in contrast, it increased the abundance of the mRNA for the ubiquitous GLUT1 (Fig. 5C). Overexpression of PPAR-γ-WT reduced the amount of GLUT1 mRNA. Whereas BRL49653 increased the abundance of both GLUT1 and GLUT4 mRNAs, TNF-α reduced only the amount of GLUT4 mRNA. Overexpression of PPAR-γΔC also significantly reduced the amounts of IR, IRS1, and IRS2 mRNAs, whereas overexpression of PPAR-γ-WT had no effect on the abundance of these transcripts (Fig. 5C). BRL49653 increased the amounts of IR and IRS2 mRNAs but not that of IRS1 mRNA, whereas TNF-α reduced only the abundance of IRS2 mRNA significantly. Finally, we examined expression of C/EBPα, which is known to play important roles in adipogenesis in concert with PPAR-γ. Both treatment with TNF-α and overexpression of PPAR-γΔC reduced the amount of C/EBPα in 3T3-L1 adipocytes (Fig. 5B).

**Effects of overexpression of PPAR-γ-WT or PPAR-γΔC on insulin-induced tyrosine phosphorylation of IRS1 and IRS2 and insulin-stimulated glucose transport in mature 3T3-L1 adipocytes.** We next investigated the effects of overexpression of PPAR-γ-WT or PPAR-γΔC on insulin signaling, specifically on the insulin-induced tyrosine phosphorylation of IRS1 and IRS2, in mature 3T3-L1 adipocytes. Cells infected with Adex-PPAR-γ-WT or Adex-PPAR-γΔC at an MOI of 60 PFU/cell were incubated in the presence of 100 nmol/l insulin for 1 min at 37°C. Cell lysates were then subjected to immunoprecipitation with polyclonal antibodies to IRS1 or to IRS2, and the resulting precipitates were subjected to immunoblot analysis with a monoclonal antibody to phosphotyrosine. Total cell lysates were also directly subjected to immunoblot analysis with antibodies to IRS1 or to IRS2 for determining the abundance of these two proteins. Overexpression of PPAR-γΔC resulted both in a decrease in the amount of IRS2 protein consistent with the effect of PPAR-γΔC on the abundance of IRS2 mRNA (Fig. 5C) and in inhibition of insulin-induced tyrosine phosphorylation of this protein (Fig. 6A). Overexpression of PPAR-γΔC had no effect either on the amount of IRS1 protein, even though it induced a small decrease in the abundance of IRS1 mRNA (Fig. 5C), or on the extent of insulin-induced tyrosine phosphorylation of this protein (Fig. 6A). Overexpression of PPAR-γ-WT had no substantial effect either on the abundance of IRS1 or IRS2 proteins or on the insulin-induced tyrosine phosphorylation of these proteins (Fig. 6A).

Treatment of cells with BRL49653 or TNF-α had no marked effect either on the expression of IRS1 or on the insulin-induced tyrosine phosphorylation of this protein (Fig. 6B). However, BRL49653 increased both the amount of IRS2 protein and the extent of insulin-induced tyrosine phosphorylation of IRS2. In contrast, TNF-α both reduced the amount of IRS2 protein and inhibited insulin-induced tyrosine phosphorylation of this protein (Fig. 6B).

Furthermore, we carried out glucose transport assay to assess the effects of the alterations of protein expressions induced by PPAR-γΔC in mature 3T3-L1 adipocytes. Overexpression of PPAR-γΔC reduced insulin-stimulated glucose transport to the extent of 77.7% at an MOI of 60 PFU/cell in 3T3-L1 adipocytes, although overexpression of PPAR-γ-WT showed no significant changes on insulin-stimulated glucose uptake (Fig. 7A). BRL49653 increased
both basal and insulin-stimulated glucose uptake by 27 and 39%, respectively. Conversely, TNF-α decreased insulin-stimulated glucose uptake to the extent of 66.3% with the significant increase of basal glucose uptake by 47% (Fig. 7B).

Finally, we compared the expression of IRS1 and IRS2 between 3T3-L1 preadipocytes and mature 3T3-L1 adipocytes. IRS1 was detected in substantial amounts in 3T3-L1 preadipocytes, and the abundance of this protein was moderately increased during adipocyte differentiation (Fig. 8). In contrast, IRS2 was barely detectable in preadipocytes, but its expression greatly increased during the differentiation of these cells to mature adipocytes. The expression of IRS2 thus seemed more closely related to the degree of adipocyte differentiation than that of IRS1 in 3T3-L1 cells.

**DISCUSSION**

To determine the functional role of PPAR-γ in mature adipocytes, we studied 3T3-L1 adipocytes that had been cultured for >15 days after the induction of differentiation. 3T3-L1 cells subjected to such long-term culture are considered to be representative of mature adipocytes, given that the expression of proteins linked to adipocyte differentiation does not change significantly after 8 days of such treatment (31). Overexpression of PPAR-γ-WT in mature 3T3-L1 adipocytes increased both cell size and the intracellular content of triglyceride. In contrast, overexpression of PPAR-γ-ΔC resulted in significant decreases in both cell size and intracellular triglyceride content. Furthermore, overexpression of PPAR-γ-ΔC in mature 3T3-L1 adipocytes promoted lipolysis, inhibited FFA uptake, reduced the amounts of mRNAs for several key proteins.
involved in lipid metabolism and insulin signaling, and inhibited insulin-stimulated glucose transport. These observations thus suggest that PPAR-\(\gamma\) plays an important role in mature 3T3-L1 adipocytes in addition to its functions in adipocyte differentiation.

Overexpression of PPAR-\(\gamma\)-ΔC in mature adipocytes reduced the abundance of mRNAs encoding proteins that participate in triglyceride and FFA metabolism, including aP2, ACS, CD36, LPL, HSL, and perilipin, effects that likely contributed to the associated enhancement of lipolysis and inhibition of FFA uptake. These observations suggest that PPAR-\(\gamma\) serves to maintain the expression of genes whose products play a role in lipid metabolism, in particular those that encode proteins that function in FFA uptake and intracellular storage of triglycerides. CD36 acts together with ACS, an enzyme that catalyzes the conversion of incorporated fatty acids to acyl-CoA derivatives, to prevent the efflux of FFAs and thereby to render FFA transport across the cell membrane unidirectional. Thus, the decrease in the abundance of CD36 and ACS mRNAs in adipocytes overexpressing PPAR-\(\gamma\)-ΔC likely underlies the reduction in the rate of FFA uptake apparent in these cells.

The changes in cell morphology and gene expression induced by overexpression of PPAR-\(\gamma\)-ΔC in mature adipocytes resembled those induced by treatment of the cells with TNF-\(\alpha\). Zhang et al. (27) previously showed that dedifferentiation of 3T3-L1 adipocytes induced by TNF-\(\alpha\) was accompanied by a decrease in the abundance of PPAR-\(\gamma\) mRNA, which preceded a reduction in the amounts of C/EBP and aP2 proteins. Xing et al. (32) also showed that exposure of fully differentiated adipocytes to TNF-\(\alpha\) resulted in the rapid disappearance of PPAR-\(\gamma\) mRNA and protein. Indeed, in the present study, RT-PCR analysis revealed that treatment of mature 3T3-L1 adipocytes with TNF-\(\alpha\) resulted in the rapid disappearance of PPAR-\(\gamma\) mRNA and protein. In the present study, RT-PCR analysis revealed that treatment of mature 3T3-L1 adipocytes with TNF-\(\alpha\) resulted in the rapid disappearance of PPAR-\(\gamma\) mRNA and protein.
cytes with TNF-α (10 ng/ml) for 48 h resulted in an ~45% decrease in the amount of endogenous PPAR-γ mRNA (data not shown). These observations suggest that the antiadipogenic effects of TNF-α, including a decrease in intracellular triglyceride content, in fully differentiated adipocytes may be mediated, at least in part, by a reduction in PPAR-γ expression.

In contrast to the effects of TNF-α or PPAR-γ-ΔC, treatment of mature 3T3-L1 adipocytes with BRL49653 induced an increase in the abundance of mRNAs for proteins involved in fatty acid and triglyceride metabolism, including those encoding αP2, ACS, CD36, HSL, and perilipin. These results are consistent with previous data showing that TZDs promote adipocyte differentiation, resulting in increased expression of fatty acid transporter protein, αP2, ACS, and perilipin. Again, these observations indicate that PPAR-γ plays an important role in FFA uptake and triglyceride storage in mature adipocytes. In contrast, however, it was recently reported that PD068235, a potent antagonist of PPAR-γ, did not affect the phenotype of terminally differentiated 3T3-L1 adipocytes. The reasons for the difference between these results and ours are unknown. A possible explanation is that PD068235 may not antagonize the endogenous PPAR-γ ligand in mature 3T3-L1 adipocytes.

Unsaturated fatty acids and eicosanoids have been identified as natural ligands for PPAR-γ. It is also likely that such molecules function as ligands of recombinant PPAR-γ-WT in the mature 3T3-L1 adipocytes of the present study. Given the marked effects of exposure of mature 3T3-L1 adipocytes to BRL49653 on the expression of genes important in lipid metabolism, the reason for the lack of effect of overexpression of PPAR-γ-WT in this regard remains to be determined.

Fat and skeletal muscle are the two major sites of insulin-stimulated glucose uptake. The maturation of adipocytes is accompanied by the acquisition of insulin sensitivity. Insulin action in adipocytes is mediated by a cascade of signaling events, which begin with the tyrosine phosphorylation of the IR, the subsequent tyrosine phosphorylation of IRS1 and IRS2, and the consequent activation of phosphoinositide 3-kinase. This last event results in the translocation of GLUT4 from intracellular vesicles to the plasma membrane to facilitate glucose uptake. We have now shown that overexpression of PPAR-γ-ΔC reduced the abundance of IR, IRS1, and IRS2 mRNAs in addition to that of the mRNA for the insulin-responsive GLUT4. The expression of these proteins is increased during adipogenesis. In contrast, overexpression of PPAR-γ-ΔC in mature 3T3-L1 adipocytes increased the amount of the mRNA for the ubiquitous GLUT1, whose expression has previously been shown to be downregulated during adipocyte differentiation. The reduction of insulin-stimulated glucose transport in 3T3-L1 adipocytes overexpressing PPAR-γ-ΔC may be partially explained by the reduced expression of IRS1, IRS2, and GLUT4. Thus, our data suggest that PPAR-γ plays an important role in maintaining insulin sensitivity in mature 3T3-L1 adipocytes by regulating the expression of genes that encode components of the insulin signaling pathway. In addition, the increase of both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes with BRL49653 treatment is thought to reflect the increased expression levels of GLUT1 and GLUT4 in these cells. The decrease of insulin-stimulated glucose uptake in 3T3-L1 adipocytes with TNF-α treatment is supposed to result from the decreased expression of GLUT4 or IRS2. The increase of basal glucose uptake in 3T3-L1 adipocytes with TNF-α treatment can be thought to reflect the amount of GLUT1 whose expression level shows a tendency to increase.

In the present study, the abundance of IRS2 mRNA in mature 3T3-L1 adipocytes was increased by exposure to BRL49653 for 48 h and was reduced by similar treatment with TNF-α. However, the amount of IRS1 mRNA was not significantly affected by either of these agents. Exposure of 3T3-L1 adipocytes to TNF-α for 72 to 96 h but not for 48 h was previously shown to induce a substantial decrease in the expression of the IR and IRS1 in 3T3-L1 adipocytes. Furthermore, exposure of 3T3-L1 adipocytes or human adipocytes to TZDs for 48 h was recently shown to increase expression of the IRS2 gene but not that of the IRS1 gene. These previous results are thus consistent with those of the present study.

Overexpression of PPAR-γ-ΔC or TNF-α treatment reduced, whereas BRL49653 treatment increased, both the abundance of IRS2 and the extent of insulin-induced tyrosine phosphorylation of this protein in mature 3T3-L1 adipocytes. We think that the detected changes of tyrosine phosphorylation of IRS2 in PPAR-γ-ΔC- or TNF-α–treated cells depend mainly on the reduced expression levels of IRS2. Not PPAR-γ-ΔC, TNF-α, or BRL49653 affected IRS1 expression or insulin-induced tyrosine phosphorylation of IRS1. These results are consistent with the markedly greater induction of IRS2 expression than of IRS1 expression in association with adipocyte differentiation. The IRS1 and IRS2 genes thus may be regulated differentially in 3T3-L1 cells.

The identification of dominant negative mutations of the PPAR-γ gene in individuals with severe insulin resistance supports an important role for PPAR-γ in the pathways responsible for systemic insulin sensitivity in humans. The accumulation of intramyocellular triglyceride has also been shown to be related to the development of insulin resistance. The activation of PPAR-γ in adipocytes and promotion of adipocyte differentiation by TZDs, resulting in increased FFA uptake and triglyceride clearance by adipose tissue, thus may redirect fatty acids
from muscle to adipose tissue and thereby relieve the fatty acid–mediated inhibition of glucose utilization in muscle cells. PPAR-γ may thus contribute to an improvement in overall insulin sensitivity by maintaining the characteristics of FFA uptake and triglyceride storage in mature adipocytes. Our results are consistent with a recent study that showed that PPAR-γ activation with the potent PPAR-γ ligand GW1929 stimulated the expression of genes involved in lipogenesis and fatty acid metabolism in adipose tissue in Zucker diabetic fatty rats (44).

In summary, our data suggest that PPAR-γ contributes to FFA uptake and triglyceride accumulation in mature adipocytes by maintaining the expression of genes whose products mediate these processes. Furthermore, PPAR-γ regulates the expression of genes whose products are responsible for insulin sensitivity, including those for the IR as well as IRS and GLUT proteins. PPAR-γ thus functions not only in inducing adipogenesis but also in maintaining the expression of genes that confer on cells the characteristics of mature adipocytes.

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