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Mechanisms of divergent effects of activated peroxisome proliferator-activated receptor-γ on mitochondrial citrate carrier expression in 3T3-L1 fibroblasts and mature adipocytes ☆

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ABSTRACT

The citrate carrier (CIC), a nuclear-encoded protein located in the mitochondrial inner membrane, plays an important metabolic role in the transport of acetyl-CoA from the mitochondrion to the cytosol in the form of citrate for fatty acid and cholesterol synthesis. Citrate has been reported to be essential for fibroblast differentiation into fat cells. Because peroxisome proliferator-activated receptor-gamma (PPARγ) is known to be one of the master regulators of adipogenesis, we aimed to study the regulation of CIC by the PPARγ ligand rosiglitazone (BRL) in 3T3-L1 fibroblasts and in adipocytes. We demonstrated that BRL up-regulated CIC mRNA and protein levels in fibroblasts, while it did not elicit any effects in mature adipocytes. The enhancement of CIC levels upon BRL treatment was reversed using the PPARγ antagonist GW9662, addressing how this effect was mediated by PPARγ. Functional experiments using a reporter gene containing rat CIC promoter showed that BRL enhanced CIC promoter activity. Mutagenesis studies, electrophoretic-mobility-shift assay and chromatin-immunoprecipitation analysis revealed that upon BRL treatment, PPARγ and Sp1 are recruited on the Sp1-containing region within the CIC promoter, leading to an increase in CIC expression. In addition, mithramycin, a specific inhibitor for Sp1-DNA binding activity, abolished the PPARγ-mediated up-regulation of CIC in fibroblasts. The stimulatory effects of BRL disappeared in mature adipocytes in which PPARγ/Sp1 complex recruited SMRT corepressor to the Sp1 site of the CIC promoter. Taken together, our results contribute to clarify the molecular mechanisms by which PPARγ regulates CIC expression during the differentiation stages of fibroblasts into mature adipocytes.

1. Introduction

The mitochondrial citrate carrier (CIC), a nuclear-encoded protein which belongs to the mitochondrial carrier family is located in the inner membrane of mitochondria [1–3] and consists of three tandemly related domains of approximately 100 amino acids in length that span the membrane six times with both the N- and C-termini protruding toward the cytosol [4,5]. CIC exports citrate from the mitochondria to the cytosol where citrate is cleaved by ATP-citrate lyase to acetyl-CoA and oxaloacetate. Acetyl-CoA is used for fatty acid and sterol biosynthesis; whereas oxaloacetate is reduced to malate, which in turn is converted to pyruvate via malic enzyme with production of NADPH plus H^+. In addition to its role in fatty acid synthesis, CIC is involved in other processes such as gluconeogenesis, insulin secretion, histone acetylation and inflammation [6–10].

In rat, CIC activity was found to be decreased in diabetic and hypothyroid animals [11,12]. Later, it has been shown that CIC gene promoter contains an active FOXA site and that FOXA1 controls glucose-stimulated insulin secretion in INS-1 cells by transcriptional regulation of the CIC gene [13]. As for other lipogenic enzymes, CIC activity and expression are controlled by various nutritional states [14–17]. For instance, dietary polyunsaturated fatty acids (PUFA) inhibited CIC expression at both transcriptional and post-transcriptional levels, while saturated and monounsaturated fatty acid-enriched diet administration to rats did not have any effects [7,17,18]. Indeed, a PUFA response region containing the binding sites for some transcription factors such as SREBP-1 (sterol regulatory element binding protein-1), Sp1 (Specificity protein1) and

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NF-Y (Nuclear Factor-Y) has been identified in the CIC gene promoter [7,19,20]. SREBP-1 activates the expression of CIC in HepG2 cells [7], hepatocytes [20] and mammary epithelium [21]. Recently, Damiano et al. have demonstrated that CIC expression, in hepatocytes and adipocytes, is regulated by ligands of peroxisome proliferator-activated receptor (PPAR) α and γ, identifying a peroxisome proliferator-activated receptor responsive element (PPRE) motif at −625 bp of CIC promoter [22].

PPARs are members of nuclear hormone receptors superfamily that function as ligand-dependent transcription factors [23]. Three PPAR isoforms, α, β/δ, and γ, are expressed in multiple species in a tissue-specific manner [24,25]. Among them, PPARγ integrates the control of energy, lipid and glucose homeostasis participating in the transcriptional activation of several genes important for adipocyte maturation, lipid accumulation, and insulin-sensitive glucose transport, as adipocyte fatty acid binding protein aP2, CCAAT/enhancer-binding protein-α (C/EBPα), Perilipin, and GLUT4 [26–29], phosphoenolpyruvate carboxykinase [30,31] and glycerol kinase [32]. Moreover, it has been demonstrated a role for PPARγ in cell differentiation, growth arrest and apoptosis in a large variety of cells [33–36]. Natural ligands of PPARγ include fatty acids and prostaglandin derivatives, while synthetic ligands of PPARγ are the insulin-sensitizing thiazolidinediones, as rosiglitazone (BRL).

Due to this knowledge, aim of our study was to define whether PPARγ may differently regulate CIC expression during the differentiation of 3T3-L1 fibroblasts into mature adipocyte cells. We have provided evidence, for the first time, that activated PPARγ up-regulates CIC expression in fibroblasts through Sp1 site present within CIC promoter region. The stimulatory effects of BRL disappear in mature adipocytes in which PPARγ/Sp1 complex recruits SMRT corepressor to the Sp1 site of the CIC promoter. Our results contribute to clarify the molecular mechanisms by which PPARγ during adipogenesis regulates CIC expression, which represents a crucial cross-point for several metabolic pathways.

2. Materials and methods

2.1. Reagents

BRL49653 (BRL) was purchased from Alexis (San Diego, CA, USA), the irreversible PPARγ antagonist GW9662 (GW) and mithramycin (M) were purchased from Sigma (Milan, Italy).

2.2. Cell culture and differentiation

The 3T3-L1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U penicillin and 100 μg/ml streptomycin (Sigma) in an atmosphere of 5% CO_2 at 37 °C. The cells were cultured until confluence had been reached, and then differentiation was induced 2 days thereafter (designated as "day 0") by adding 3-isobutyl-1-methylxanthine, dexamethasone and insulin (Sigma) to make their final concentrations of 0.5 mM, 1 μM and 1 μg/ml, respectively. After 72 h, the medium was changed to maturation medium supplemented with 1 μM dexamethasone and 1 μg/ml insulin. Cells were fed with maturation medium every 48 h, obtaining adipocytes at an early stage of differentiation (preadipocytes) after 7 days and mature adipocytes after 14 days from day 0. Cell differentiation was monitored by evaluating cell morphology under phase-contrast microscopy. Cells were considered to be adipocytes when numerous lipid droplets were observed in the cytoplasm. More than 90% of cells expressed the adipocyte phenotype. Cells were switched to serum-free medium the day before each experiment and then treated as indicated.

2.3. Mitochondrial isolation

3T3-L1 fibroblasts and mature adipocytes were grown in 10-cm dishes and exposed to treatments in serum-free medium as indicated before fractionation. Mitochondria were isolated as described previously [37]. Briefly, cells were washed with ice cold PBS, collected by scrapping in cold PBS and, after centrifugation (600 ×*g*, 4 °C, 10 min), resuspended in 200 mM sucrose, 10 mM Tris–MOPS and 1 mM EDTA/Tris, pH 7.4 (STE buffer). Cells were homogenized by glass Potter homogenization and mitochondria were then isolated by serial centrifugations. The mitochondrial pellet was resuspended in lysis buffer for immunoblotting analysis and in STE buffer for transport measurements.

2.4. Immunoblot analysis

Cells were grown in 10-cm dishes to 70% to 80% confluence and exposed to treatments in serum-free medium as indicated. Cells were then harvested in cold PBS and resuspended in lysis buffer containing 50 mM Tris–HCl (pH 8), 150 mM NaCl, 2 mM EDTA, 20% glycerol, 1% NP-40, and inhibitors (0.1 mM sodium orthovanadate, 1% phenylmethylsulfonylfluoride, and 20 mg/ml aprotinin). Equal amounts of total protein lysates or mitochondrial extracts, isolated as described above, were resolved on 10% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane (Amersham Biosciences, Milan, Italy), and probed with the antibody directed against human C terminal-CIC [5] or PPARγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). A mouse monoclonal antibody against the β-subunit of human F1-ATPase (β-ATPase) (BD Biosciences, San Josè, CA, USA) was used as a loading control to ensure that any differences in protein expression between pre- and post-differentiation cells were not due to the increase in number of mitochondria, typically occurring to mature adipocytes during differentiation. Antigen–antibody complexes were detected using anti-rabbit or anti-mouse IgG-coupled horseradish peroxidase (Pierce, Rockford, IL, USA) and revealed using the ECL Western Blotting Analysis System (Amersham). The bands of interest were quantified by the Scion Image laser densitometry scanning program.

2.5. Mitochondria reconstitution and transport measurements

Isolated mitochondria from 3T3-L1 fibroblasts and mature adipocytes were solubilized in a buffer containing 3% Triton X-114, 4 mg/ml cardiolipin, 10 mM Na_2SO_4, 0.5 mM EDTA, and 5 mM PIPES, pH 7 using modifications of the method described previously by Jordens et al. [38]. After incubation for 20 min at 4 °C, the mixture was centrifuged at 138,000 ×*g* for 10 min. The supernatant was incorporated into phospholipid vesicles by cyclic removal of the detergent [39]. The reconstitution mixture consisted of 0.04 mg protein solution, 10% Triton X-114, 10% phospholipids (egg lecithin from Fluka, Milan, Italy) as sonicated liposomes, 10 mM citrate, 0.85 mg/ml cardiolipin (Sigma) and 20 mM PIPES; pH 7.0. The mixture was recycled 13 times through an Amberlite column. All phases were performed at 4 °C, except for the passages through Amberlite, which were carried out at room temperature. To measure citrate transport, external substrate was removed from the proteoliposomes on Sephadex G-75 columns pre-equilibrated with buffer A (50 mM NaCl and 10 mM PIPES, pH 7.0). Transport at 25 °C was started by the addition of 0.5 mM [^{14}C]citrate (Amersham) to the eluted proteoliposomes and terminated by the 'inhibitor-stop' method with the addition of 20 mM 1,2,3-benzene-tricarboxylate [40,41]. In control samples, the inhibitor was added simultaneously to the labeled substrate. Finally, the external radioactivity was removed from the Sephadex G-75 columns and radioactivity in the liposomes was measured [39]. Transport activity was calculated by subtracting the control values from the experimental values.

2.6. RT-PCR assay

Total RNA was extracted from 3T3-L1 fibroblasts, pre-adipocytes and mature adipocytes using a Trizol reagent (Invitrogen, Milan, Italy) according to the manufacturer's protocol. RNA was quantified spectrophotometrically and its quality was checked by electrophoresis through agarose gels stained with ethidium bromide. CIC and PPARγ expression were analyzed by the real-time polymerase chain reaction (RT-PCR) method as described previously [42], using the following primers: CIC forward 5′-CTGTCAGGTTTGGGATGTTC-3′ and reverse 5′-GTGGGTTCATAGGTTTGTTG-3′; PPARγ forward 5′ GGTGA AACTCTGGGAGATTC-3′ and reverse 5′-CAACCATTGGGTCAGCTCTT-3′; β-actin forward 5′-AGGCATCCTGACCCTGAAGTAC-3′ and reverse 5′-TC TTCATGAGGTAGTCTGTCAG-3′. PCR was performed for 34 cycles for CIC (94 °C 1 min, 66 °C 1 min, 72 °C 1 min), 32 cycles for PPARγ (94 °C 1 min, 67 °C 1 min, 72 °C 1 min) and 24 cycles for β-actin (94 °C 1 min, 60 °C 1 min, 72 °C 1 min).

2.7. Plasmid and reporter vector construction

The plasmid pCIC1437 containing the rat CIC gene promoter region spanning from −1473 to +35 bp was amplified from Rat Genomic DNA (Novagen, Merck Bioscience, Germany) by nested PCR as previously described [42] using the following primers: sense 5′-AGAGCTCCAGAC CATGTGC-3′ and antisense 5′-AGTTTGGCTTTCCCGGACC-3′; nested-sense (pCIC1473for) 5′-TGAGGTACCAACAAGCCCCTCAGAGGCTG-3′ and nested-antisense (pCICrev) 5′-TGAAAGCTTTCGACCTCGGGTCCGAGCC-3′. The amplified DNA fragment was digested with *KpnI* and *HindIII* and then cloned into the pGL3 basic vector (Promega, Milan, Italy). The pCIC1473 plasmid was used as template to generate the different deleted constructs: pCIC284 (−284 to +35 bp), pCIC145 (−145 to +35 bp), pCIC115 (−115 to +35 bp) and pCIC82 (−82 to +35 bp).

Forward primers are listed in Table 1; the reverse primer was pCICrev for all the constructs.

The mutation of Sp1 site included from −115 to −82 region was obtained by site-directed mutagenesis using QuickChange kit (Stratagene, La Jolla, CA) performed on pCIC115 plasmid. The mutagenic primers to construct the pCIC115-Sp1mut are listed in Table 1. The plasmid pCIC-3xSp1, containing a threefold repeat of wild type responsive Sp1 site, was constructed by annealing between the following forward and reverse primers: forward: 5′-CATGGTACCTAATGCGGGGCGGATG CGGGGCGGAAGCGGGGCGGATCCAAGCTTTAG-3′; reverse: 5′-CTAAAG CTTGGATCCGCCCCGCTTCCGCCCCGCATCCGCCCCGCATTAGGTACCATG-3′. The fragment obtained by annealing was used as template in a PCR reaction conducted with the following forward and reverse primers 5′-CATGGTACCTAATGCGGG-3′ and 5′-CTAAAGCTTGGATCCGCC-3′, respectively. The DNA fragment was digested with *KpnI* and *HindIII* and then cloned into the pGL3 basic vector (Promega, Milan, Italy).

The sequence of the different constructs was verified by nucleotide sequence analysis.

2.8. Transient transfection assays

3T3-L1 fibroblasts were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen, Milan, Italy) with the described rat CIC promoter constructs for 18 h. After transfection, cells were treated as described for 12 h. Thymidine kinase–Renilla luciferase plasmid was used to normalize the efficiency of the transfection. Firefly and Renilla luciferase activities were measured with the Dual Luciferase Kit (Promega) according to the manufacturer's recommendations.

Table 1
Oligonucleotides used for CIC promoter constructs.

Construct	Oligonucleotide sequence
pCIC1473	5′-TGAGGTACCAACAAGCCCCTCAGAGGCTG-3′
pCIC284	5′-TGAGGTACCTACCCGCTTTGGCAAAGAGTTGC-3′
pCIC145	5′-TAGGGTACCAGTTTCCCGGCTGGCAC-3′
pCIC115	5′-TAGGGTACCGGCGGGGCTCAGCTCAG-3′
pCIC82	5′-TAGGGTACCCCGGGGAGCTGACGTGA-3′
pCIC115Sp1mut For	5′-GCTCAGGCCACGCGGATCCGAGCCGGGGAGCTGAC-3′
pCIC115Sp1mut Rev	5′-GTCAGCTCCCCGGCTCGGATCCGCGTGGCCTGAGC-3′

2.9. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts from 3T3-L1 fibroblasts were prepared as previously described [43]. The probe was generated by annealing single-stranded oligonucleotides labeled with [^{32}P]ATP and tyrosine polynucleotide kinase and then purified using Sephadex G-50 spin columns (Sigma). The DNA sequence used as probe or as cold competitor was as follows (the nucleotide motif of interest is underlined and mutations are shown as lowercase letters): Sp1 5′-AGGCCA CGCGGGGCGGAGCCCGGGA-3′, mutated Sp1 5′-AGGCCACGCGattaGG AGCCCGGGA-3′.

The protein-binding reactions were carried out in 20 μl of buffer [20 mM HEPES (pH 8), 1 mM EDTA, 50 mM KCl, 10 mM dithiothreitol, 10% glycerol, 1 mg/ml BSA, 50 μg/ml poly(dI/dC)] with 50,000 cpm of labeled probe, and 5 μg of fibroblast nuclear protein. The mixtures were incubated at room temperature for 20 min in the presence or absence of unlabeled competitor oligonucleotides. For the experiments involving anti-PPARγ and anti-Sp1 antibodies (Santa Cruz Biotechnology), the reaction mixture was incubated with these antibodies at 4 °C for 30 min before addition of the labeled probe. The entire reaction mixture was electrophoresed through a 6% polyacrylamide gel in 0.25× Tris-borate-EDTA for 3 h at 150 V. Gel was dried and subjected to autoradiography at −80 °C.

2.10. Chromatin immunoprecipitation (ChIP) and re–ChIP assays

3T3-L1 fibroblasts and mature adipocytes were grown in 10-cm dishes to 50%–60% confluence, starved with serum-free medium for 24 h and then treated with BRL. Thereafter, cells were washed twice with PBS and cross-linked with 1% formaldehyde and sonicated. Supernatants were immunocleared with salmon sperm DNA/protein A agarose for 1 h at 4 °C. The precleared chromatin was immunoprecipitated with specific anti-PPARγ, anti-Sp1, or anti-polymerase II (POLII) antibodies (Santa Cruz Biotechnology). The anti-PPARγ samples were reimmunoprecipitated with anti-Sp1, anti-ARA70, anti-PCG1α (Santa Cruz Biotechnology), anti-SMRT or anti-NCoR (Novus Biologicals, Milan, Italy) antibodies. The anti-Sp1 samples were reimmunoprecipitated with anti-SMRT antibody. A normal mouse serum IgG was used as negative control. Pellets were washed, eluted with elution buffer (1% SDS, 0.1 mol/l $NaHCO_3$), and digested with proteinase K. DNA was obtained by phenol/chloroform/isoamyl alcohol extractions and was precipitated with ethanol. Five microliters of each sample and input were used for PCR with the primers flanking the Sp1 sequence present in the CIC promoter region: 5′-TAGCGTTGCTGTCCGGAGACCA-3′ and 5′-GAGACCAC GACCAATTCTGGT-3′. The amplification products obtained were analyzed in 2% agarose gel and visualized by ethidium bromide staining.

2.11. RNA silencing

3T3-L1 fibroblasts and mature adipocytes were transfected with RNA duplex of stealth siRNA targeted for the mouse SMRT mRNA sequence (Ambion, ID:s74031) or with a control siRNA used as a control for non-sequence-specific effects to a final concentration of 100 nM using Lipofectamine 2000 as recommended by the manufacturer. After 5 h the transfection medium was changed with serum-free medium and then the cells were exposed to treatments.

2.12. Statistical analysis

Statistical analysis was performed using ANOVA followed by Newman–Keuls' testing to determine differences in means. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Functional characterization of CIC in 3T3-L1 cells

We first aimed to investigate the expression and activity of CIC in mitochondrial extracts from 3T3-L1 fibroblasts (F) and mature adipocytes (A, 14 days after differentiation induction). Immunoblot analysis, using an antibody raised against the carboxy-terminus of the mature CIC protein, revealed a weak immunoreactive band in fibroblasts at 34 kDa, corresponding to the mitochondrial CIC, while a 4.5-fold increase in band intensity was observed in mature adipocytes (Fig. 1A). A similar pattern of CIC expression was also found in total extracts of both fibroblasts and adipocytes (Fig. 1B). The activity of CIC in mitochondrial extracts from 3T3-L1 fibroblasts and mature adipocyte cells was tested by assaying the rate of the [^{14}C]citrate/citrate exchange in reconstituted liposomes [40,41]. As shown in Fig. 1C, the uptake of radioactive L-citrate in liposomes reconstituted with mitochondrial extracts from fibroblasts was approximately 45% lower compared to liposomes reconstituted with the mitochondrial extracts from mature adipocyte cells (132 ± 14.4 versus 238 ± 25.0 nmol citrate/mg protein, respectively).

3.2. The PPARγ ligand BRL up-regulates CIC expression in 3T3-L1 fibroblasts

Since PPARγ is considered to be one of the master regulators of adipocyte differentiation, we evaluated the involvement of this nuclear receptor in the modulation of CIC expression during adipocyte differentiation. We tested the effects of BRL49653 (BRL), a synthetic and specific ligand of PPARγ, in 3T3-L1 fibroblasts (F), in pre-adipocytes (P), which are adipocytes at an early stage of differentiation, and in mature adipocytes (A). The results obtained demonstrated that BRL treatment up-regulated CIC mRNA expression in fibroblasts and to a lesser extent in pre-adipocytes, while it did not elicit any effects on mature adipocytes (Fig. 2A). As previously reported [44], the expression level of PPARγ mRNA was enhanced in BRL-treated fibroblasts and pre-adipocytes and reduced in BRL-treated mature adipocytes (Fig. 2A). Moreover, CIC protein content in fibroblasts increased 4-fold after treatment with BRL for 24 h compared to untreated fibroblasts (Fig. 2B). This up-regulation was abrogated by GW9662 (GW), an irreversible PPARγ antagonist, demonstrating a direct involvement of PPARγ (Fig. 2B). As expected, in mature adipocytes BRL treatment did not modulate CIC protein levels, while it down-regulated PPARγ protein expression, which was reversed in the presence of GW (Fig. 2C).

Finally, we investigated the effects of the PPARγ ligand BRL on CIC activity in mitochondrial extracts from fibroblasts and adipocytes. We found that the uptake of [^{14}C]citrate in BRL-treated fibroblasts was enhanced as compared to untreated cells (192 ± 21.2 versus 130 ± 15 nmol citrate/mg protein, respectively), reaching the CIC activity levels measured in mature adipocytes (235 ± 24 nmol citrate/mg protein), while BRL did not exert any effects in mature adipocytes (Fig. 2D). Taken together, these data suggest that activated PPARγ is able to induce CIC expression and increase CIC activity only in fibroblasts.

3.3. BRL transactivates CIC gene promoter in 3T3-L1 fibroblasts

The aforementioned observations prompted us to investigate whether BRL is able to modulate CIC transcriptional activity. Thus, we performed functional assays by transiently transfecting 3T3-L1

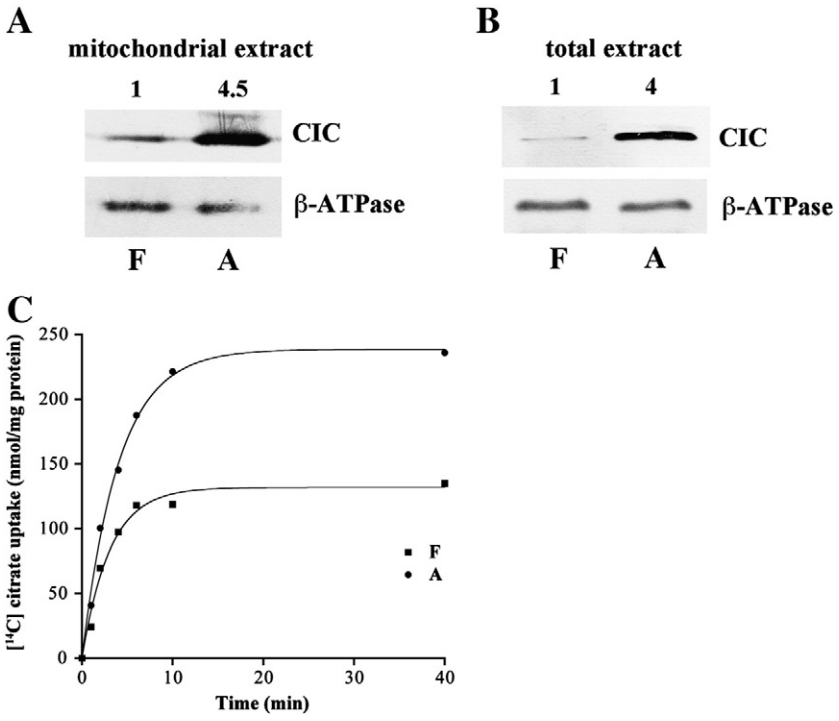

Fig. 1. CIC expression and activity in 3T3-L1 cells. Immunoblots for CIC expression from mitochondria (A) and total extracts (B) of fibroblasts (F) and mature adipocytes (A). Beta subunit of mitochondrial ATPase (β-ATPase) was used as loading control. Numbers represent the average fold change of CIC/β-ATPase levels. C: Rate of [^{14}C]citrate/citrate exchange in fibroblast and adipocyte mitochondria. Transport was initiated by adding 0.5 mM [^{14}C]citrate to proteoliposomes containing 10 mM citrate and reconstituted with mitochondria isolated from either fibroblasts (square) or mature adipocytes (circle). The transport reaction was stopped at the indicated times. The data represent means of three independent experiments.

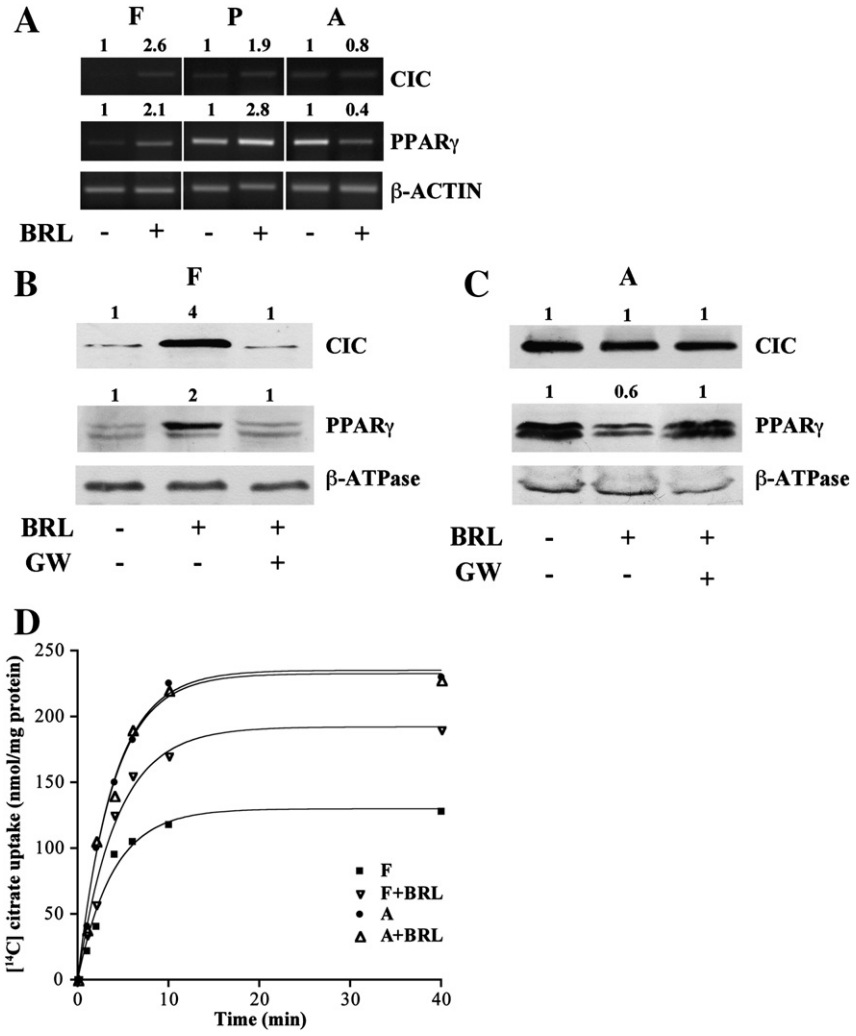

Fig. 2. Activated PPARγ up-regulates CIC expression and activity in fibroblasts. A: CIC and PPARγ mRNA expression in 3T3-L1 fibroblasts (F), preadipocytes (P) and mature adipocytes (A) untreated (−) or treated with 10 μM BRL for 24 h. β-ACTIN was used as loading control. Numbers represent the average fold change of CIC or PPARγ/β-ACTIN levels. Immunoblots for CIC and PPARγ expression from total extracts of fibroblast (B) and mature adipocyte cells (C) untreated (−) or treated with 10 μM BRL in the presence or not of 10 μM GW for 24 h. β-ATPase was used as loading control. Numbers represent the average fold change of CIC or PPARγ/β-ATPase levels. D: [^{14}C]citrate/citrate exchange in fibroblast and adipocyte mitochondria untreated or treated with BRL. Transport was initiated by adding 0.5 mM [^{14}C]citrate to proteoliposomes containing 10 mM citrate and reconstituted with mitochondria isolated from untreated fibroblasts (square), BRL-treated fibroblasts (down-pointing triangle), adipocytes (circle) and BRL-treated adipocytes (up-pointing triangle). The transport reaction was stopped at the indicated times. The data represent means of three independent experiments.

fibroblasts with a plasmid containing rat CIC regulatory sequence pCIC1473 (−1473/+35) and found that BRL significantly induced luciferase activity (Fig. 3A). This effect was no longer noticeable in the presence of GW, confirming that the transactivation of CIC by BRL occurred in a PPARγ-dependent manner (data not shown). The rat CIC promoter contains multiple responsive elements for different transcription factors, including glucocorticoid receptor (GR), estrogen receptor alpha (ERα), PPARγ and α, c/EBPα and Sp1 (Fig. 3A). To identify the region within the CIC promoter responsible for the BRL-induced transactivation, the activity of the different CIC promoter–deleted constructs pCIC284 (−284/+35), pCIC145 (−145/+35), pCIC115 (−115/+35) and pCIC82 (−82/+35) was tested. In transfection experiments performed using the aforementioned plasmids pCIC284, pCIC145 and pCIC115, responsiveness to BRL was still observed (Fig. 3A). Of note, BRL was able to transactivate all tested constructs independently of the PPRE site, which was recently identified at −625 bp [22]. In contrast, in cells transfected with the promoter–deleted construct pCIC82 we did not detect any increase in luciferase activity (Fig. 3A). Consequently, the region from −115 to −82, which contains the Sp1 motif, was the minimal region of CIC promoter responsible for BRL induction.

Thus, we performed site-directed mutagenesis on the minimal responsive Sp1 domain (pCIC115-Sp1mut) within the CIC promoter (Fig. 3A). Mutation of this domain abrogated BRL effects (Fig. 3A) demonstrating that the integrity of Sp1-binding site is necessary for PPARγ modulation of CIC promoter activity. To strengthen the importance of the Sp1 site in CIC promoter modulation by BRL, we performed transfection experiments using a construct (pCIC-3xSp1) bearing threefold repeat of wild type responsive Sp1 site located in the minimal region of CIC promoter. BRL treatment induced a 1.7 fold increase in luciferase activity respect to untreated cells (Fig. 3B).

In addition, functional experiments and RT-PCR analysis were performed using mithramycin that binds to GC boxes and prevents sequential Sp1 binding to its consensus sequence [45]. Our results showed that mithramycin was able to abrogate the BRL-induced CIC transcriptional activity as well as its mRNA expression in fibroblast cells (Fig. 3C and D).

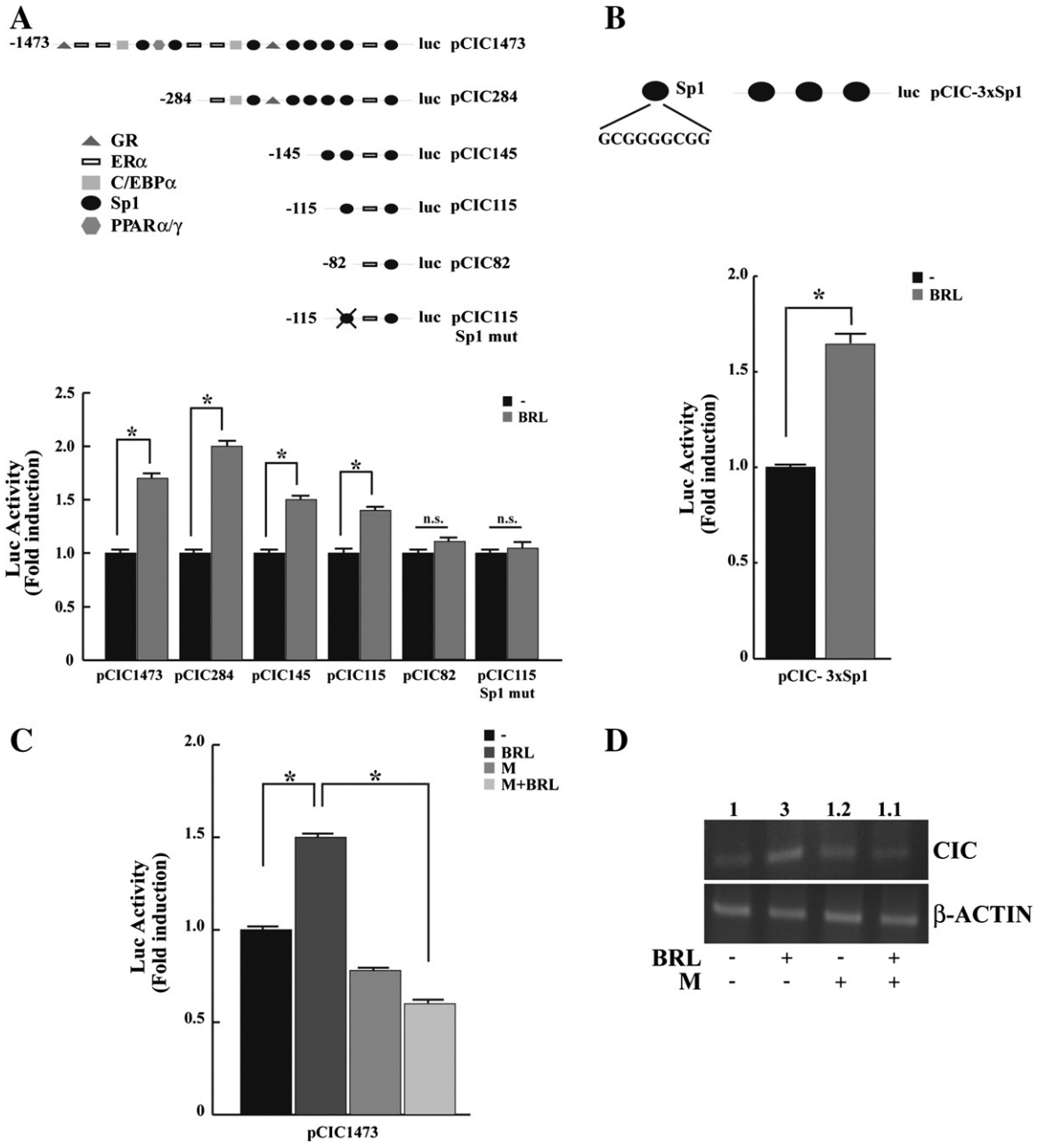

Fig. 3. BRL transactivates CIC transcriptional activity in fibroblasts. A:*Upper panel*, schematic representation of the CIC promoter constructs used in this study. *Lower panel*, 3T3-L1 fibroblasts were transiently transfected with luciferase plasmids containing the CIC promoter (pCIC1473), its deletions (pCIC284, pCIC145, pCIC115 and pCIC82) or pCIC115-Sp1mut mutated in Sp1 site and then untreated (−) or treated with 10 μM BRL for 12 h. B: *Upper panel*, schematic representation of the pCIC-3xSp1 construct used in this study. *Lower panel*, cells transiently transfected with pCIC-3xSp1were untreated (−) or treated with 10 μM BRL for 12 h. C: Cells transiently transfected with pCIC1473 were untreated (−) or treated with 10 μM BRL and/or 100 nM mithramycin (M) for 12 h. Luciferase activity of untreated cells was set as 1-fold induction, upon which treatments were calculated. Columns are the means ± S.D. of three independent experiments performed in triplicate.*P<0.05, n.s. = non significant. D: CIC mRNA expression in 3T3-L1 fibroblasts untreated (−) or treated with 10 μM BRL and/or 100 nM mithramycin (M) for 24 h. Numbers represent the average fold change of CIC/β-ACTIN levels.

3.4. BRL enhances recruitment of PPARγ and Sp1 to the CIC promoter in 3T3-L1 cells

To further support the role of the Sp1 site in mediating the BRL-induced up-regulation of CIC, we performed electrophoretic mobility shift assay (EMSA) using as a probe the Sp1 sequence present in the minimal CIC regulatory region. We observed the formation of a protein complex in nuclear extracts from fibroblast cells (Fig. 4A, lane 1), which was abrogated by a 100-fold molar excess of unlabeled probe, demonstrating the specificity of the DNA binding complex (Fig. 4A, lane 2). This inhibition was no longer observed using a mutated oligodeoxyribonucleotide as competitor (Fig. 4A, lane 3). In cells treated with BRL, we observed an increase in the specific band compared with control samples (Fig. 4A, lane 4). Of note, in the presence of anti-PPARγ and anti-Sp1 antibodies, the specific band was immunodepleted (Fig. 4A, lane 5) and supershifted (Fig. 4A, lane 6), respectively, suggesting the presence of both proteins in the complex. Non-specific IgG used as a control did not generate either an immunodepleted or a supershifted band (Fig. 4A, lane 7). The functional interaction of PPARγ and Sp1 with the CIC promoter region was further elucidated by ChIP and Re-ChIP assays (Fig. 4B). Using anti-PPARγ, anti-Sp1, or anti-RNA polymerase II (POLII) antibodies,

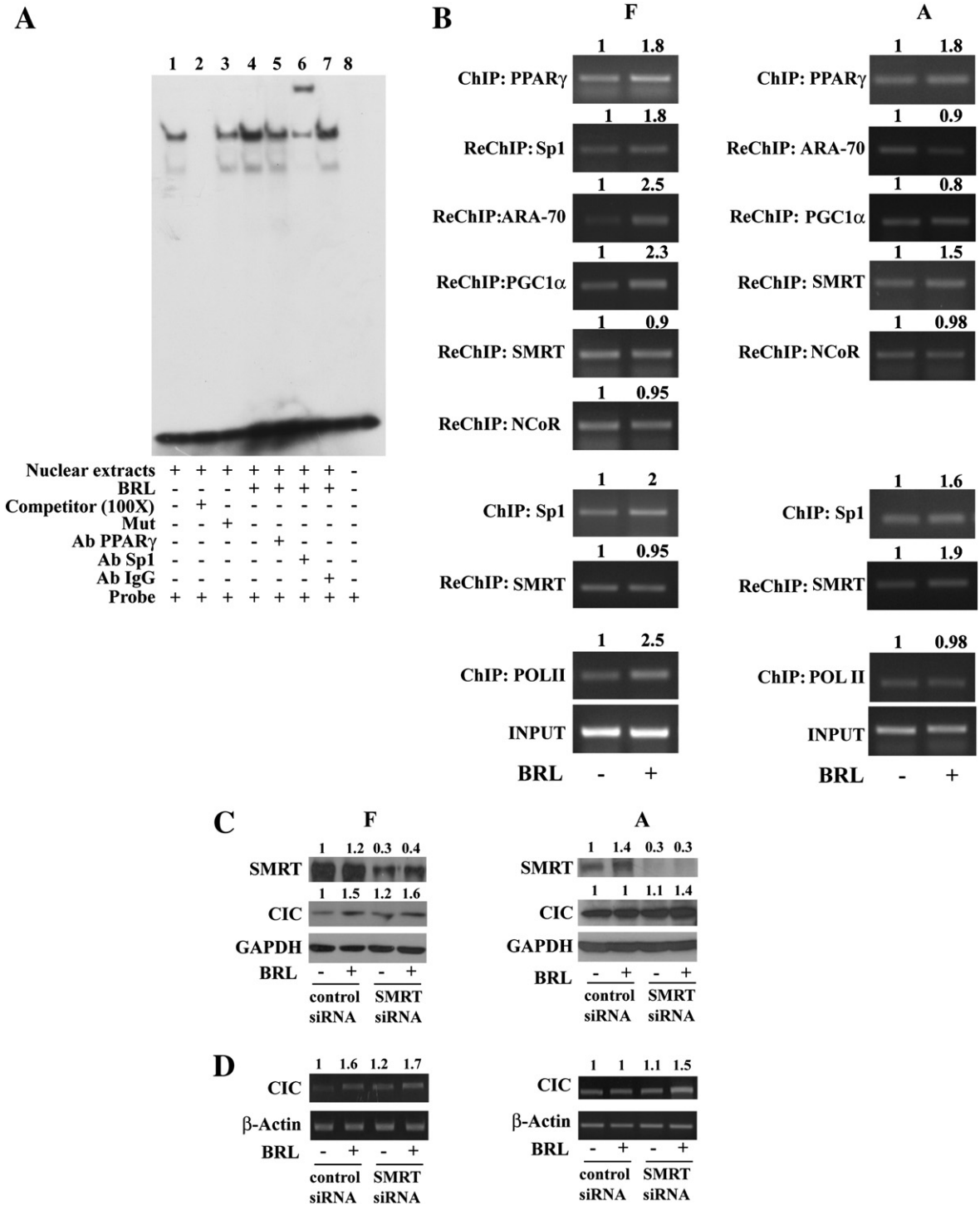

Fig. 4. PPARγ/Sp1 complex binds to the Sp1 site in the CIC promoter in 3T3-L1 cells. A: Nuclear extracts from fibroblasts (lane 1) were incubated with a double-stranded Sp1 sequence probe labeled with [^{32}P] and subjected to electrophoresis in a 6% polyacrylamide gel. Competition experiments were performed adding as competitor a 100-fold molar excess of unlabeled (lane 2) or mutated (Mut) Sp1 probe (lane 3). In lane 4, nuclear extracts from cells treated with 10 μM BRL for 6 h. Anti-PPARγ (lane 5), anti-Sp1 (lane 6) or IgG (lane 7) antibodies were incubated with nuclear extracts treated with BRL. Lane 8 contains probe alone. B: 3T3L1 fibroblasts (F) and adipocytes (A) were untreated (−) or treated with 10 μM BRL for 1 h. The soluble chromatin was immunoprecipitated with the anti-PPARγ, anti-Sp1, anti-RNA Pol II antibodies. Chromatin immunoprecipitated with the anti-PPARγ antibody was re-immunoprecipitated with the anti-Sp1, anti-ARA-70, anti-PCG1α, anti-SMRT and anti-NCoR antibodies (ReChiP). Chromatin immunoprecipitated with the anti-Sp1 antibody was re-immunoprecipitated with the anti-SMRT antibody (ReChiP). The CIC promoter sequence containing the Sp1 site was detected by PCR with specific primers (see Materials and methods). For control input DNA, the CIC promoter was amplified from 30 μl initial preparations of soluble chromatin (before immunoprecipitations). C: Immunoblots for SMRT and CIC experiments in fibroblasts (F) and adipocytes (A) transfected and untreated or treated with 10 μM BRL for 24 h as indicated. GAPDH was used as loading control. Numbers represent the average fold change of SMRT/GAPDH and CIC/GAPDH levels. D: CIC mRNA expression in fibroblasts (F) and adipocytes (A) transfected and untreated or treated with 10 μM BRL for 24 h as indicated. β-Actin was used as loading control. Numbers represent the average fold change of CIC/β-Actin levels.

protein-chromatin complexes were immunoprecipitated from fibroblasts treated for 1 h with vehicle or BRL. The PPARγ immunoprecipitated chromatin was re-immunoprecipitated with anti-Sp1 antibody. PCR was used to determine the occupancy of PPARγ, Sp1 and POLII to the CIC promoter region containing the Sp1 site. We showed that both PPARγ and Sp1 transcription factors were constitutively bound to

the CIC promoter in untreated cells and that this recruitment was increased upon BRL exposure (Fig. 4B, left panel). Similar results were also obtained by PPARγ/Sp1 Re-ChIP assay (Fig. 4B, left panel). In addition, the positive regulation of the CIC transcriptional activity induced by BRL was demonstrated by an increased recruitment of RNA POLII (Fig. 4B, left panel). Although protein–chromatin complexes from adipocytes treated with BRL showed an enhanced recruitment of PPARγ and Sp1 to the CIC regulatory region, no changes in the association of RNA POLII to the Sp1 site were detected (Fig. 4B, right panel).

To assess whether the divergent effects exerted by BRL on CIC expression during adipocyte differentiation might be caused by the cooperative interaction between PPARγ and positive (PCG1α and ARA-70) or negative (SMRT and NCoR) transcriptional regulators, we performed Re-ChIP assays in both cell lines. We found, after BRL exposure, an enhanced recruitment of PCG1α and ARA-70 coactivators in the Sp1-containing region of the CIC promoter in fibroblast cells (Fig. 4B, left panel), while an increased SMRT occupancy was observed in adipocyte cells (Fig. 4B, right panel). Finally, to better define the role of SMRT in the PPARγ-dependent modulation of the CIC mRNA and protein levels, RNA silencing technologies were used to knockdown the expression of endogenous SMRT in both fibroblast and adipocyte cells. SMRT expression was effectively silenced as revealed by immunoblot analysis after 24 h of siRNA transfection in both cell lines (Fig. 4C). As expected, silencing of the SMRT gene had no effects on the up-regulation of CIC protein content and mRNA levels (Fig. 4C and D, left panels) induced by the specific PPARγ ligand in fibroblast cells. In contrast, BRL was able to increase CIC expression in SMRT silenced adipocyte cells (Fig. 4 C and D, right panels) highlighting a crucial role of SMRT corepressor in regulating CIC activity under adipocyte differentiation.

4. Discussion

In this study, we have demonstrated that activated PPARγ modulates the expression and the activity of the mitochondrial CIC during the differentiation stages of fibroblasts into adipocytes.

PPARγ, a ligand-activated transcription factor, plays a key role in adipocyte biology by regulating their differentiation, maintenance, and lipid metabolism [46–48]. Actually, PPARγ is considered the master regulator of adipogenesis participating in the transcriptional activation of several adipogenic and lipogenic genes [49,50]. It is known that cellular fat synthesis is regulated at various steps [51,52]. Particularly, the regulation of fatty acid synthesis via CIC or dicarboxylate carriers is essential for adipocyte differentiation from the early differentiation stage of 3T3-L1 fibroblasts into mature fat cells [53].

Using the cultured 3T3-L1 cell system, we have shown that BRL up-regulated CIC expression and activity in fibroblasts through PPARγ activation, while BRL was not able to modulate CIC levels in mature adipocytes. These data contradict previous findings indicating that PPARγ ligands increased CIC expression in adipocytes [22], although the latter measurements were performed at 7 days after differentiation induction.

From our study, the specific involvement of PPARγ in up-regulating CIC expression in fibroblasts was proved by the observation that the PPARγ effect was completely abrogated in the presence of GW, a potent and selective antagonist of PPARγ. The molecular events responsible for CIC induction by the PPARγ ligand BRL were consistent with the enhanced transcriptional activation of this gene as it raised by the capability of BRL to activate CIC promoter. Multiple transcription factor binding sites within the rat and human CIC promoter have been described, including FOXA, SRE, GR, C/EBP, ER and Sp1 binding sequences. Functional studies using different CIC-promoter-deleted constructs identified the region of CIC promoter, spanning from −115/−82, as the minimal region responsible for BRL induction. Of note, this region of rat CIC promoter shows a very high degree of sequence similarity with the corresponding portion of the mouse CIC gene**s** (approximately 94% identity). Although it has been recently demonstrated that CIC expression is regulated by PPARγ ligands through a PPRE site, identified at −625 bp of the CIC promoter [22], our results showed that CIC transactivation occurs independently of the PPRE site, suggesting that other transcription factors are involved in PPARγ-mediated CIC induction. Indeed, analysis of the minimal CIC promoter region reveals the presence of a GC-box sequence, and deletion as well as mutation of this site results in the abrogation of PPARγ transactivating activity. The ability of activated PPARγ to transactivate CIC promoter through Sp1 was confirmed in functional assays using a construct carrying threefold repeat of the cognate Sp1 motif within the minimal region of CIC gene promoter. It could be clearly seen that luciferase expression was significantly increased upon BRL treatment.

Furthermore, when Sp1-DNA binding activity was blocked by a selective inhibitor, both PPARγ-mediated transactivation and induction of CIC expression were subsequently abolished. In line with our results, an interesting observation is that in the presence of the Sp1 mutation at −92 bp the basal activity of CIC promoter is reduced when compared with the transcriptional activity of the wild-type CIC promoter [20].

Sp1 has been considered traditionally as a ubiquitous factor associated closely with core promoter activities; it has recently been observed that it participates in the regulation of gene transcription triggered by multiple signaling pathways and metabolic or differentiation conditions. Moreover, Sp1 interacts physically and cooperates functionally with several sequence-specific activators including NF-kB, GATA, YY1, E2F1, Rb, SREBP-1 and PPARγ [54–59] to modulate gene expression. In addition, it has been shown that the activation of CIC gene expression by Sp1 is virtually abolished by methylation of the Sp1-binding elements which are present in the promoters of all CIC genes sequenced in mammals within the CpG island located immediately upstream the translocation start codon [19]. For the first time, our results have revealed a novel important regulatory role of Sp1 in regulating CIC promoter activity. In nuclear extracts from fibroblast cells treated with BRL, EMSA showed a strong increase in the Sp1-DNA binding that was immunodepleted by anti-Sp1 and anti-PPARγ antibodies, suggesting the presence of both proteins in the complex. In addition, ChIP and Re-ChIP assays demonstrated that PPARγ/Sp1 occupancy of the Sp1-containing promoter region induced by BRL treatment was concomitant with an increase in RNA-Pol II, addressing a positive CIC transcriptional regulation mediated by PPARγ.

It is known that members of the nuclear hormone receptor superfamily, including PPARγ, once activated, can interact physically and modulate target gene transcription. PPARγ can regulate transcription by several distinct mechanisms, and its function seems to depend not only on ligand binding, which is known to regulate receptor conformation, but also on the context of the gene and associated promoter factors that contribute to create a gene-specific topography, achieving specific profiles of gene expression. Several studies have examined the role of coregulators in adipogenesis and demonstrated that coactivators such as PGC-1α or steroid receptor coactivators (SRCs) are essential [60]; whereas NCoR, SMRT and histone deacetylases act as negative regulators of differentiation [61–63]. The physiological relevance of their implication in metabolic regulation has been demonstrated in the context of PPARγ-mediated adipogenesis, during which they promote a target-gene specific repression of PPARγ activity [64]. A negative action of SMRT and NCoR on fat storage has been suggested by the enhanced adipogenesis and increased expression of proadipogenic PPARγ target genes after RNAi-mediated inhibition of these corepressors [62].

Our results evidenced in fibroblast cells, after BRL stimulation, an enhanced recruitment of PGC-1α and ARA-70 on the Sp1 site of the CIC promoter. In contrast, we observed that mature adipocytes treated with BRL showed an increased recruitment of SMRT corepressor to the Sp1 site within the CIC promoter along with no changes in the occupancy of RNA POLII. Finally, we demonstrated a direct involvement of SMRT in the loss of CIC promoter responsiveness to the BRL in mature adipocytes using a specific SMRT siRNA.

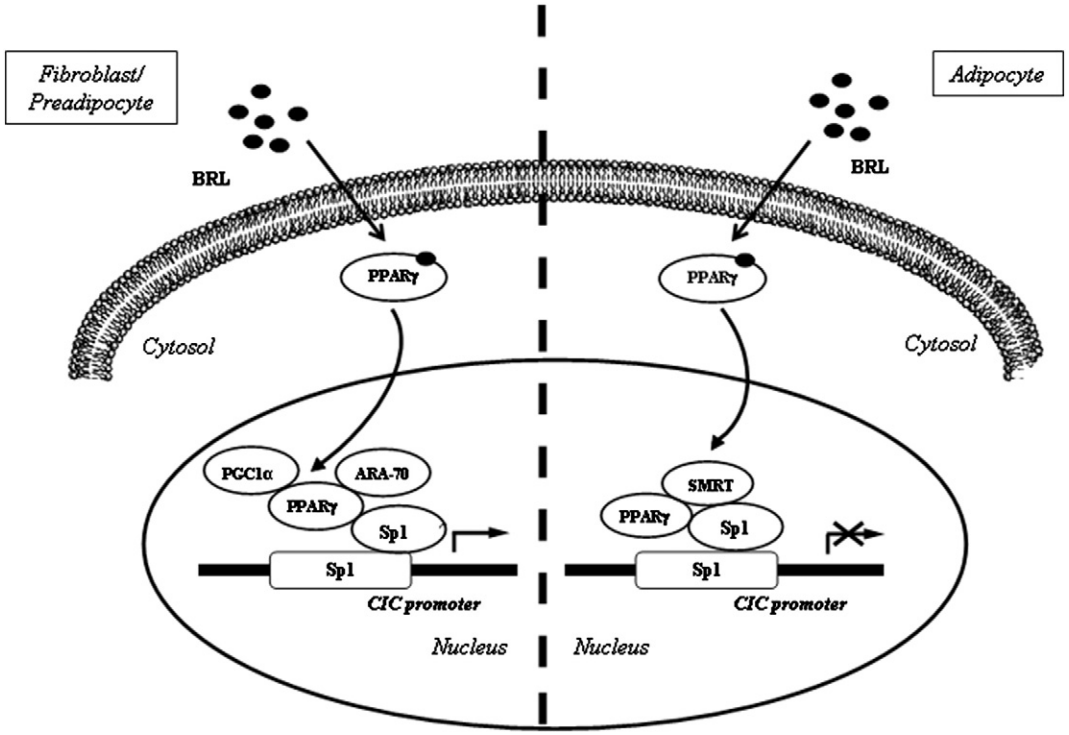

Fig. 5. Proposed working model of the PPARγ-mediated regulation of CIC expression in fibroblasts and adipocytes. In fibroblasts, upon BRL treatment, PPARγ/Sp1 complex is recruited on the Sp1-containing region of CIC promoter along with PGC1-α and ARA70 coactivators, leading to an increase in CIC expression. In adipocytes, the formation of PPARγ/Sp1 complex is associated with the recruitment of SMRT corepressor, resulting in an inhibition of CIC transcription.

In conclusion, our study identifies a novel molecular mechanism through which PPARγ modulates CIC expression, a crucial mitochondrial carrier for glucose and lipid metabolism and for energy homeostasis regulation. The divergent mechanisms through which PPARγ activation may switch the modulation of CIC expression during adipocyte differentiation are schematically shown in Fig. 5. We propose a model in which: i) in fibroblasts treated with BRL, PPARγ/Sp1 complex along with PGC1α and ARA-70 coactivators are recruited on the Sp1-containing region of the CIC promoter, thereby increasing CIC expression; ii) in adipocytes treated with BRL, PPARγ/Sp1 complex is associated with an enhanced recruitment of SMRT corepressor on the Sp1 site of CIC promoter resulting in an inhibition of CIC transcription.

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