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Isolation of up- or down-regulated genes in PPARγ-expressing NIH-3T3 cells during differentiation into adipocytes

Masaaki Okuno[a], Emi Arimoto[a,b], Makoto Nishizuka[a,b], Tsutomu Nishihara[a], Masayoshi Imagawa[b,*]

[a] *Laboratory of Environmental Biochemistry, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-Oka, Suita, Osaka 565-0871, Japan*

[b] *Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467-8603, Japan*

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Abstract Adipocyte differentiation is a complex process in which the expression of many transcription factors and adipocyte-specific genes is regulated under a strict program. The peroxisome proliferator-activated receptor γ (PPARγ), a member of the steroid/thyroid nuclear hormone receptor superfamily of ligand-activated transcription factors, is an important regulator of adipocyte gene expression and differentiation. In this study, we tried to identify downstream target genes of PPARγ, by using PPARγ-expressing cells and a subtractive cloning strategy, and isolated cDNA clones which were up-regulated or down-regulated by PPARγ. Northern blot analyses revealed that the expression levels of the aldehyde dehydrogenase-2-like, type VI collagen alpha 3 subunit, cellular retinoic acid binding protein 1 and thrombospondin 1 are changed during the differentiation of mouse 3T3-L1 preadipocyte cells, indicating that these genes might be downstream targets of PPARγ in adipocyte differentiation.

Key words: Peroxisome proliferator-activated receptor γ; Adipocyte differentiation; Gene expression; Obesity; Subtraction method

1. Introduction

Obesity is associated with type 2 diabetes mellitus characteristic of insulin (Ins) resistance, and both diseases are serious health problems in developed countries. Obesity results from an imbalance between energy intake and energy expenditure, and this imbalance leads to a pathologic accumulation of adipose tissue. Adipocyte differentiation is a complex process in which the expression of many transcription factors and adipocyte-specific genes is programmatically regulated. Evidence is accumulating that three families of transcription factors, peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding proteins and sterol regulatory element-binding protein-1, are important regulators for the differentiation of preadipocytes into adipocytes [1–3].

PPARγ is a member of the steroid/thyroid nuclear hormone receptor superfamily of ligand-activated transcription factors. PPARγ forms a heterodimer with the retinoid X receptor α and binds to common consensus response elements called PPAR response elements (PPREs) consisting of a direct repeat of two hexanucleotides spaced by one nucleotide (DR1, 5′-AGGTCA A AGGTCA-3′) [4]. PPREs have been reported to exist in the promoter region of the genes encoding lipoprotein lipase [5], adipocyte fatty acid binding protein (aP2) [6], phosphoenolpyruvate carboxykinase [7] and acylCoA synthase [8]. Ectopic expression of PPARγ in NIH-3T3 fibroblasts leads to adipogenic conversion in the presence of its ligands [9,10]. Moreover, loss-of-function experiments using PPARγ$^{-/-}$ mice or embryonic stem cells have confirmed the requirement of PPARγ for adipogenesis in vivo and in vitro [11–13].

However, the mechanism of adipocyte differentiation through PPARγ is not fully understood, and the genes regulated by PPARγ remain to be identified. In this paper, therefore, we tried to isolate downstream target genes of PPARγ by using PPARγ-expressing fibroblastic cells and a subtractive cloning strategy.

2. Materials and methods

2.1. Cells and Cell Culture

Mouse NIH-3T3 (clone 5611, JCRB 0615, Japanese Cancer Research Bank) fibroblasts and mouse 3T3-L1 (ATCC CL173) preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (CS). PT-67, a packaging cell line derived from NIH-3T3 cells (Clontech Lab. Inc., Palo Alto, CA, USA), was maintained in DMEM containing 10% fetal bovine serum (FBS).

2.2. 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 Biomedicals, Kusatsu, Japan) was used as a retroviral vector. A full-length cDNA encoding PPARγ derived from PPARγ2/pSVSPORT [6] was inserted into pDON-AI. The PT-67 packaging cell line was cultured in DMEM containing 10% FBS and transfected at 80% confluence by calcium phosphate coprecipitation [14] with either pDON-AI-PPARγ or empty pDON-AI. The viruses transiently expressed were harvested 72 h after transfection and applied to the NIH-3T3 cells in DMEM containing

*Corresponding author. Fax: (81)-52-836-3455.
E-mail address: imagawa@phar.nagoya-cu.ac.jp (M. Imagawa).

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; aP2, adipocyte fatty acid binding protein; CRABP-1, cellular retinoic acid binding protein 1; CS, calf serum; Dex, dexamethazone; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IBMX, isobutyl methyxanthine; Ins, insulin; PCR, polymerase chain reaction; TSP1, thrombospondin 1

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10% CS and 4 μg/ml of polybrene for the infection. The resultant cells were split 1:4 24 h after infection and replated in DMEM containing 10% CS and 0.5 mg/ml of G418 to eliminate uninfected cells for 10 days.

2.3. Cell culture for induction of differentiation

Following the drug selection, stable virally infected cells were cultured to confluence in DMEM containing 10% CS and 5 μg/ml of Ins. At 2 days after reaching confluence, the cells were exposed to DMEM supplemented with 10% FBS, 1 μM dexamethazone (Dex) and 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 SmithKline Beecham Pharmaceuticals) for 48 h. After this treatment, the medium was replaced with DMEM containing 10% FBS and 0.5 μM BRL49653. At 8 days postinduction, the cells were fixed with formalin and stained with Oil Red O.

For differentiation of 3T3-L1 preadipocytes, 2 day postconfluent preadipocytes were cultured with 10 μg/ml of Ins, 1 μM Dex and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) in DMEM supplemented with 10% FBS. After 48 h, the culture medium was replaced with DMEM supplemented with 10% FBS and 5 μg/ml of Ins.

2.4. Subtraction cloning

2 μg each of Poly $(A)^+$ RNA from untreated NIH-vector cells (a stable cell line integrated with the pDON-AI vector) or NIH-PPARγ cells (a stable cell line integrated with pDON-AI-PPARγ) incubated with inducers described above for 1 day was used for cDNA synthesis. After *Rsa*I digestion of cDNAs, the cDNA fragments were used for subtractive hybridization and polymerase chain reaction (PCR) amplification according to the PCR-select cDNA subtraction protocol (Clontech Lab. Inc.).

The enriched cDNA fragments were digested with *Rsa*I. The resultant fragments were recovered with DE81 paper, subcloned into *Eco*RV-digested and dephosphorylated pBluescript KS (+) (Stratagene Cloning Systems, La Jolla, CA, USA), and transformed into DH5α competent cells. The sequences of the inserts were determined by ABI PRISM 310 (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA).

2.5. RNA isolation and Northern blot analysis

Total RNA was isolated using TRIzol (Gibco BRL Life Technologies, Gaithersburg, MD, USA). Poly $(A)^+$ RNA was purified from total RNA using Oligotex-dT30 (Daiichi Pure Chemicals, Tokyo, Japan), and used for the subtraction cloning described above. For Northern blot analysis, 25 μg of total RNA was electrophoresed on a 1.0% agarose gel containing 2% formaldehyde, and then transferred to a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech Ltd.). The filter was hybridized with each probe which was labeled with [α-^{32}P]dCTP using a random labeling kit (Takara Biomedicals). The filter was washed and then exposed to X-ray film.

3. Results and discussion

3.1. Establishment of PPARγ-expressing stable transformants

To identify the downstream target genes of PPARγ, we produced a stable cell line expressing PPARγ. The full-length cDNA encoding PPARγ was inserted into the retrovirus vector pDON-AI. This plasmid and the parental expression vector were transfected into the packaging cell line PT-67, and the resulting viruses produced were used for infection of NIH-3T3 cells. The infected cells were then selected by treatment with the antibiotic, G418. In this manner, stable cell lines were isolated that contained the parental expression vector (NIH-vector cells) or expressed PPARγ (NIH-PPARγ cells).

To analyze the expression of the integrated PPARγ gene, we performed Northern blot analysis using total RNA prepared from NIH-PPARγ and NIH-vector cells. In both cell lines, the endogenous expression of PPARγ was not observed. While the exogenous transcript encoding PPARγ derived from the virus was produced in NIH-PPARγ cells, NIH-vector cells did not express the mRNA of exogenous PPARγ (Fig. 1A). To convert these cell lines into adipocytes, the cells were exposed to DMEM supplemented with FBS, Dex and BRL49653 (high-affinity ligand for PPARγ) as described under Section 2. As

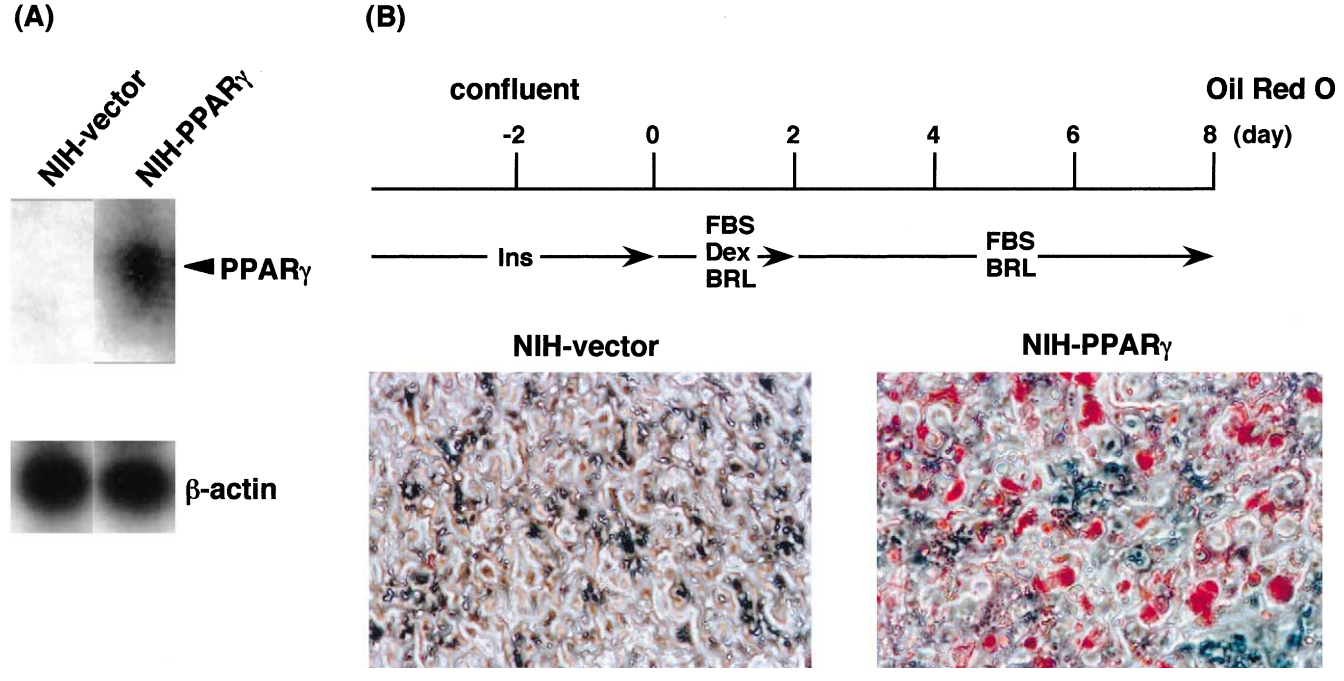

Fig. 1. Ectopic expression of PPARγ in NIH-3T3 cells and conversion to adipocytes. A: NIH-3T3 cells were infected with retroviruses carrying pDON-AI or pDON-AI-PPARγ. Total RNA (25 μg per lane) was isolated from NIH-vector cells and NIH-PPARγ cells, blotted to nylon membrane, and hybridized with ^{32}P-labeled PPARγ or β-actin cDNA. The arrowhead shows the mRNA band derived from the exogenous expression of PPARγ. B: Stable transformants of NIH-vector and NIH-PPARγ cells were cultured in differentiation medium. At day 8 postinduction, the cells were fixed and stained with Oil Red O.

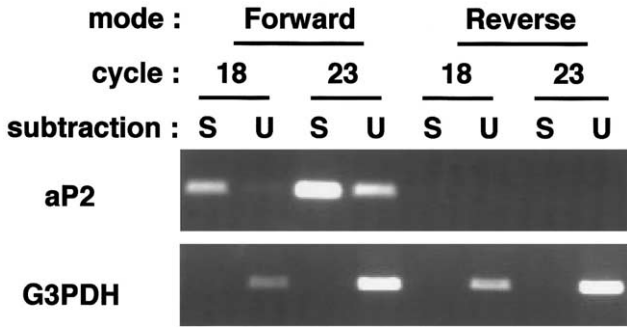

Fig. 2. Subtraction efficiency for cloning of differentially expressed genes. For checking the efficiencies of the forward and reverse subtraction, PCR was performed using primers for aP2 and G3PDH, and subtracted (S) and unsubtracted (U) PCR products were compared.

shown in Fig. 1B, 50–60% of the NIH-PPARγ cells were stained with Oil Red O, indicating the storage of droplets of oil, but no lipid-containing cells were seen among the NIH-vector cells. When cultured in the differentiation medium without Dex or BRL49653, NIH-PPARγ cells could not differentiate into adipocytes (data not shown).

3.2. Subtraction cloning for isolation of downstream target genes of PPARγ

By using the stable transformant expressing PPARγ which gains the potential to differentiate into adipocytes, we next isolated the genes up-regulated or down-regulated in this stable transformant in the presence of the inducers. For the forward subtraction (for the isolation of up-regulated genes), the tester cDNAs were prepared from NIH-PPARγ cells incubated with inducers (FBS, Dex and BRL49653) for 1 day, and the driver cDNAs were prepared from untreated NIH-vector cells. For the reverse subtraction (for the isolation of down-regulated genes), the tester cDNAs were prepared from untreated NIH-vector cells and the driver cDNAs were prepared from NIH-PPARγ cells incubated with inducers (FBS, Dex and BRL49653) for 1 day. Accordingly, the forward-subtracted cDNAs contain genes whose expression is increased in the NIH-PPARγ cells, and the reverse-subtracted cDNAs contain genes whose expression is decreased in the untreated NIH-vector cells.

For checking the enrichment efficiencies of differentially expressed genes, we performed PCR analyses using the aP2 gene which is known to be a target of PPARγ [6] and the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene which is a housekeeping gene and expressed constitutively. As shown in Fig. 2, the fragment of aP2 was concentrated after the forward subtraction, but not the reverse subtraction. On the other hand, the expression of G3PDH was not detected after either the forward or reverse subtraction, indicating that the subtraction efficiencies were high.

Using this method, we isolated the subtracted cDNAs and subcloned then. However, unfortunately, it is well known that many false positive clones are obtained by this protocol. Therefore, we next performed Northern blot analyses for the isolated clones, and the results are shown in Fig. 3. Out of 98 clones isolated from the forward subtraction, 17 clones were identified as genes up-regulated, and 10 clones out of 75 clones were identified as genes down-regulated during the differentiation of NIH-PPARγ cells. These clones were sequenced and their names are listed in Fig. 3. Finally, we identified 10 of 17 forward-subtracted clones as known genes.

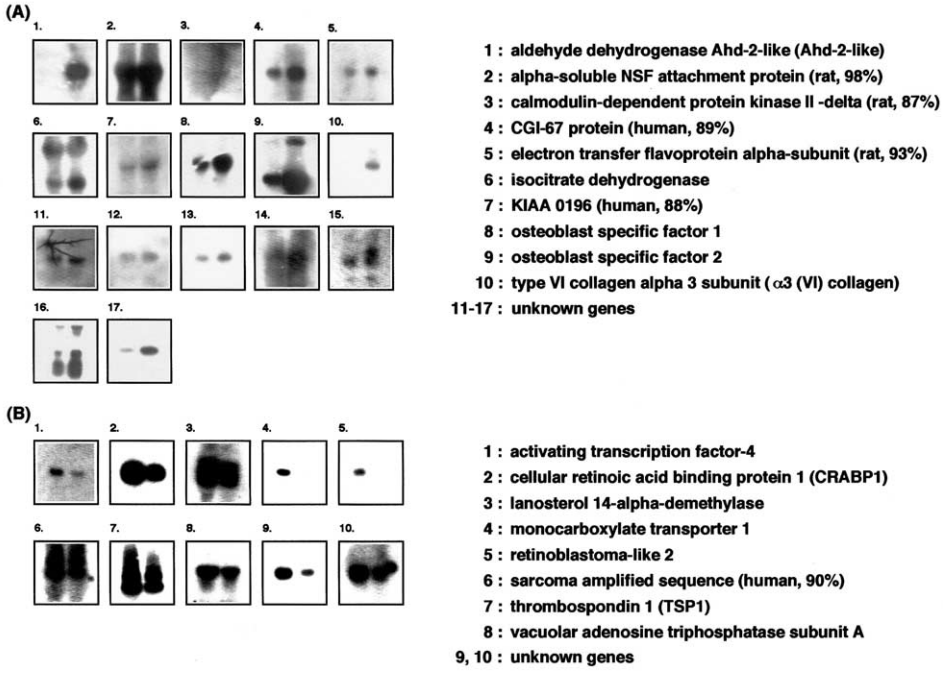

Fig. 3. Northern blot analyses of up-regulated or down-regulated genes by the addition of differentiation inducers to the PPARγ-expressing cells. Forward-subtracted (A) and reverse-subtracted (B) clones are shown. Total RNA was isolated from untreated NIH-vector cells (left lane) and NIH-PPARγ cells treated with differentiation inducers for 1 day (right lane), and 25 μg of the RNA was analyzed by Northern blotting. The filter was hybridized with each probe. The sequences of isolated clones were run through DNA databases, and names are shown when identical to mouse clones. If the clones showed high similarity to other species, the name of the source and the similarity value are shown in parentheses.

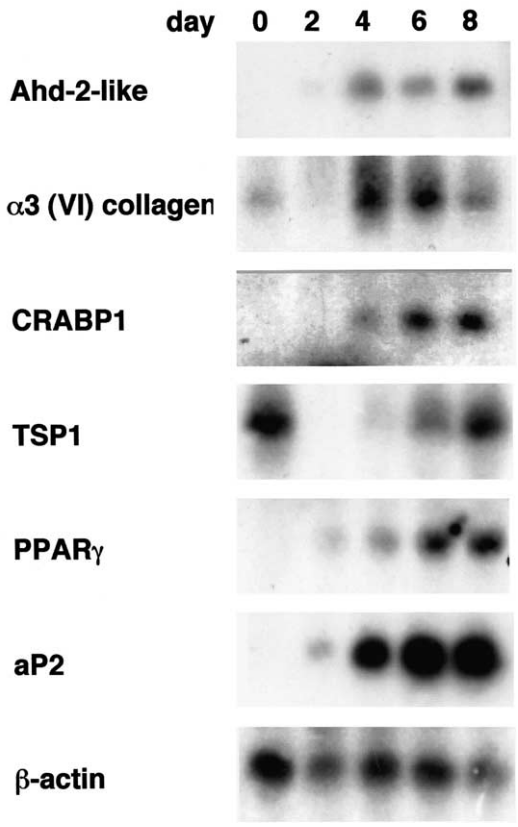

Fig. 4. Time course of mRNA expression of Ahd-2-like, α3 (VI) collagen, CRABP1 and TSP1 during 3T3-L1 adipocyte differentiation. Total RNA was prepared from 3T3-L1 cells at various time points after treatment with inducers, and 25 μg of the RNA was loaded for Northern blot analysis. The filter was hybridized with each probe. The expression pattern of PPARγ and aP2 was shown as differentiation markers. β-Actin as a control is also shown.

Seven clones remain as unknown genes that were not in the database. We also identified 8 of 10 reverse-subtracted clones as known genes and two as unknown genes.

3.3. Expression pattern of isolated genes during adipocyte differentiation of 3T3-L1 cells

3T3-L1 preadipocyte cells, which are fibroblastic cells destined to become adipocytes, are widely used to investigate the mechanisms of adipocyte differentiation [15]. When treated with inducers including Ins, Dex, IBMX and FBS, they undergo differentiation into mature fat cells over a 6–8 day period. Since PPARγ is expressed during adipogenesis and plays a key role in this process, we examined the time course of the expression of the subtracted genes and also of PPARγ during adipocyte differentiation. We showed that the expression of PPARγ started to elevate at 2 days, and that the expression level of some of these genes changed during the differentiation process (Fig. 4).

Aldehyde dehydrogenase-2-like (Ahd-2-like) mRNA increased during the differentiation, and this induction coincided with that of a well-known target of PPARγ, aP2. The type VI collagen alpha 3 subunit (α3 (VI) collagen) was also increased at 4 days, but decreased at 8 days. α3 (VI) collagen had already been identified as a product whose gene is differentially expressed during adipocyte development [16].

Unexpectedly, although a cellular retinoic acid binding protein 1 (CRABP-1) cDNA clone was isolated by reverse subtraction, the expression of this clone was elevated relatively late compared with Ahd-2-like. When Dex was added to NIH-PPARγ cells, CRABP-I mRNA expression markedly decreased, but this clone was induced to express when Dex was omitted from the medium at 2 days postinduction (data not shown). Therefore, CRABP-I mRNA might be increased during 3T3-L1 adipocyte differentiation regardless of the reverse subtracted clone. CRABP-I is a member of the gene superfamily that encodes small proteins involved in the binding, transport, and metabolism of hydrophobic ligands [17]. Since retinoic acid is known to inhibit the adipocyte differentiation [18], it is possible that CRABP-1 has some functional role in the conversion to adipocytes. This possibility remains to be tested.

The expression of thrombospondin 1 (TSP1) decreased at 2 days and then markedly increased at 6 days. TSP1 is a large, trimeric, multidomain extracellular matrix molecule, which is intimately involved in cell proliferation, cell attachment and neurite outgrowth [19,20].

In this study, we newly isolated genes using PPARγ-expressing cells and a PCR-mediated subtraction protocol. The results strongly suggest that Ahd-2-like, α3 (VI) collagen, CRABP-I and TSP1 are the downstream targets of PPARγ in adipocyte differentiation. Further study including promoter analyses, is necessary to clarify whether the genes for these four targets are directly regulated by the transcription factor, PPARγ.

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