Genetic Analysis of Adipogenesis through Peroxisome Proliferator-activated Receptor γ Isoforms

Elisabetta Mueller‡‡, Stavit Drori‡, Anita Aiyer‡, Junming Yie‡‡, Pasha Sarraf‡, Hong Chen‡, Stefanie Hauser‡‡, Evan D. Rosen‡ ‡‡, Kai Ge§§, Robert G. Roeder§§, and Bruce M. Spiegelman‡‡

From the #Dana-Farber Cancer Institute and the Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115, #Millenium Pharmaceutical, Inc., Cambridge, Massachusetts 02139, and §§Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, New York 10021

Peroxisome proliferator-activated receptor (PPAR) γ is a nuclear receptor that is a key regulator of adipogenesis and is present in two isoforms generated by alternative splicing, PPARγ1 and PPARγ2. Studies of the ability of each isoform to stimulate fat differentiation have yielded ambiguous results, in part because PPARγ stimulates its own expression. We have thus undertaken a formal genetic analysis using PPARγ-null fibroblast cell lines to assess the specific role of each individual isoform in adipogenesis. We show here that both PPARγ1 and PPARγ2 have the intrinsic ability to stimulate robust adipogenesis. Adipose cells stimulated by either PPARγ1 or PPARγ2 express a similar gene profile and show similar responses to insulin. However, in response to low ligand concentrations, PPARγ2 shows a quantitatively greater ability to induce adipogenesis. Analyses involving coactivator binding and transcriptional assays indicate that PPARγ2 has an enhanced ability to bind components of the DRIP/TRAP complex, coactivators required for fat differentiation.

The peroxisome proliferator-activated receptor (PPAR) γ is a member of the nuclear hormone receptor superfamily that is expressed at high levels in fat cells. It was independently identified as a transcriptional regulator of a fat cell-specific enhancer element (1) and as a novel member of the PPAR family (2). A key regulatory role for PPARγ in fat differentiation was demonstrated by gain of function experiments, which showed that ectopic expression and activation of this factor in fibroblasts or myoblasts promoted adipogenesis (3–5). More recently, it has been shown that PPARγ is necessary as well as sufficient for fat cell differentiation in cultured cells and mice (6–8).

PPARγ activity is regulated by the binding of ligands and by the subsequent docking of coactivators. Several natural occurring ligands of relatively low affinity have been identified, including various fatty acids and prostaglandins (9, 10); the biological significance of their function with PPARγ is largely unknown. Synthetic ligands such as the anti-diabetic thiazolidinediones (pioglitazone, rosiglitazone, and troglitazone) and certain tyrosine analogs are effective agonists and bind with $K_d$ values between 5 and 750 nM (11, 12).

The binding of agonist ligands to nuclear receptors promotes the docking of coactivator proteins such as members of the p160/SRC family, p300/CBP, and p300/CBP-associated factor, all of which exhibit histone-acetyltransferase activity (13). In addition PPARγ can also dock the tissue-selective coactivators PGC-1α and β, although these docking events are not ligand-gated (14, 15). PPARγ also binds to PBP/DRIP205/TRAP220, initially identified as a PPARγ-binding protein and subsequently characterized as a component of the mediator-like co-activator complex DRIP/TRAP/ARC (16–18). This protein complex is essentially devoid of histone-acetyltransferase activity and is believed to associate directly with the general transcription factors of the preinitiation complex. Recent studies illustrate that PBP/TRAP220/DRIP205 is absolutely required for PPARγ-mediated adipogenesis (19).

PPARγ is present in two isoforms, PPARγ1 and PPARγ2, generated by alternative promoter usage; PPARγ2 has an additional 30 N-terminal amino acids relative to PPARγ1. The expression of PPARγ2 is restricted mainly to fat, whereas PPARγ1 is expressed in fat and many other tissues. Our earlier report indicated that both PPARγ1 and PPARγ2 could stimulate adipogenesis when introduced into fibroblastic cells (3). However, conclusions concerning the inherent adipogenic potential of these individual receptors are compromised by the fact that ectopic expression of PPARγ1 turns on endogenous PPARγ2 (3). In an attempt to circumvent this problem, one recent report has utilized engineered artificial transcriptional suppressors of the endogenous PPARγ gene in combination with ectopic expression of the individual isoforms. This study concluded that PPARγ2 is adipogenic and PPARγ1 is not (20). To avoid problems associated with these engineered suppressors and to investigate this key question in a more formal genetic way, we now make use of cells completely lacking a functional PPARγ gene to reassess the adipogenic action of PPARγ1 and PPARγ2. Our studies clearly illustrate that both PPARγ isoforms can drive the differentiation of fully functional fat cells. However, under limiting levels of a PPARγ ligand, PPARγ2 has an enhanced ability to promote differentiation and to interact with PBP/DRIP205/TRAP220.

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PPARγ1 and PPARγ2 isoforms were cloned into PMSCV-puro retroviral vector for infections. PPARγ1 and PPARγ2 baculovirus constructs were obtained by subcloning full-length PPARγ1 and PPARγ2 into pAcGHLT (Pharmingen).

Cell Culture—Cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin/streptomycin in 5% CO2. For differentiation assays, cells were treated with 0.5 mM 3-isobutyl-1-methylxanthine, 5 μg/ml insulin, and 1 μM dexamethasone for 2 days, and, subsequently, cells were kept in maintenance medium consisting of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum supplemented with penicillin/streptomycin and insulin (5 μg/ml). Oil Red O staining of differentiated adipocytes was performed as described previously (3).

Transient Transfections and Infections—95% confluent U2OS cells were transiently transfected with 25 ng of PPARγ1 or PPARγ2 retroviral constructs or empty vector, with 100 ng of DR1-luc, using FuGene 6 (Roche Molecular Biochemicals) according to manufacturer’s instructions. 24 h after transfection, cells were treated with various ligand concentrations, and 24 h later, the luciferase levels were measured. TRAP220−/− cells (19) were cotransfected with 25 ng of PPARγ1 or PPARγ2 and 100 ng of DR1-luc in the absence or presence of 400 ng of TRAP220. After 24 h, cells were treated with 10 μM rosiglitazone, and 48 h after transfection, cells were harvested for luciferase measurements. For retrovirus preparation, the Bosc23 packaging cell lines were transfected with PMCV retroviral PPARγ1 and PPARγ2 constructs or vector, according to the protocols described previously (14). PPARγ knockout cells were plated the day before infection and infected with the viral supernatant at 70% confluence. After an 8–12-h incubation with virus and 5 μg/ml Polybrene, cells were split 1:3 and selected with 2 μg/ml puromycin for 1 week.

RESULTS

Adipogenic Functions of PPARγ Isoforms in PPARγ−/− Cells—We investigated the adipogenic action of PPARγ isoforms utilizing PPARγ-deficient cells. We infected immortalized 3T3 fibroblasts null for PPARγ (24) with an empty retroviral vector or with vectors expressing murine PPARγ1 or PPARγ2. Both RNA and protein for the two PPARγ isoforms were present in undifferentiated cells at virtually identical levels after drug selection for stable viral integration (Fig. 1A). These levels of PPARγ are approximately equal to those seen in differentiated adipocytes (data not shown). The cells were then stimulated to differentiate according to different protocols. In the first set of experiments, strong induction stimuli were used: cells were treated for 2 days with a mixture commonly used to stimulate differentiation of 3T3-L1 cells, including dexamethasone, insulin, and isomethylbutyloxanthine, plus the additional induction of the PPARγ ligand rosiglitazone. At day 7 after induction, we observed extensive lipid accumulation in cell lines expressing either PPARγ1 or PPARγ2 (Fig. 1B). In addition to showing similar morphological changes, these cell lines also expressed similar levels of mRNA for molecular markers of white fat differentiation such as aP2 and CD36 (Fig. 1C). No induction of endogenous PPARγ2 protein was observed in differentiated PPARγ1 cells, confirming the total ablation of the PPARγ gene in these cells (data not shown). Similar effects on differentiation and gene expression were obtained when these cells were induced to differentiate with dexamethasone, insulin, and isomethylbutyloxanthine in the absence of ligand (data not shown).

The development of insulin-sensitive glucose uptake is a very important part of the adipose differentiation program. The results shown in Fig. 2A indicate that insulin-stimulated glucose uptake is similar in cells induced to differentiate by either PPARγ1 or PPARγ2. We also measured the uptake of fatty acids in these cell lines and found that it is also similar in both cell lines (Fig. 2B).

Transcriptional Cascades Activated by PPARγ1 and PPARγ2 Isoforms—To investigate whether PPARγ1 and PPARγ2 can regulate similar sets of genes, we performed global transcriptional profiling using mRNA isolated from cells differentiated through PPARγ1 and PPARγ2 expression and activation. As...
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TABLE I
Genes induced in PPARγ−/− cells after reexpression of PPARγ1 or PPARγ2

| Gene                                           | Accession Number | G1-G2 |
|------------------------------------------------|------------------|-------|
| Pentose phosphate pathway                      |                  |       |
| Glutokinase                                    | M58755           | 0.9   |
| Hexokinase II                                  | M68972           | 1.3   |
| Glucose-6-phosphate 1-dehydrogenase X          | Q00612           | 1.0   |
| 6-phosphogluconate dehydrogenase               | P52209           | 1.1   |
| Transketolase                                   | U90889           | 0.9   |
| Glyceraldehyde-3-phosphate dehydrogenase       |               |       |
| Phosphoglycerate kinase                         | P09411           | 2.8   |
| Lipid metabolism                               |                  |       |
| Acyl-CoA carboxylase                            | P11497           | 1.2   |
| Glycerol-3-phosphate dehydrogenase             | D50430           | 1.4   |
| Acyl-CoA desaturase 2                           | P13011           | 2.3   |
| Lipoprotein lipase precursor                   | P11152           | 0.8   |
| Fatty acid synthase                            | P19096           | 0.9   |
| Hormone-sensitive lipase                       | U40001           | 1.1   |
| DGAT                                           | AP078752         | 1.0   |
| CD36                                           | L23108           | 0.8   |
| β Oxidation                                    |                  |       |
| Carnitine O-acetyltransferase                  | P47934           | 1.0   |
| Acyl-CoA dehydrogenase, medium chain-specific  | P459532          | 0.7   |
| Acyl-CoA dehydrogenase, long chain-specific    | P51174           | 0.9   |
| Peroxisomal acyl-CoA oxidase                   | AP006688         | 0.8   |
| Adipogenesis                                   |                  |       |
| Adipsin                                        | P03953           | 0.5   |
| ACRP30                                         | U37222           | 0.9   |
| Glucose transporter type                       | P19357           | 1.7   |
| Uncoupling protein 2                           | P56500           | 0.9   |
| Caveolin-1                                     | P49817           | 0.8   |
| Transcription factors                          |                  |       |
| C/EBPα                                         | P53566           | 1.0   |
| STAT5                                          | P42230           | 1.0   |
| Pyruvate metabolism                            |                  |       |
| NADP-dependent malic enzyme                    | P06801           | 1.1   |
| Lactate dehydrogenase A-4                      | M17516           | 1.7   |
| Pyruvate dehydrogenase protein X component     | O00330           | 0.9   |
| Pyruvate dehydrogenase E1 component β subunit | P49432           | 1.0   |
| Pyruvate carboxylase                            | P11498           | 1.0   |
| Krebs cycle                                    |                  |       |
| Aconitase hydrolase                            | P16276           | 0.9   |
| Fumarate hydrolase                             | P14408           | 0.9   |
| Mitochondrial dicarboxylate carrier            | AJ223355         | 1.1   |
| Respiratory chain                              |                  |       |
| Electron transfer flavoprotein α subunit       | M22030           | 1.0   |
| Electron transfer flavoprotein-ubiquinone      | Q16134           | 0.8   |
| Rodoxidase                                     |                 |       |
| Ubiquinol-cytochrome c reductase complex       | P31800           | 0.9   |
| Protein I                                      |                 |       |
| ATP synthase β chain                           | P56480           | 1.0   |
| ATP synthase lipid-binding protein P3          | P56384           | 1.0   |
| Mitochondrial trifunctional enzyme α subunit   | Q04428           | 0.9   |
| NADH-ubiquinone oxidoreductase chain 1         | P03888           | 0.8   |
| Carnitine metabolism                           |                  |       |
| 4-trimethylaminobutyraldehyde dehydrogenase    | AF170919         | 0.9   |
| Amino acid metabolism                          |                  |       |
| Neutral amino acid transporter B                | P51912           | 0.7   |
| Alanine aminotransferase                       | P25409           | 0.9   |
| Amino acid transporter 1                       | P56064           | 0.9   |
| Ketogenesis                                    |                  |       |
| 3-Hydroxyisobutyrate dehydrogenase             | P29266           | 0.8   |

Fig. 2. Induction of insulin-stimulated glucose uptake and fatty acid uptake by PPARγ isoforms. A, glucose uptake measured in fully differentiated PPARγ1 or PPARγ2 cells in response to 8 min of stimulation with 100 nM insulin. B, radiolabeled oleic acid uptake in fully differentiated PPARγ1 and PPARγ2 cells at time 0 and after 3 and 6 h of incubation with this fatty acid. The number of total counts is 1 × 106. C, Northern blot analysis of several mRNAs differentially regulated by PPARγ isoforms in PPARγ−/− cells. Lane −/− represents the control cell line, which was infected with empty vector.

Table I shows most of the genes associated with adipogenic differentiation or lipid metabolism are induced similarly by both isoforms, indicating that both PPARγ1 and PPARγ2 are competent to activate an adipogenic gene program. Fig. 2C illustrates that genes involved in the Krebs cycle, such as aconitase precursor and malate dehydrogenase, appear to be similarly induced in cells differentiated by the two different isoforms. However, a few genes appear to be regulated differently, at least quantitatively. For example, genes of the glycolytic pathway, such as phosphofructokinase and phosphoglycerate kinase, are induced to a greater extent in PPARγ1 cells, whereas certain other genes associated with differentiation, such as adipin, are induced at higher levels in PPARγ2 cells.

Differentiation Capacity of PPARγ Isoforms under Higher Stringency—Whereas the data presented above illustrate clearly that both PPARγ isoforms can drive adipogenesis, it is reasonable to suppose that the extracellular influences may not be as consistently pro-adipogenic as the conditions that we have used. We therefore stimulated cells more gently, using only insulin and a titration of the PPARγ ligand levels. Fig. 3A shows that PPARγ1 and PPARγ2 drive cells to differentiate to differing extents when the ligand concentrations are submaxi-
Adipogenesis through PPARγ1 and PPARγ2 at low ligand concentrations. A, oil red O staining of PPARγ−/− cells expressing PPARγ1 or PPARγ2 after 7 days of treatment with low amounts of rosiglitazone. B, Northern blot analysis of the expression of the adipocyte-specific marker aP2 after 7 days of ligand treatment at the doses shown. C, transient transfections of PPARγ1 and PPARγ2 retroviral vectors in U2OS cells. 24 h after transfection, cells were treated with different doses of rosiglitazone (10 nM, 100 nM, and 1 μM), as shown in the figure, and luciferase analysis was performed 24 h later.

To determine whether these differences in adipogenic action correlated with differences in transcriptional function of the two isoforms, their transcriptional activities were examined at low ligand concentrations of rosiglitazone. As shown in Fig. 3C, PPARγ2 can activate a multimerized PPAR response element linked to luciferase more effectively than PPARγ1, both in the absence of rosiglitazone and at low doses of rosiglitazone. This difference, which is 2–3-fold at 10 nM rosiglitazone, is <30% at 1 μM rosiglitazone.

Adipogenic Actions of PPARγ Isoforms Correlate with PBP/DRIP/TRAP220 Docking—The differences in adipogenic and transcriptional activity between PPARγ1 and PPARγ2 observed at low ligand concentrations could be due to differential affinity for agonists or could reflect other aspects of receptor function. To investigate the molecular basis of these differences, we first produced both PPARγ isoforms as full-length proteins in insect cells using baculovirus vectors. These were subsequently purified and used to investigate ligand binding capacity. As shown in Fig. 4A and B, PPARγ1 and PPARγ2 bind rosiglitazone and 15dΔ12,14PGJ2 with essentially identical affinities.

Because nuclear receptors are known to activate transcription through docking of specific coactivator proteins, we assessed whether the quantitative differences in the ability of the two PPARγ isoforms to induce differentiation could reflect a difference in their ability to recruit known coactivators. We therefore performed in vitro interaction assays in the presence or absence of a low concentration of ligand using baculovirus purified full-length proteins and coactivators produced as in vitro-translated proteins in reticulocyte lysates. As shown in Fig. 5A, the interactions of the two PPARγ isoforms with both PGC-1α and SRC-1 are comparable in the presence or absence of rosiglitazone. In contrast, two members of the DRIP/TRAP complex, PBP/DRIP205/TRAP220 and DRIP150/TRAP170, appear to have a quantitative preference for PPARγ2 in both the absence and presence of added ligand. PPARγ2 docks DRIP150/TRAP170 independently of added ligand, consistent with the idea that this subunit of the DRIP/TRAP complex may bind to the N terminus of PPARγ, as shown previously for the glucocorticoid receptor (25).

To critically analyze the role of the DRIP/TRAP complex in the differential effects of PPARγ1 and PPARγ2, we utilized fibroblasts lacking the DRIP205/TRAP220 subunit of the DRIP/TRAP complex. Mice lacking DRIP205/TRAP220 die embryonically at day 11.0, and a DRIP205/TRAP220 −/− cell line was obtained by immortalizing cells isolated from embryonic day 10.0 littermate embryos (19). We utilized this cell
line to analyze first whether the differences in transcriptional activity between PPARγ1 and PPARγ2 are dependent on TRAP220. As shown in Fig. 5B, in the absence of TRAP220, both PPARγ1 and PPARγ2 show an identical ability to transactivate the multimerized PPAR response element reporter gene in either the absence or presence of a low concentration (10 nM) of rosiglitazone. The addition of exogenous DRIP205/TRAP220 coactivator partially restores differential transcriptional activation observed between the two isoforms, with or without ligand addition. These data suggest a functional significance in the preferential interaction between DRIP205/TRAP220 and PPARγ2.

DISCUSSION

A key question regarding the PPARγ isoforms is whether they can both stimulate fat cell differentiation in culture and in vivo. Whereas the latter cannot be answered with confidence, we provide definitive genetic evidence here that PPARγ1 and PPARγ2 can individually drive a full program of adipogenesis in culture. Both isoforms can stimulate lipid accumulation, the development of insulin-sensitive glucose uptake, and a pattern of gene expression that is very similar. Translating these results to an in vivo setting must be purely speculative. It is notable that a difference in relative adipogenic action of the two PPARγ isoforms becomes clear at lower ligand concentrations, with PPARγ2 more adipogenic at 10–50 nM rosiglitazone, approximating the Km of the receptor. If we assume that ligand levels are limiting in vivo, these results imply that the PPARγ2 isoform is quantitatively more adipogenic. This might provide a biological rationale for the adipose-selective expression of PPARγ1.

These data are quite different from those of Ren et al. (20), who conclude that the PPARγ1 protein has no adipogenic action. Their experiments, like ours, used strong adipogenic conditions, including treating the cells with the mixture containing 3-isobutyl-1-methylxanthine, insulin, and dexamethasone. The suppression of expression of the endogenous PPARγ gene in 3T3-L1 cells was achieved by using an engineered repressor and subsequently reexpressing each individual PPARγ protein for comparison. It is possible that the effects observed by Ren et al. (20) could be due to a differential level of expression of the PPARγ1 isoform relative to the PPARγ2 isoform apparent in their protein data. Alternatively, it is possible that some other nonspecific effects of their artificial repressor blocked the adipogenic action of PPARγ1 through unknown mechanisms.

In our study, the greater adipogenic activity of PPARγ2 compared with PPARγ1 correlates with better binding to PBP/DRIP205/TRAP220 and DRIP150/TRAP170 and, presumably, the entire DRIP/TRAP/ARC complex. Furthermore, the higher transcriptional activity of PPARγ2 compared with PPARγ1 seems to be dependent on the presence of the DRIP205/TRAP220 subunit. Because recent data have indicated an absolute genetic requirement for DRIP205/TRAP220 for the adipogenic action of PPARγ, this may provide a mechanistic basis for the quantitatively greater adipogenic action of PPARγ2.

At the present time, very little is known about the precise function of the N terminus of PPARγ or any nuclear receptor, for that matter. The present study, combined with earlier work showing that a substantial portion of adipogenic action of PPARγ is carried in the N terminus (26), suggests a real need to determine how this part of PPARγ functions in mechanistic detail.

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