Isolation of Genes Regulated by Peroxisome Proliferator-Activated Receptor γ (PPARγ) by Two-Dimensional Electrophoresis and Mass Spectrometry

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To isolate the genes regulated by peroxisome proliferator-activated receptor γ (PPARγ), we first developed a stable transformant expressing PPARγ. Using cytosol and nuclear fractions prepared from this cell line, we performed two-dimensional electrophoresis. A comparison of the electrophoretic patterns with those from control cells revealed many spots to be up-regulated or down-regulated proteins. Some spots were subjected to mass spectrometric analyses, and triosephosphate isomerase and galectin-3 were identified as proteins down-regulated by PPARγ. Moreover, Northern blot analyses revealed that the expression level of both genes decreased in PPARγ-expressing cells. These results strongly suggest that triosephosphate isomerase and galectin-3 are target genes whose expression is mediated by PPARγ.

Key words ——— peroxisome proliferator-activated receptor γ, two-dimensional electrophoresis, mass spectrometry, adipocyte differentiation, transcription factor, gene expression

INTRODUCTION

The peroxisome proliferator-activated receptor (PPAR) belongs to a nuclear hormone receptor superfamily which comprises over 150 different proteins and is involved in transcriptional responses to extracellular signals.[1] PPAR was originally identified as a receptor activated by peroxisome proliferator and at present at least three gene subtypes of PPARs (PPARα, PPARγ, PPARδ) are known.[2] PPARδ is also called PPARβ and NUC1. PPARs are implicated in a variety of functions in health and disease including cellular differentiation, apoptosis and tumorigenesis.[2,3] The expression of PPARs differs among tissues; PPARα is highly expressed in liver, kidney, and heart. PPARδ shows a ubiquitous expression, while PPARγ is expressed predominantly in adipose tissue and the immune system.[2,4]

Adipocyte differentiation is a complex process in which the expression of many transcription factors and adipocyte-specific genes is programmatically regulated. Several lines of evidence suggest that three families of transcription factors, PPARγ, CCAAT/enhancer-binding proteins (C/EBPs) and sterol regulatory element-binding protein-1 (SREBP-1), are important regulators of the differentiation of preadipocytes into adipocytes.[5–8]

Among these three families, PPARγ is a most important transcription factor in adipogenesis.[8] Two main isoforms, PPARγ1 and PPARγ2, are produced using different promoters from a single gene,[9–11] and PPARγ2 participates in adipocyte differentiation.[11]

PPARγ2 forms a heterodimer with the retinoid X receptor α (RXRα) and binds to a common consensus response element called the PPAR response element (PPRE) consisting of a direct repeat of two hexanucleotides spaced by one nucleotide (DR1, 5′-AGGTCA A AGGTCA-3′) in various gene promoters.[1] For example, lipoprotein lipase,[12] adipocyte

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fatty acid binding protein (aP2),[13] phosphoenolpyruvate carboxykinase[14] and acylCoA synthase[15] are known as targets of PPARγ.

However, the mechanism of adipocyte differentiation through PPARγ is not fully understood, and the genes regulated by PPARγ remain to be identified. Proteome analysis by mass spectrometry (MS) is a powerful technology for the identification of proteins.[16–19] In this paper, we established a PPARγ-expressing stable transformant. Using this cell line, we performed two-dimensional electrophoresis (2DE), and found that the expression of many proteins is regulated by PPARγ. Some spots were identified using matrix-assisted laser desorption/ionization-time-of-flight MS (MALDI-TOF MS).

MATERIALS AND METHODS

Cells and Cell Culture —— Mouse NIH-3T3 (clone 5611, JCRB 0615, Japanese Cancer Research Bank, Japan) fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). PT67, a packaging cell line derived from NIH-3T3 cells (Clontech Lab. Inc., Palo Alto, CA, U.S.A.), was maintained in DMEM containing 10% fetal bovine serum (FBS).

Establishment of PPARγ-Expressing NIH-3T3 Cell Lines —— A stable transformant expressing PPARγ was established using the Retro-X System (Clontech Lab. Inc.) according to the manufacturer's instructions, except that pDON-AI (TAKARA BIO Inc., Otsu, Japan) was used as a retroviral vector. A full-length cDNA encoding PPARγ derived from PPARγ2/pSVSPORT[13] was inserted into pDON-AI. The PT67 packaging cell line was cultured in DMEM containing 10% FBS and transfected at 80% confluence by calcium phosphate coprecipitation[20] with either pDON-AI-PPARγ or empty pDON-AI. The viruses transiently expressed were harvested 72 hr after transfection and applied to the NIH-3T3 cells in DMEM containing 10% CS and 4 μg/ml of polybrene for the infection. The resultant cells were split 1 : 4 24 hr after infection and replated in DMEM containing 10% CS and 0.5 mg/ml of G418. After 8–10 days incubation, a G418-resistant colony was removed and stored. As a control, the empty vector, pDON-AI, was transfected in the same way, and a G418-resistant control stable transformant was established.

Cell Culture of Stable Transformants —— PPARγ–expressing stable transformant and control stable transformant cells were cultured to confluence in DMEM containing 10% CS. Two days after the cells reached confluence, the medium was changed to a fresh one with or without 0.5 μM BRL49653 (5-(4-[2-(N-methyl-N-(2-pyridyl)amino) ethoxy] benzyl)thiazolidine-2,4-dione maleic acid salt, a gift from GlaxoSmithKline), a ligand for PPARγ. After 48 hr incubation, the cells were harvested and cytosol and nuclear fractions were prepared as described below.

RNA Isolation and Northern Blot Analysis —— Total RNA was isolated using TRIzol (Gibco BRL Life Technologies, Gaithersburg, MD, U.S.A.). For Northern blot analysis, 25 μg of total RNA was electrophoresed on a 1% agarose gel containing 2% formaldehyde, and then transferred to a Hybond-N^+ nylon membrane (Amersham Biosciences Crop., Piscataway, NJ, U.S.A.). The filter was hybridized with each probe which was labeled with [α-^{32}P]dCTP using a random labeling kit (TAKARA BIO Inc.). The filter was washed and then exposed to X-ray film.

Preparation of Cytosol and Nuclear Fractions —— After harvesting the cells, the cell pellet was rinsed with PBS, and suspended in lysis buffer [20 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM spermidine, 1 mM dithiothreitol (DTT), 10% glycerol, and protease inhibitors (1 mM AEBSF, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin A, and 0.3 μg/ml of antipain)]. NP40 was added at a final concentration of 0.03% to the suspension, and kept on ice. After centrifugation at 12000 rpm and 4°C, the resultant supernatant was dialysed against 0.1 M HM buffer [25 mM Hepes (pH 7.9), 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, and 0.1 M KCl] to obtain the cytosol fraction. The pellet after centrifugation was resuspended in the lysis buffer, KCl was added at a final concentration of 1 M, and the suspension was kept on ice. After centrifugation at 98000 rpm for 30 min at 4°C, the resultant supernatant was dialysed against 0.1 M HM buffer, and stored as a nuclear fraction.

2DE —— As the first-dimension step, isoelectric focusing (IEF) was performed using Immobiline DryStrip (pH 3–10, 11 cm) and Multiphor II (Amersham Biosciences) according to the manufacturer's instructions. Twenty micrograms of the cytosol or nuclear fraction in 0.1 M HM buffer was mixed with an equal volume of sample solution [8 M urea, 2% mercaptoethanol, 2% pharmalyte pH 3–10, 0.5% Triton X-100, and bromophenol blue (BPB)], electrophoresed, and separated based on iso-

electric points (pIs). The DryStrip after IEF was immersed in buffer A [50 mM Tris–HCl (pH 6.8), 6 M urea, 30% glycerol, 1% SDS, and 16 mM DTT] for 10 min, and buffer B [50 mM Tris–HCl (pH 6.8), 6 M urea, 30% glycerol, 1% SDS, and 243 mM iodoacetamide] for 10 min. This DryStrip was laid on the 10% polyacrylamide gel (PAGE) and the second-dimension step, SDS-PAGE was performed. The gel was stained with silver using Silver Stain II Kit Wako (Wako Pure Chemical, Osaka, Japan).

MS —— For the identification of protein spots, Immobiline DryStrip (pH 3–10, 18 cm) was used, and 2DE was performed as described above, except that sixty micrograms of proteins were applied. The gel was stained with silver by the method of Shevchenko *et al.*[21] After cutting the gel, the gel piece was destained, dried, digested with trypsin, and finally suspended in 5% acetonitrile and 0.1% TFA.[22] For MS, Voyager Elite DE MALDI-TOF MS (Applied Biosystems, Foster city, CA, U.S.A.) was used. As standard proteins, angiotensin I, ACTH, and α-cyano-4-hydroxycinnamic acid (CHCA) were utilized. The identification of protein was performed by the mass finger-print method using MASCOT.

RESULTS

Establishment of PPARγ-Expressing NIH-3T3 Cell Lines

To identify the target genes of PPARγ, we first established a PPARγ-expressing cells by introducing the PPARγ expression vector into NIH-3T3 cells. The exogenous expression of PPARγ in the stable transformant was observed by Northern blot analysis, while the endogenous PPARγ expression was detected in neither PPARγ-expressing stable transformant cells nor control cells harboring empty vector (Fig. 1A). Next we performed a Northern blot analysis on the expression of aP2 which is a target gene of PPARγ.[13] As shown in Fig. 1B, aP2 expression increased gradually in the presence of BRL49653, which is a ligand for PPARγ. These results indicated that we had established a PPARγ-expressing stable transformant, which is responsive to PPARγ ligand.

2DE Analyses of Nuclear and Cytosol Fractions Obtained from PPARγ-Expressing Cells and Control Cells

PPARγ-expressing cells and control cells were cultured to confluence in DMEM containing 10% CS. At 2 days after confluence was reached, the medium was renewed and incubation continued for another 2 days. In the PPARγ-expressing cells, the culture was done with or without 0.5 μM BRL49653, a ligand for PPARγ. Then, the cells were harvested and nuclear and cytosol fractions were prepared from three cells; PPARγ-expressing cells with ligand, PPARγ-expressing cells without ligand, and control cells.

Using these protein fractions, we next performed 2DE analyses, and stained the gels with silver. Many proteins in the nuclear fraction prepared from PPARγ-expressing cells without ligand were up- or down-regulated by PPARγ expression (Figs. 2A and 2B). When the ligand for PPARγ was added to the PPARγ-expressing cells, different proteins were identified as up- or down-regulated (Figs. 2B and 2C). The spot A in Fig. 2D was identified by MS as described below.

Similarly, it was found that the expression of lots of proteins in the cytosol fraction was up- or down-regulated: the PPARγ-expressing cells without ligand versus control cells (Figs. 3A and 3B), and the PPARγ-expressing cells with ligand versus the PPARγ-expressing cells without ligand (Figs. 3B and 3C). The spots B–G in Fig. 3D were identified by MS as described below.

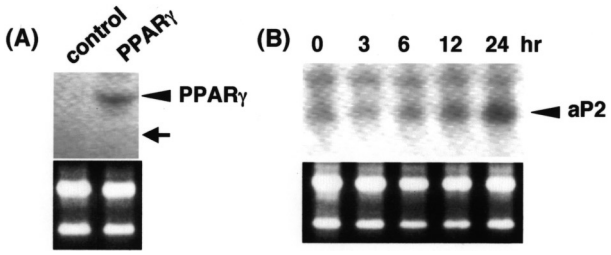

Fig. 1. Establishment of PPARγ-Expressing Stable Transformant

(A) The ectopic expression of PPARγ in NIH-3T3 stable transformants. Northern blot analyses were performed for 25 μg of RNAs prepared from two stable transformants; control, with the empty vector only, and PPARγ, with the PPARγ-expressing vector in NIH-3T3 cells. The blots were hybridized with PPARγ. The arrowhead and arrow indicate the exogenous and endogenous expression of PPARγ, respectively, although the endogenous expression was not observed in both cells. Staining with ethidium bromide (EtBr) for ribosomal RNA is shown as a control. (B) Time course of aP2 expression in PPARγ-expressing cells. PPARγ-expressing cells were cultured in the presence of PPARγ ligand, 0.5 μM BRL49653. Total RNA was isolated from cells at various time points of incubation, and 25 μg of RNA was used for Northern blot analysis. Staining with EtBr for ribosomal RNA is shown as a control.

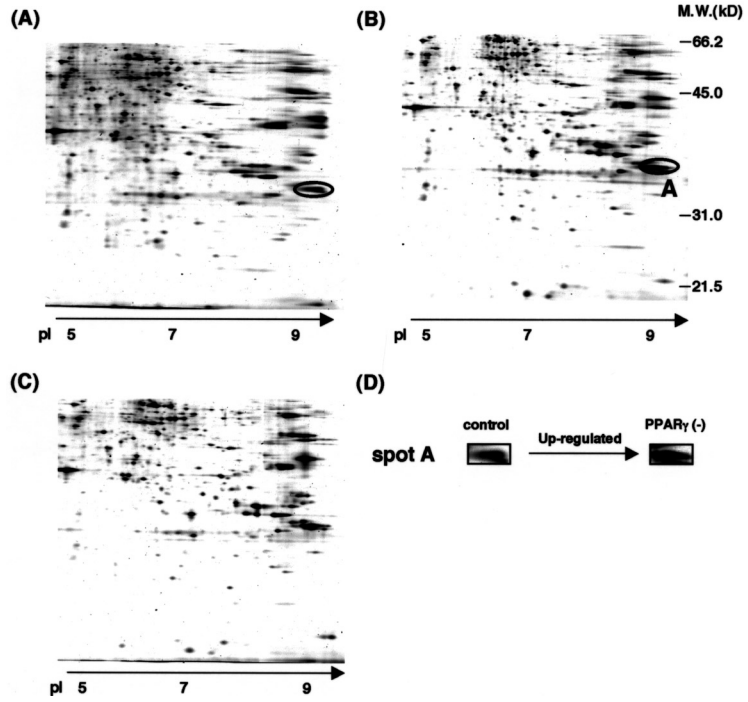

Fig. 2. 2DE Pattern of Nuclear Fraction from PPARγ-Expressing Cells and Control Cells

The nuclear fractions were prepared from PPARγ-expressing cells and control cells cultured with or without ligand. Twenty μg of the nuclear fraction was separated by 2DE, and stained with silver. (A) control cells without ligand. (B) PPARγ-expressing cells without ligand. (C) PPARγ-expressing cells with ligand. Open symbol revealed the up-regulated protein by PPARγ, and this spot was magnified in panel (D) and subjected to MALDI-TOF MS analysis for the identification. PPARγ(–); PPARγ-expressing cells without ligand.

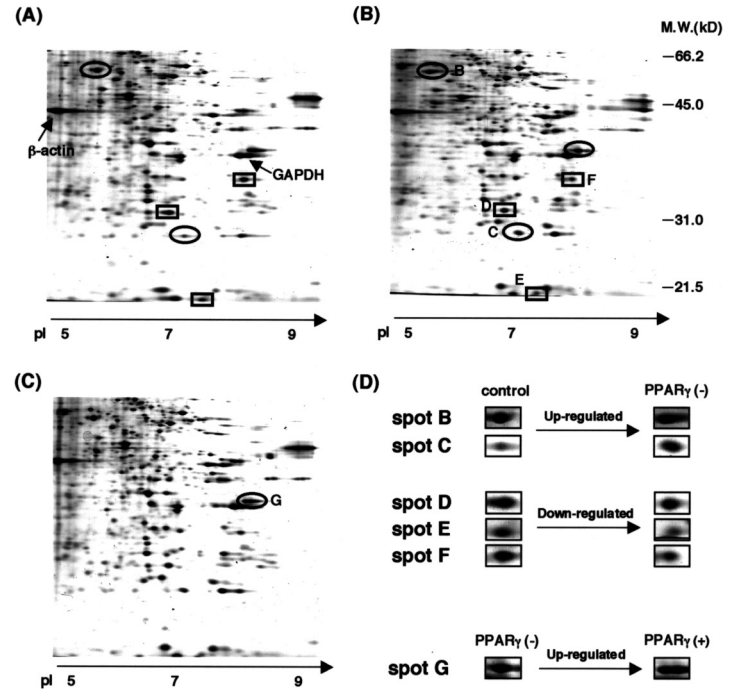

Fig. 3. 2DE Pattern of Cytosol Fraction from PPARγ-Expressing Cells and Control Cells

The cytosol fractions were prepared from PPARγ-expressing cells and control cells cultured with or without ligand. Twenty μg of the nuclear fraction was separated by 2DE, and stained with silver. (A) control cells without ligand. (B) PPARγ-expressing cells without ligand. (C) PPARγ-expressing cells with ligand. Open and square symbols revealed the up-regulated and down-regulated proteins by PPARγ or ligand, respectively. (D) Some spots were magnified and subjected to MALDI-TOF MS analyses for the identification. PPARγ(–); PPARγ-expressing cells without ligand, PPARγ(+); PPARγ-expressing cells with ligand. GAPDH and β-actin were also identified as the unchanged proteins, and the spots were shown in panel (A).

Table 1. List of Identified Proteins which are Up- or Down-Regulated by PPARγ Expression or Ligand for PPARγ

spot	fraction	expression	regulator	protein identified
A	nuclear	up-regulated	PPARγ	hnRNP A2/B1
B	cytosol	up-regulated	PPARγ	similar to ER-60 protease
C	cytosol	up-regulated	PPARγ	osteoblast specific factor 3 (OSF-3)
D	cytosol	down-regulated	PPARγ	triosephosphate isomerase
E	cytosol	down-regulated	PPARγ	cyclophilin A
F	cytosol	down-regulated	PPARγ	galectin-3
G	cytosol	up-regulated	ligand	mitochondrial malate dehydrogenase

Identification of Up- or Down-Regulated Proteins by PPARγ

We next performed MALDI-TOF MS analyses to identify the proteins whose expression was found to be up- or down-regulation by PPARγ in the 2DE analyses. The proteins were digested with trypsin in the presence of *n*-octylglucoside, and the resultant peptide mixtures were subjected to MALDI-TOF MS. A search of the database with the peptide mass finger-print method identified several proteins.

Although many spots were subjected to MALDI-TOF MS, only 7 spots (Figs. 2D and 3D) were identified, probably due to the low amount of protein or the low sensitivity of MS. The identified genes were listed in Table 1. Of these, malate dehydrogenase (spot G in Fig. 3) is known to be a PPARγ-target gene and the promoter of this gene has a DR1-like sequence. Moreover, this gene is thought to be a marker gene in the late stages of adipocyte differentiation.[22,23] In our experiment, malate dehydrogenase was identified as a protein up-regulated by PPARγ ligand in PPARγ-expressing cells.

Thus, although it seemed to work well in this experiment, 2DE analysis has low quantitative value. Therefore, we next determined the expression level of the genes using Northern blot analyses. As shown in Fig. 4, the expression of triosephosphate isomerase and galectin-3 (34 kDa β-galactoside-binding lectin) (spots D and F, respectively, in Fig. 3) was down-regulated in PPARγ-expressing cells, strongly indicating that triosephosphate isomerase and galectin-3 are target proteins negatively regulated by PPARγ expression. The expression level of osteoblast specific factor 3 (OSF-3) and cyclophlin A was not significantly changed (data not shown).

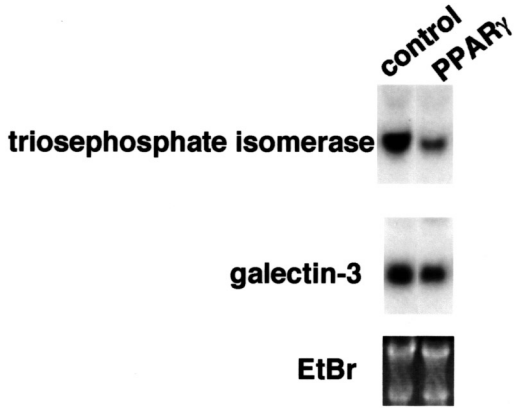

Fig. 4. Northern Blot Analyses of Genes Identified by 2DE-MS
PPARγ-expressing cells in the presence of PPARγ ligand, 0.5 μM BRL49653 and control cells were cultured for two days. Total RNA was isolated from these cells, and 18 μg of RNA was used for Northern blot analysis. Staining with EtBr for ribosomal RNA is shown as a control.

DISCUSSION

PPARγ binds to various promoters including lipoprotein lipase,[12] aP2,[13] phosphoenolpyruvate carboxykinase[14] and acylCoA synthase,[15] which are activated during adipogenesis. PPARγ also represses the expression of leptin.[24] Thus, PPARγ is known to be quite an important transcription factor which functions as an activator or a repressor during adipocyte differentiation. Moreover, the ligand for PPARγ is reported to improve insulin resistance.[3]

The molecular mechanisms of adipocyte differentiation and the improvement of insulin resistance remain to be resolved. One reason for this seems to be that the number of isolated and characterized genes as targets of PPARγ is not very large. Therefore, we performed 2DE-MS analyses for the identification of genes regulated by PPARγ.

In this paper, we used the single clone of transformant. Therefore, it is possible that the differences of protein expression were caused by changed characteristics of stable transformant, not by PPARγ expression or the addition of the ligand. Therefore, we next performed Northern blot analyses whether the expression of these genes is regulated by PPARγ or not, and showed that triosephosphate

isomerase and galectin-3 were really down-regulated by PPARγ expression.

The malate dehydrogenase was identified as a positively regulated protein. Its gene was already characterized as a target of PPARγ whose promoter contains the PPARγ-binding sequence DR1.[22,23] However, the known other genes, such as aP2 and lipoprotein lipase, regulated by PPARγ expression were not identified, probably because of the low quantitative value of 2DE analysis. It is also possible that the low amount of protein or the low sensitivity of MS failed to identify the proteins.

We identified triosephosphate isomerase and galectin-3 as proteins negatively regulated by PPARγ expression. The Northern blot analyses revealed that expression levels of these two genes decreased in PPARγ-expressing cells, suggesting regulation at the mRNA level.

Triosephosphate isomerase (D-glyceraldehyde-3-phosphate ketol-isomerase, E.C.5.3.1.1) is an enzyme which has catalytic activity responsible for the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate in the glycolytic and gluconeogenic pathways.[25,26] Although a promoter analysis was conducted for the human gene, a DR1-like sequence was not found.[25] A partial sequence of the mouse triosephosphate isomerase gene was submitted to the NCBI database (accession number; L31777). The search for *cis*-acting elements revealed the existence of a DR1-like sequence in the intron. A DNA-binding analysis and a reporter analysis are required for the characterization of this sequence, and also the gene promoter.

Galectin-3 is known as advanced glycation endproducts (AGE)-binding receptor-3, and is a multifunctional lectin with (anti)adhesive and growth-regulating properties. Galectin-3 contributes to the development of diabetic glomerular disease, and is associated with chemotaxis, angiogenesis, oxidative stress, cell proliferation and programmed cell death.[27–29] Although promoter analyses of the human and mouse galectin-3 gene were reported, a DR1-like sequence is yet to be found. Rather, Jun and NFκB may contribute to the regulation of galectin-3 expression.[30–32]

The expression level of many genes were changed in PPARγ-expression cells without ligand (Fig. 2). However, it does not mean the independence of the ligand for PPARγ activation. The undetectable endogenous ligand may activate the PPARγ activity, or it is also possible that the enough amount of PPARγ expressed in PPARγ-expressing cells does not require the activation by ligand. These possibilities should be clarified by detailed analyses.

In this paper, we newly identified triosephosphate isomerase and galectin-3 as a negative PPARγ-target. Further characterization including promoter analysis is needed to elucidate the mechanisms behind the negative regulation of these two genes by PPARγ.

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