The insulin receptor: a new anticancer target for peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) and thiazolidinedione-PPAR\(\gamma\) agonists

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Abstract

The peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) is a member of the nuclear hormone receptor superfamily. Ligand activation of PPAR\(\gamma\) is associated with differentiation and inhibition of proliferation in the normal and malignant cells. Herein, we studied the effects of PPAR\(\gamma\) and the PPAR\(\gamma\) agonists thiazolidinediones (TZDs) on the insulin receptor (IR), a cell membrane tyrosine kinase receptor protein, whose role is of paramount importance in mediating the metabolic and growth-promoting effects of the peptide hormone insulin. Overexpression of the PPAR\(\gamma\)1 in human hepatocellular (HepG2) cells was associated with decreased IR gene transcription and protein expression levels, and these reductions were more evident in the presence of TZDs. Since no PPAR\(\gamma\) response elements were identified on the IR promoter, we postulated that PPAR\(\gamma\) adversely affects the IR gene transcription by perturbing the assembly and stability of the transcriptionally active multiprotein-DNA complex identified previously, which includes the high-mobility group A1 protein, the ubiquitously expressed transcription factor (Sp1), the CAAT enhancer-binding protein (C/EBP\(\beta\)), and, in some cell lines, the developmentally regulated activator protein-2 (AP-2) transcription factor. Using glutathione S-transferase pull-down assays combined with electrophoretic mobility shift assay and chromatin immunoprecipitation, we demonstrated that by interacting with Sp1, C/EBP\(\beta\), and AP-2, PPAR\(\gamma\) can prevent Sp1/AP-2 protein–protein association and inhibit binding of Sp1 and C/EBP\(\beta\) to DNA, thus reducing IR gene transcription. Our results demonstrate that IR is a new target gene of PPAR\(\gamma\), and support a potential use of TZDs as anti-proliferative agents in selected neoplastic tissues overexpressing IRs.

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Introduction

The peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) is a member of a subfamily of nuclear receptors involved in the control of several aspects of lipid metabolism, including fatty acid transport, uptake by the cells, intracellular binding and activation, as well as catabolism and storage (Desvergne & Wahli 1999, Kliwer et al. 2001, Berger & Moller 2002). It is highly expressed in adipose cells and its induction precedes the activation of most adipose-specific genes (Rosen et al. 1999, Keller et al. 1993a,b). Although PPAR\(\gamma\) is mainly expressed in the adipose tissue, its expression also occurs in many normal cells...
(Vidal-Puig et al. 1997), and in several cancer cell lines and tissues, including breast, prostate, colon, lung, and B-lineage cells (Padilla et al. 2000, Allred & Kilgore 2005, Nunez et al. 2005). Besides a fundamental role in adipogenesis, PPARγ plays a critical role in placental, cardiac, and embryonic development (Barak et al. 1999, Maloney & Rees 2005) and influences the production of cytokines, growth factor release, and cell cycle progression leading to differentiation-inducing, anti-inflammatory, and anti-proliferative effects (Kersten et al. 2000, Tan et al. 2005, Chou et al. 2007). The PPARγ generally exerts its biological function as heterodimer with the retinoid X receptor, which binds to a specific cis-acting sequence on DNA, a peroxisome proliferator response element, initiating gene transcription (Desvergne & Wahli 1999, Tan et al. 2005). The PPARγ activators identified so far include some inflammatory activators, like a prostaglandin J2 metabolite (15-deoxy-D12,14-PGJ2), and oxidized LDL particles (9-and 13-HODE; Desvergne et al. 1999, Kersten et al. 2000), and the synthetic thiazolidinedione (TZD) compounds (Lehmann et al. 1995). The TZDs are a new class of insulin-sensitizing drugs widely used in the treatment of human type 2 diabetes mellitus, including rosiglitazone, pioglitazone, and ciglitazone (Lehmann et al. 1995, Saltiel & Olefsky 1996). However, despite the positive role of TZDs in the control of glucose homeostasis, increasing evidences support a potential use of these drugs as anti-proliferative agents.

The peptide hormone insulin is a major regulator of glucose homeostasis and cell growth. The first step in insulin action is the binding of the hormone to the insulin receptor (IR), a phylogenetically ancient receptor protein embedded in the plasma membrane of insulin target cells. The IR belongs to the tyrosine kinase growth factors receptor superfamily and consists of two identical extracellular α-subunits that bind insulin, and two transmembrane β-subunits with intrinsic tyrosine kinase activity (Ullrich et al. 1985, Goldfine 1987, White & Kahn 1994). When insulin binds to the IR, the receptor is first activated by tyrosine autophosphorylation and then the IR tyrosine kinase phosphorylates various effector molecules, like the insulin receptor substrate-1 (IRS-1), leading to hormone action (Ullrich et al. 1985, Goldfine 1987, White & Kahn 1994). In target cells, the IR has been shown to be under the regulation of hormones, metabolites, and differentiation (Brunetti et al. 1989, Mamula et al. 1990). To understand the molecular mechanisms controlling IR gene regulation, many groups including our own group have previously identified and characterized the 5′-flanking region of the IR gene (Araki et al. 1987, Seino et al. 1989, Brunetti et al. 1993). Later, we have provided evidence that transcriptional activation of the human IR promoter requires the assembly of a transcriptionally active multiprotein-DNA complex which includes, in addition to the architectural factor high-mobility group A1 (HMGA1), the transcription factors Sp1 and C/EBPβ. Functional integrity of this nucleoprotein complex is required for full transactivation of the IR gene by Sp1 and C/EBPβ in cells readily expressing IRs (Brunetti et al. 2001, Foti et al. 2003, 2005). Recently, we published data showing that the nuclear transcription factor activator protein-2 (AP-2), by interacting with Sp1 and HMGA1, may stabilize this multiprotein complex at the level of the IR promoter, leading to IR over-expression in breast cancer cells (Paonessa et al. 2006).

In this paper, we demonstrate for the first time that PPARγ and its ligands inhibit IR gene transcription. We show that PPARγ physically interacts with Sp1, AP-2, and C/EBPβ in vitro, and this protein–protein interaction significantly reduces the transactivation of the IR gene by Sp1, C/EBPβ, and AP-2 in vivo, in the absence of a consensus sequence for PPARγ on the IR promoter.

Materials and methods

Cells and protein extracts

HepG2 human hepatoma cells, 3T3-L1 mouse fibroblasts, and MCF-7 human breast cancer cells (American Type Culture Collection, Manassas, VA, USA) were maintained in DMEM (GIBCO-BRL) supplemented with 10% fetal bovine serum. The 3T3-L1 fibroblasts were differentiated into 3T3-L1 adipocytes as described elsewhere (Foti et al. 2003). Nuclear and cytoplasmic extracts were prepared from cultured cells as previously described (Brunetti et al. 2001). For each nuclear extract, an equal number of nuclei were homogenized, and the final protein concentration in the extracts was determined by the modified Bradford method (Bio-Rad Laboratories).

RT-PCR, immunoprecipitation, and western blot analysis

Total cellular RNA was extracted from cells using the RNAqueous-4PCR kit and subjected to DNase treatment (Ambion, Austin, TX, USA). The cDNA was synthesized from total RNA with the RETROscript first strand synthesis kit (Ambion) and used for PCR amplification. The PCR products were electrophoretically resolved on 2% agarose gel, visualized by
ethidium bromide staining, and quantified by densitometry. The values obtained were then normalized to those of the tubulin gene. Western blot analyses of IR, PPARγ, HMGA1, Sp1, C/EBPβ, and AP-2 were done on total cellular lysates or nuclear extracts as previously described (Brunetti et al. 2001, Foti et al. 2003, 2005). For immunoprecipitation studies with IR antibody, aliquotes of HepG2 cytoplasmic extracts were incubated for 12 h with rotation at 4 °C with 10 μl IR antibody-coupled protein A beads. Covalent coupling of antibody was performed as previously described (Paonessa et al. 2006). Beads were recovered by gentle centrifugation and washed thrice with 500 μl NETN wash buffer (0.1% NP40, 150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 8.0)) for 5 min. Protein was removed from the beads by boiling in sample buffer for 5 min and analyzed by SDS-PAGE and immunoblotting. Antibodies used for these studies were as follows: anti-HMGA1 (Brunetti et al. 2001), anti-PPARγ1 (H-100), anti-Sp1 (PEP 2), anti-C/EBPβ (C-19), anti-AP-2α (H-79), and anti-IRβ (C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### GST pull-down assay

The 35S-labeled HA-tagged HMGA1, Sp1, C/EBPβ, and AP-2 (Foti et al. 2003, Paonessa et al. 2006) were synthesized in vitro using the TNT-T7 quick-coupled transcription/translation system (Promega). The glutathione S-transferase (GST) fusion protein expression vectors pGEX4T-3-human PPARγ2 (kindly provided by H Nishizawa and T Funahashi, University of Osaka; Nishizawa et al. 2002) and the pGEX-2TK control vector (Amersham Pharmacia Biotech) were transformed into the BL21(D3) strain of Escherichia coli (Stratagene, La Jolla, CA, USA), expanded in suspension culture, and induced for 2 h with 0.5 mM isopropyl-β-thiogalactopyranoside (Sigma). Bacteria were pelleted, sonicated in ice-cold PBS lysis buffer containing 1% NP-40, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg pepstatin A, leupeptin, and aprotinin/ml, and 0.4 mg lysozyme/ml and centrifuged. The resultant supernatant was then added to 300 μl glutathione-agarose beads, mixed on a rotating wheel at 4 °C for 2 h and centrifuged. The bound GST-fused proteins in the pellet were washed five times with lysis buffer and resuspended in 300 μl binding buffer (50 mM NaCl, 20 mM Tris–HCl (pH 8.0), 0.05% NP-40, 0.25% BSA, 1 mM PMSF, 1 mM DTT). The bound protein was quantitated with the Coomassie protein assay reagent (Pierce Co., Rockford, IL, USA), and 0.5 μg of each GST-protein bound to glutathione-agarose beads was incubated with 7 μl in vitro-translated 35S-labeled protein in 150 μl binding buffer at 4 °C for 2 h. Reactions were terminated by centrifugation, the precipitate was washed three times with protein-binding buffer and subjected to a 10% PAGE (SDS-10% PAGE), and proteins were visualized by autoradiography.

### Plasmids, transfections, and electrophoretic mobility shift assay (EMSA)

Recombinant chloramphenicol acetyltransferase (CAT) plasmids (pIR-CAT, pCAT-C2 and pCAT-E3) containing different fragments of the human IR gene promoter have been described previously (Brunetti et al. 1993, Foti et al. 2003, Paonessa et al. 2006). The human PPARγ1 expression vector was obtained using standard techniques after PCR amplification and cloning of PPARγ full-length cDNA into the expression vector pcDNA3. The pC-hu/AP-2α expression vector was kindly provided by K Fujimori (Osaka Bioscience Institute). The IR-CAT reporter plasmids, together with effector vectors for PPARγ and/or AP-2, in the absence or presence of the PPARγ agonist rosiglitazone (a kind gift of Glaxo Smith Kline) or ciglitazone (Vinci-Biochem Alexis, Vinci, Italy) were transiently transfected into cultured cells by the calcium phosphate precipitation method, and CAT activity was assayed 48 h later, as previously described (Brunetti et al. 1993). The pSV-β-galactosidase control vector served as an internal control of transfection efficiency, together with measurements of protein expression levels (Brunetti et al. 2001). For EMSA, the C2 sequence (300 bp) of the human IR gene promoter was generated by PCR amplification, using the recombinant plasmid pCAT-C2 (Brunetti et al. 1993). The 32P-labeled C2 was used in gel shift assays as previously described (Brunetti et al. 2001).

### Chromatin immunoprecipitation (ChIP)

ChIP assay was done as described previously (Foti et al. 2005), using HepG2 cells transfected with the AP-2α expression vector and untransfected MCF-7 cells. Formaldehyde-fixed DNA–protein complex was immunoprecipitated with anti-HMGA1, anti-Sp1, anti-C/EBPβ, or anti-AP-2α antibody. Primers for the IR sequence (Paonessa et al. 2006) were used for PCR amplification of immunoprecipitated DNA (30 cycles) using PCR ready-to-go beads (Amersham Pharmacia Biotech). The PCR products were electrophoretically resolved on 1.5% agarose gel and visualized by ethidium bromide staining.

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Statistical analysis

All experiments were performed at least three times. The mean and s.e.m. were calculated for each set of results and the significance of the difference between values was assessed by the Student’s t-test. For all analyses, P < 0.05 was considered significant.

Results

PPARγ and TZDs reduce IR protein and mRNA expression in HepG2 cells

To see whether PPARγ had any effect on IR expression, we first measured the IR protein content in HepG2 cells, before and after forced expression of exogenous PPARγ1. The HepG2 cells were considered ideally suited for studying the effects of PPARγ on IR expression since IRs are relatively abundant in this cell line, while endogenous PPARγ is barely detectable.

Using immunoprecipitation and western blot analyses, we showed that IR protein content was considerably reduced in cells overexpressing PPARγ (∼50% less than control cells), and this reduction was more pronounced in PPARγ-overexpressing cells exposed to 10^{-5} M TZD (rosiglitazone; Fig. 1). It is notable that rosiglitazone alone also reduced IR protein content, and it is likely that this occurred, at least in part, by stimulating endogenous PPARγ (Tontonoz et al. 1994). As measured by RT-PCR, the abundance of IR mRNA transcripts in HepG2 cells closely correlated with IR protein content in every condition analyzed, indicating that changes at the protein level reflected differences in IR mRNA (Fig. 1).

PPARγ and TZDs reduce IR gene transcription in cultured cells

In order to reveal whether PPARγ and its agonists inhibited the transcriptional activation of IR gene, we performed reporter gene analysis using the pIR-CAT recombinant vector that contains the entire 5′-flanking region of the human IR gene (Foti et al. 2003). Forced expression of PPARγ1 in HepG2 cells transfected with pIR-CAT determined a 40–50% reduction in CAT activity that was similar to the reduction observed in cells exposed to rosiglitazone alone, and resulted more marked in PPARγ1-overexpressing cells simultaneously treated with TZD rosiglitazone (Fig. 2A). The possibility that PPARγ could theoretically decrease IR gene transcription by reducing the intracellular expression of HMGA1, Sp1 or C/EBPβ was excluded by western blot analysis of nuclear extracts showing that no changes were observed in the endogenous levels of these proteins under these experimental conditions (Fig. 2A). The hypothesis that PPARγ and its agonists could negatively interfere with the crosstalk between these various transcription factors in the context of the IR gene was substantiated further by transiently cotransfecting the AP-2-null cell line HepG2 with the pIR-CAT promoter, together with an expression vector encoding the wild-type AP-2. As shown in Fig. 2A, overexpression of AP-2 in HepG2 cells efficiently induced IR gene transcription. However, this effect was totally abolished by PPARγ and TZDs that reverted gene transcription to levels similar to those observed in the absence of AP-2 (Fig. 2A). These findings were confirmed in PPARγ overexpressing MCF-7 cells, a cell line naturally expressing only detectable levels of PPARγ (Tontonoz et al. 1994), and 3T3-L1 adipocytes producing relatively high amounts of endogenous PPARγ (Mueller et al. 1998). As shown in Fig. 2B and C, exposure of both cell lines to either rosiglitazone and/or ciglitazone reduced IR gene transcription in a dose-dependent manner.

We have previously shown that transcriptional activation of the human IR gene by HMGA1, Sp1, and C/EBPβ requires the assembly and cooperation among these various nuclear factors at the levels of two AT-rich sequences of the IR gene promoter, C2 and E3 (−674 to −874 and −1662 to −1818 bp upstream of the IR ATG codon respectively), which have a significant ability to drive transcription when introduced into mammalian cells (Brunetti et al. 1993, 2001, Foti et al. 2003). As demonstrated before, the
lack of any of these factors, or a scarcity of one or more, may severely perturb IR gene transcription in cells and tissues readily expressing IR (Brunetti et al., 2001, Foti et al., 2003, 2005). When the recombinant plasmid pCAT-C2 was transfected into HepG2 cells (and other cell lines), PPARγ, and TZDs, either alone or in combination, inhibited pCAT-C2 activity to the same extent than they did in HepG2 cells transfected with the full-length pCAT-IR promoter (Fig. 2D), indicating that interference of PPARγ and agonists with IR gene-transcription machinery occurs at the level of the proximal promoter region, C2. This conclusion was supported by the observation that in experiments using the pCAT-E3 reporter vector, containing the more distant E3 sequence of the IR gene, no effects on CAT activity were observed in cells exposed to PPARγ and/or TZDs (Fig. 2D).

**PPARγ physically interacts with Sp1, C/EBPβ, and AP-2, in vitro, in the absence of DNA**

In our studies, no peroxisome proliferator response elements (PPRE) have been identified on the promoter region of the IR gene. A similar observation has been previously provided by us for the transcription factor AP-2, for which DNA-binding activity was undetectable with the IR gene promoter, and transactivation of the IR gene by AP-2 occurred indirectly through physical and functional cooperation with HMGA1 and Sp1 (Paonessa et al., 2006). Therefore, in an attempt to identify the biochemical mechanisms underlying the negative effects

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**Figure 2** Functional significance of PPARγ and TZDs for IR gene transcription. (A) HepG2, (B) MCF-7, and (C) 3T3-L1 cells were transfected with a CAT reporter plasmid containing the IR promoter element (pCAT-IR, 2 μg), in the absence or presence of PPARγ (2 μg) and/or AP-2 (1 μg) expression vector, with or without treatment with rosiglitazone or ciglitazone at the indicated molar concentrations. For each cell line, data represent the means ± S.E.M. for three separate experiments; values are expressed relative to the CAT activity obtained in transfections with the pCAT-IR alone that is assigned an arbitrary value of 1. *P < 0.05 versus cells transfected with the pCAT-IR alone; **P < 0.01 versus cells transfected with the pCAT-IR plus AP-2 effector vector. In some experiments (D), HepG2 cells were transfected as above, using the CAT reporter vector containing the proximal C2 (pCAT-C2) or distal E3 (pCAT-E3) element of the IR gene promoter. *P < 0.05 versus cells transfected with the pCAT-C2 alone. White bars, mock (no DNA); black bars, pCAT-basic (vector without an insert). Shown are representative western blots of PPARγ, Sp1, C/EBPβ, and HMGA1 from nuclear extracts of transfected HepG2 cells.
of PPARγ on IR gene transcription, we performed experiments designed to investigate whether PPARγ could affect this promoter function by physically interacting with HMGA1, Sp1, C/EBPβ or AP-2, thus preventing the interaction between these factors in the preinitiation complex at the IR promoter. To this end, we performed GST pull-down assays, in which in vitro-translated 35S-labeled proteins were analyzed for their ability to be specifically retained by a GST-PPARγ affinity resin. As shown in Fig. 3, under our experimental conditions, no interaction was observed between 35S-labeled HA-tagged HMGA1 and GST-PPARγ, excluding a physical contact between these two proteins in vitro. Conversely, the 35S-labeled Sp1 was retained by GST-PPARγ but not GST alone, indicating that Sp1 physically interacts with PPARγ. Similarly, the 35S-labeled AP-2 and to a lesser extent labeled C/EBPβ were also specifically bound by GST-PPARγ, but not by GST alone (Fig. 3), indicating that PPARγ directly interacts with these two proteins as well. A bona fide interaction between these factors and GST-PPARγ was proved by the use of high concentrations of ethidium bromide (not shown), which has been shown to disrupt DNA-dependent protein–protein contact (Foti et al. 2003).

**Binding of Sp1, AP-2, and C/EBPβ to the IR promoter is reduced by PPARγ**

Physical interaction between PPARγ, Sp1, AP-2, and C/EBPβ has been characterized further in vitro by EMSA, using the previously described radiolabeled fragment C2 of the human IR promoter gene as probe (Brunetti et al. 1993). As shown in Fig. 4, binding of Sp1 and HMGA1 was easily detected in nuclear extracts from untreated HepG2 cells, in which only trace amounts of PPARγ are normally expressed. Binding of Sp1 was reduced in nuclear extracts from HepG2 cells overexpressing PPARγ1, and was further decreased after treatment of these cells with TZD rosiglitazone. Under these conditions, no changes were observed with HMGA1-DNA binding. When EMSA was performed by nuclear extracts from HepG2 cells overexpressing AP-2, a co-complex containing Sp1, HMGA1, and AP-2 was also detected, in addition to Sp1 and HMGA1 complexes (Fig. 4). The reduction in Sp1-DNA binding, in cells overexpressing PPARγ1, paralleled the reduction in the co-complex-DNA binding formation and these interactions were further reduced by the addition of rosiglitazone. Similar results were obtained in the MCF-7 cells transfected with PPARγ expression vector, either in the absence or in the presence of TZD. Like Sp1 and AP-2, binding of endogenous C/EBPβ to DNA was reduced in nuclear extracts from cells overexpressing PPARγ1 and was further decreased by treatment of cells with rosiglitazone (Fig. 4). The presence of HMGA1 in the more rapidly migrating complex was verified by its supershifting in the presence of the anti-HMGA1 antibody, whereas the presence of Sp1, AP-2, and C/EBPβ in the slowly migrating complexes was verified by their supershifting in the presence of Sp1-, AP-2-, and C/EBPβ-specific antibodies respectively. Since no effect on the electrophoretic migration of DNA–protein complexes was observed in the presence of control-unrelated antibodies (data not shown), the reduction of protein–DNA binding in supershift assays suggests that these factors are simultaneously binding the same labeled probe. The above findings were substantiated in vivo by ChIP analyses. As shown in Fig. 5, binding of Sp1, AP-2, and C/EBPβ to the C2 promoter region of

![Figure 3](image_url)  
**Figure 3** Physical association between PPARγ and IR-DNA binding proteins. SDS-PAGE of 35S-labeled HA-tagged HMGA1, Sp1, AP-2, and C/EBPβ bound to GST-PPARγ resin. In the first lane of each panel, 5% of the labeled protein was added directly onto the gel without binding to and elution from GST protein resin (input). Specifically bound proteins were visualized by autoradiography.

![Figure 4](image_url)  
**Figure 4** Nuclear protein–DNA interactions within the C2 region of the IR promoter. EMSAs of radiolabeled fragment C2 (0.2 ng) with 0.5 μg nuclear protein from (A) wild-type HepG2 cells or (B) AP-2-overexpressing HepG2 cells, and (C) wild-type MCF-7 cells, in the presence of DNA. In supershift assays, nuclear protein was preincubated with 1 μg polyclonal antibody (Ab) to HMGA1, Sp1, AP-2, or C/EBPβ before addition of the probe (none: probe alone).
transformation and increased cell growth occur in cultured breast cells overexpressing the IR (Osborne et al. 1978, Milazzo et al. 1992, Paonessa et al. 2006). Overexpression of functional IRs has also been involved in thyroid carcinogenesis (Farid et al. 1994). The IR can exert its oncogenic potential in malignant cells via abnormal stimulation of multiple cellular signaling cascades, enhancing growth factor-dependent proliferation and/or by directly affecting cell metabolism. Both breast and thyroid neoplastic cells express PPARγ, and PPARγ agonists have been shown to inhibit proliferation in these and other cell systems (Tontonoz et al. 1994, Martelli et al. 2002, Grommes et al. 2004). In this light, we undertook to investigate whether IR expression could be affected by PPARγ. Our study shows that IR gene transcription and receptor protein content were reduced in cells with forced PPARγ1 overexpression, or TZD-induced PPARγ activation. Although these results apparently run contrary to what might have been predicted based on the known insulin-sensitizing effects of TZD, seeming to exclude the possibility that TZDs may act as insulin sensitizers through the IR, they are compatible with the pleiotropic effects of PPARγ. In this regard, the IR may be considered a new target gene that accounts for the anti-mitogenic response to PPARγ and its agonists, and this is the first description of a tyrosine kinase receptor involved in PPARγ-induced anti-proliferative mechanisms. To unravel the molecular basis underlying the decrease in IR gene expression produced by PPARγ, we performed protein–protein and DNA–protein interaction studies together with ChIP analysis, combined with transient transcription assays in the living cells expressing variable amounts of the IRs. Since no PPRE have been detected within the IR gene promoter and no reduction in the expression of HMGA1, Sp1, or C/EBPβ was observed in cells after PPARγ or TZD stimulation, we propose that PPARγ acts as a negative regulator of IR transcription by adversely affecting binding of Sp1, C/EBPβ, or AP-2 to the IR gene. In other PPARγ-responsive genes that carry a PPRE consensus in their sequence, a functional cooperation between Sp1 and PPARγ has been described (Krey et al. 1995). On the other hand, functional cooperation between C/EBPβ (and other members of the C/EBP family of proteins) and PPARγ has been reported in the nutrient signaling system during fetal development (Maloney & Rees 2005), regulation of vascular inflammation (Takata et al. 2002), and inhibition of adhesive interaction between multiple myeloma and bone marrow stromal cells (Wang et al. 2007). Herein, we show that PPARγ physically interacts with

![Figure 5 ChIP assay. ChIP of the IR promoter gene in AP-2x-producing HepG2 cells and MCF-7 cells both induced to overexpress PPARγ, either in the absence or in the presence of TZD (rosiglitazone). ChIP was done using antibodies (Ab) against either HMGA1, Sp1, AP-2, or C/EBPβ.](image)
Sp1, C/EBPβ, and AP-2 reducing the IR gene transcription in the absence of PPARγ DNA-binding sites on the IR gene. We suggest that, in the absence of PPRE in the context of the IR promoter, this nuclear receptor may produce its adverse effects on IR gene transcription by interacting physically with these factors, thus reducing their availability to the basic transcription machinery of the IR gene. With a similar mechanism involving Sp1/PPARγ protein interaction, PPARγ has been shown to exert an anti-proliferative role by suppressing transcription of the thromboxane receptor, the cyclin-dependent kinase inhibitor p21, and the fibronectin genes (Sugawara et al. 2002, Hong et al. 2004, Han et al. 2005). The molecular mechanism herein described is therefore in agreement with the increasing repertoire of ‘non-canonical’ PPARγ target genes that now encompasses non-PPREs containing genes (Tan et al. 2005).

Many potential target genes of PPARγ have been already reported, including bcl-2, β-catenin, and the PTEN tumor suppressor gene (Elstner et al. 1998, Patel et al. 2001, Mulholland et al. 2005). However, how PPARγ and its agonists may induce their anti-proliferative effects is not fully understood yet. Recently, non-genomic crosstalks between PPARγ and cytoplasmic proteins, like extracellular signal-regulated kinase (ERK) 1/2 and MAPK kinases, have been reported in cancer cells and functional importance has been given to the subcellular localization of PPARγ (Burgermeister & Seger 2007, Papageorgiou et al. 2007). However, the last three decades of medical research examining the molecular pathogenesis of cancers have provided compelling evidence for the universal disruption of the cell cycle in human tumors, and recent studies have demonstrated a critical interface between hormonal signaling and the cell cycle (Hilakivi-Clarke et al. 2004). In this context, mitogens like insulin, via the IR, may promote the progression through the G1 phase by inducing competence of the cyclin D1/cyclin-dependant kinase 4 (CDK4) complex. It has been previously demonstrated that the PPARγ agonists inhibit cyclin D1 (Wang et al. 2001). Our findings support the conclusion that PPARγ and TZDs may interfere with the hormonal control of the cell cycle, at least in part, through the inhibition of the IR.

Over the last decade, PPARγ has emerged as an important drug target in type 2 diabetes mellitus (Savkur & Miller 2006), and TZDs are widely used for treatment of diabetic patients. However, conflicting results on the pro-carcinogenic and anti-tumorigenic effects of TZDs in humans with diabetes can be found in the literature. For instance, whereas a population-based report showed that TZDs were associated with reduced risk of lung cancer in patients with diabetes (Govindarajan et al. 2007), a possible association between cancer and the use of TZD has been reported later in type 2 diabetic patients (Ramos-Nino et al. 2007). Taken together, our results consistently support
the conclusion that IR gene may be considered a new anticancer target for PPARγ, providing further evidence for the use of TZDs as anti-proliferative agents in selected tumors overexpressing the IR.

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