A Role for C/EBPβ in Regulating Peroxisome Proliferator-activated Receptor γ Activity during Adipogenesis in 3T3-L1 Preadipocytes*

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The differentiation of 3T3-L1 preadipocytes is regulated in part by a cascade of transcriptional events involving activation of the CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ (PPARγ) by dexamethasone (DEX), 3-isobutyl-1-methylxanthine (MIX), and insulin. In this study, we demonstrate that exposure of 3T3-L1 preadipocytes to DEX and insulin fails to induce adipogenesis as indicated by a lack of C/EBPα, PPARγ2, and adipose protein 2/fatty acid-binding protein expression; however, PPARγ1 is expressed. Treatment of these MIX-deficient cells with a PPARγ ligand, troglitazone, induces C/EBPα expression and rescues the block in adipogenesis. In this regard, we also show that induction of C/EBPα gene expression by troglitazone in C3H10T1/2 cells ectopically expressing PPARγ occurs in the absence of ongoing protein synthesis, suggesting a direct transactivation of the C/EBPα gene by PPARγ. Furthermore, ectopic expression of a dominant negative isoform of C/EBPβ (liver-enriched transcriptional inhibitory protein (LIP)) inhibits the induction of C/EBPα, PPARγ2, and adipose protein 2/fatty acid-binding protein by DEX, MIX, and insulin in 3T3-L1 cells without affecting the induction of PPARγ1 by DEX. Exposure of LIP-expressing preadipocytes to troglitazone along with DEX, MIX, and insulin induces differentiation into adipocytes. Additionally, we show that sustained expression of C/EBPα in these LIP-expressing adipocytes requires constant exposure to troglitazone. Taken together, these observations suggest that inhibition of C/EBPβ activity not only blocks C/EBPα and PPARγ2 expression, but it also renders the preadipocytes dependent on an exogenous PPARγ ligand for their differentiation into adipocytes. We propose, therefore, an additional role for C/EBPβ in regulating PPARγ activity during adipogenesis, and we suggest an alternative means of inducing preadipocyte differentiation that relies on the dexamethasone-associated induction of PPARγ1 expression.

The differentiation of adipocytes into mature fat cells is regulated by a cascade of transcription factors that interact in a complex fashion to control expression of several hundred adipogenic genes (1, 2). Many different nuclear factors have been shown to influence the adipogenic process, but two families of factors in particular have received the most attention as follows: the CCAAT enhancer-binding proteins (C/EBPs)1 and the peroxisome proliferator-activated receptor (PPAR) family of nuclear hormone receptors. Three members of the C/EBP family, α, β, and δ, have been shown to play important roles in regulating adipose tissue development in mice and preadipocyte differentiation in vitro (3). In contrast, only the γ form of the PPAR family is considered to regulate adipogenesis in vitro and in vivo (4). The temporal pattern of expression of these important adipogenic factors, and control of their activity during adipogenesis, is dependent on a variety of biological effectors and other transcription factors (5–7).

The C/EBPs belong to a larger family of basic leucine zipper (bZIP) transcription factors, which have a C-terminal leucine zipper domain for dimerization and a basic domain for binding to DNA. There are at least six members of this family, α, β, δ, γ, ε, and ζ, that can both homodimerize and heterodimerize with each other and bind to the same C/EBP regulatory element in the promoters/enhancers of many different genes. In addition, each family member can give rise to several isoforms by a process of selective use of translational start sites within each mRNA or by proteolysis of a larger precursor protein (8). The C/EBPβ mRNA, for instance, gives rise to at least four isoforms corresponding to the following peptides, 38, 34, 30, and 20 kDa. The 34-kDa protein is often referred to as LAP (liver-enriched transcriptional activator protein) since it has been shown to be a potent transactivator of liver gene expression (9). The 20-kDa polypeptide, however, can inhibit hepatic gene expression and is, therefore, referred to as LIP (liver-enriched transcriptional inhibitory protein) (9). This LIP isoform of C/EBPβ corresponds to the C-terminal portion of the LAP protein that lacks the transactivation domain but contains the basic leucine zipper region. Consequently, LIP can act as a potent dominant negative repressor of C/EBPβ activity. In fact, ectopic expression of LIP, resulting in a LAP/LIP ratio of −1, blocks adipogenesis in preadipocytes in culture (10).

PPARγ exists as two protein isoforms, γ1 and γ2, that are generated by alternative splicing of at least three different mRNAs, which are transcribed from the same gene (11, 12). PPARγ1 and γ2 share almost all the same exon sequences, except γ2 contains an additional 30 amino acids at the N terminus. Both isoforms of PPARγ form an obligatory heterodimer with the retinoid X receptor to bind to regulatory elements within the promoters/enhancers of many genes associated with lipid metabolism. Activation of PPARγ-RXR complexes requires association with a series of ligands that include...
RXR ligands such as 9-cis-retinoic acid as well as ligands for PPARγ (13). The latter includes polyunsaturated fatty acids and their derivatives as well as the thiazolidinedione family of insulin sensitizers such as troglitazone (14–16). Transcription from the PPARγ gene has been detected in many tissues in which the γ1 isoform is the predominant transcript (17). In contrast, transcription from the PPARγ2 promoter is highly adipose tissue-selective giving rise to abundant production of the PPARγ2 polypeptide in addition to the more ubiquitous PPARγ1 isoform (18). Ectopic expression of PPARγ2 or γ1 in non-adipogenic fibroblasts under appropriate hormonal conditions results in potent induction of adipocyte differentiation (19).

Various mouse cell lines have been used to delineate the many different processes involved in regulating adipogenesis. Most notable are 3T3-L1 preadipocytes, which can be induced to differentiate into mature fat cells following exposure to a mixture of hormonal inducers including dexamethasone (DEX), isobutylmethylxanthine (MIX), insulin, and FBS. MIX and DEX induce expression of C/EBPβ and C/EBPδ, respectively, which in turn activate C/EBPα and PPARγ expression (10, 20, 21). PPARγ and C/EBPα are then capable of cross-activating each other as well as governing expression of the mature adipocytic phenotype (22, 23). The normal differentiation of preadipocytes in culture does not require addition of an exogenous PPARγ ligand. In contrast, non-adipogenic fibroblasts that ectopically express a C/EBP or PPARγ require exposure to a potent PPARγ ligand to undergo conversion into adipocytes (24, 25). Preadipocytes have likely acquired the ability to produce an appropriate ligand of PPARγ. The molecular mechanisms that regulate production of such molecules are not known. Earlier studies by others (26) have suggested a role for the sterol regulatory element-binding proteins (SREBPs).

We demonstrate that attenuation of C/EBPβ activity by omitting MIX from the culture medium or ectopically expressing a dominant negative form of C/EBPβ (LIP) renders 3T3-L1 preadipocytes dependent on an exogenous PPARγ ligand for their differentiation into adipocytes. These studies have also uncovered an alternative pathway of adipogenesis, which involves a glucocorticoid-associated induction of PPARγ1 in the absence of C/EBPβ activity. Furthermore, activation of PPARγ1 in the LIP-expressing cells with troglitazone directly activates C/EBPγ gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone, 3-isobutyl-1-methylxanthine, insulin, purumycin, aprotinin, leupeptin, and digitonin were purchased from Sigma. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were supplied by Life Technologies, Inc. [α-32P]dCTP and [γ-32P]ATP were purchased from PerkinElmer Life Sciences. Klow fragment of DNA polymerase I was obtained from Promega (Madison, WI). Troglitazone was from Parke-Davis/Warner Lambert. The fragment of DNA polymerase I was obtained from Promega (Madison, WI). Troglitazone was from Parke-Davis/Warner Lambert.

**Plasmids and Stable Cell Lines**—The BOSC 23 packaging cells (27), pBabe-Puro and pBabe-PPARγ-Puro retroviral expression vectors (19), were kind gifts of Dr. Bruce Spiegelman (Dana Farber Cancer Institute, Harvard Medical School). The pBabe vector expressing either the LAP or LIP isoforms of C/EBPβ were constructed by subcloning corresponding PCR products of the C/EBPβ cDNA (20) into the BamHI and EcoRI sites of the pBabe-puro vector. The LAP PCR fragments were generated using the following primers: cβ-1 (5′-CCCGGATCCGCCCGGT-3′) and cβ-3 (5′-GGTCGACCTAGCTGAGCCGGGT-3′) and the LIP PCR fragments were generated using cβ-2 (5′-CCGGGATCCGCCCGGT-3′) and cβ-3 primers. Transfection of BOSC 23 packaging cells and subsequent infection of target cells were performed as described by others (19, 27). Infected target cells were selected for 6–10 days in medium containing 2.0 μg/ml puromycin.

**Cell Culture**—Murine 3T3-L1 preadipocytes were cultured, maintained, and differentiated as described previously (28, 29). Briefly, cells were plated and grown for 2 days post-confluence in DMEM supplemented with 10% calf serum. Differentiation was then induced (Day 0) by changing the medium to DMEM containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, and 1.67 μM insulin for 48 h, cells were transferred in DMEM containing 10% FBS. 3T3-L1 cells expressing either C/EBPβ LIP or control vector and 10T1/2 cells expressing PPARγ were differentiated by the same protocol for 3T3-L1 cells, except growth medium was DMEM containing 10% FBS and 2.0 μg of puromycin, and the cells were differentiated and maintained in the presence or absence of 10 μM troglitazone, except as noted. Oil Red O Staining—Oil Red O staining was performed following the procedure described previously (29). The cells were then photographed using phase contrast microscopy.

**Preparation of Whole Cell Extracts**—At the indicated times, cultured cells grown in 10-cm dishes were rinsed with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 8.1 mM Na2HPO4, pH 7.4) and then harvested in 1 ml of ice-cold buffer containing 50 mM Tris (pH 7.4), 100 mM NaCl, 1% sodium deoxycholate, 4% Nonidet P-40, 0.4% SDS, 5 μM aprotinin, and 50 μM leupeptin. Lysates were vortexed for 1 min and centrifuged for 15 min at full speed (13,000 rpm) in a microcentrifuge. Pellets were discarded and supernatants stored at −80 °C. Protein content of supernatants was determined using the BCA kit (Amersham Pharmacia Biotech).

**Electrophoretic Mobility Shift Assay**—Proteins were separated in SDS-polyacrylamide (acylamide from American BioAnalytical) gels as described previously (23) and transferred to polyvinylidene difluoride membrane (Bio-Rad) in 25 mM Tris, 192 mM glycine. After transfer, the membrane was blocked with 4% nonfat dry milk in PBST for 1 h at room temperature. After incubation with the primary antibodies specified above, horseradish peroxidase-conjugated secondary antibodies (Sigma) and an enhanced chemiluminescence (ECL) substrate kit (PerkinElmer Life Sciences) were used for detection.

**RNA Analysis**—Total RNA was harvested according to the procedure of Chomczynski and Sacchi (30). Cells were lysed in buffer containing 4 M guanidinium isothiocyanate. Lysates were extracted with acid phenol/chloroform, and RNA was precipitated in 50% isopropanol alcohol overnight at −20 °C. Northern blot analysis was performed using each sample RNA as described. cDNA probes for C/EBPα and PPARγ were labeled using Klown fragment of DNA polymerase I and [α-32P]dCTP by random priming.

**Preparation of Nuclear Protein Extracts**—Nuclear protein extracts were prepared essentially as described. Cells were washed twice with ice-cold phosphate-buffered saline and then lysed in nuclease buffer (50 mM Tris [pH 7.6], 10 mM EDTA, 0.5 mM MgCl2, 0.5% Nonidet P-40). Samples were spun at low speed in a clinical centrifuge. Supernatants were discarded, and nuclei were lysed in nuclear extraction buffer (20 mM HEPES (pH 7.9), 350 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA (pH 8.0), 25% glycerol). Nuclear extracts were incubated on ice for 15 min and centrifuged at full speed (13,000 rpm) at 4 °C. The resulting supernatants were stored at −80 °C. Protein concentrations were determined using the BCA protein assay kit (Amersham Pharmacia Biotech).

**Gel Electrophoresis and Immunoblotting**—Proteins were separated in acrylamide/bisacrylamide gels at 200 V for 1 h. Labeled probes on ice for 30 min and resolved on nondenaturing 6% polyacrylamide (39.5:0.5 acrylamide/bisacrylamide) gels at 80 °C.

**Northern Blot Analysis**—Northern blot analysis was performed on 20 μg total RNA. RNA was denatured and electrophoresed through 8% polyacrylamide gels at 200 V for 2–2.5 h at 4 °C in TBE buffer (80 mM Tris borate, 2 mM EDTA (pH 8.0)). Northern blots were incubated at room temperature for 1.5 h. Reactions were mixed with labeled probes on ice for 30 min and resolved on nondenaturing 6% polyacrylamide (39:5:0.5 acrylamide/bisacrylamide) gels at 200 V for 2–2.5 h at 4 °C in TBE buffer (80 mM Tris borate, 2 mM EDTA (pH 8.0)). Gels were vacuum-dried for 1 h before exposure to Biomax MR-1 autoradiography film (Eastman Kodak Co.). Antibodies used for C/EBP Western blots were the same as described above. Goat anti-rabbit IgG antibodies (Sigma) were used in negative control experiments.

**RESULTS**

To understand the roles of various inducers and the C/EBPs in regulating adipogenesis, we generated 3T3-L1 cell lines expressing vector DNA alone (designated L1-V cells) or the dominant negative C/EBPβ, LIP (designated L1-LIP cells). To determine the effect of the different inducers on expression of
PPARγ and C/EBPα, we stimulated L1-V cells to differentiate by exposure to different combinations of insulin, dexamethasone (DEX), and isobutylmethylxanthine (MIX) in the presence or absence of 10 μM troglitazone. Cells were maintained according to the procedure described under “Experimental Procedures,” and total protein was harvested 4 days after induction. Fig. 1, lane 7, shows the abundant expression of PPARγ, C/EBPα, and aP2 expression. I, 1.67 μM insulin; D, 1 μM dexamethasone; M, 0.5 mM isobutylmethylxanthine; CRM, cross-reaction material.

PPARγ and C/EBPα expression. 3T3-L1 cells transfected with vector DNA alone were induced to differentiate in media containing insulin and FBS in the presence or absence of dexamethasone (D), isobutylmethylxanthine (M), or 10 μM troglitazone (Trog). Four days later, whole cell extracts were prepared, and 100 μg of each sample was subjected to Western blot analysis for PPARγ, C/EBPα, and aP2 expression. I, 1.67 μM insulin; D, 1 μM dexamethasone; M, 0.5 mM isobutylmethylxanthine; CRM, cross-reaction material.

Fig. 1. Effect of different effectors on PPARγ and C/EBPα expression. 3T3-L1 cells transfected with vector DNA alone were induced to differentiate in media containing insulin and FBS in the presence or absence of dexamethasone (D), isobutylmethylxanthine (M), or 10 μM troglitazone (Trog). Four days later, whole cell extracts were prepared, and 100 μg of each sample was subjected to Western blot analysis for PPARγ, C/EBPα, and aP2 expression. Lane 1, 1.67 μM insulin; Lane 2, 1 μM dexamethasone; Lane 3, 0.5 mM isobutylmethylxanthine; Lane 4, cross-reaction material.

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Fig. 2. Time course of protein expression during adipogenesis with and without MIX and troglitazone. At the indicated times following induction of differentiation of L1-vector cells in the presence or absence of isobutylmethylxanthine (ID versus DIM) and troglitazone (Trog), total protein extracts were collected and analyzed by Western blot analysis for expression of PPARγ, C/EBPα, and C/EBPβ.

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C/EBPβ Regulation of PPARγ Activity

Fig. 3. PPARγ induces C/EBPα mRNA expression in the absence of ongoing protein synthesis. 10T1/2 cells ectopically expressing PPARγ2 were exposed to DMEM containing 1 μM DEX, 0.5 mM MIX, 1.67 μM insulin, and 10% FBS for 48 h. Cells were maintained in 10% FBS for an additional 24 h and then treated with or without cycloheximide (CHX, 5 μg/ml), in the presence or absence of 5 μM troglitazone (Trog). Total RNA was harvested at the indicated times post-treatment and analyzed by Northern blot for C/EBPα and PPARγ mRNA expression.

A

B

L1-V

L1-LIP

V LAP LIP

CRM

34 kD C/EBPβ

20 kD C/EBPβ

-Trog +Trog

Fig. 4. Ectopic expression of a dominant negative isoform of C/EBPβ (LIP) inhibits adipogenesis in 3T3-L1 preadipocytes. A, total proteins harvested from proliferating preadipocytes, which ectopically express either vector (V), LAP, or LIP were subjected to Western blot analysis using an anti-C/EBPβ antibody. CRM, cross-reacting material. B, confluent L1-vector and L1-LIP cells were induced to differentiate in the presence or absence of troglitazone (Trog). At day 7, cells were fixed and stained for neutral lipids with Oil Red O.

4 with Fig. 1, lanes 3 and 4). In contrast, exposure of LIP cells to MIX and insulin only slightly enhances PPARγ1 with no PPARγ2 expression, and when troglitazone is added under these conditions it does not induce adipogenesis (i.e. minimal aP2 and C/EBPα expression). This pattern of gene expression differs significantly from that observed in the absence of LIP. Specifically, MIX induces both PPARγ1 and -2 in the L1-vector cells, and consequently, exposure of these cells to troglitazone promotes adipogenesis (compare Fig. 1, lanes 5 and 6, with Fig. 4, lanes 5 and 6). Taken together, these data are consistent with a model in which DEX is capable of priming the preadipocytes to be responsive to troglitazone even in the absence of C/EBPβ; this likely involves induction of PPARγ1 expression. MIX, however, is only capable of a similar priming process if C/EBPβ is actively expressed in the absence of LIP. These data also strongly suggest that inhibiting C/EBPβ activity blocks production of an endogenous activator of PPARγ, which renders the 3T3-L1 preadipocytes dependent on an exogenous PPARγ ligand for their differentiation into adipocytes.

To gain more insight into the ligand dependence of these LIP-expressing preadipocytes, we analyzed the temporal pattern of gene expression following exposure to troglitazone as well as determining the optimum dose of troglitazone required to induce adipogenesis. In the experiment shown in Fig. 6, confluent L1-LIP cells were exposed to DEX, MIX, and insulin in the presence or absence of troglitazone, and total cellular proteins were subjected to Western blot analysis. The combination of DEX, MIX, and insulin is capable of initiating the early phase of adipogenesis in these LIP-expressing cells as indicated by induction of C/EBPβ as well as PPARγ1 (compare lane 2 and 4 with lane 1). Exposure of these cells to troglitazone appears to have no significant effect on this pattern of gene expression during the first 2 days. After this time, however, troglitazone is essential for the induction of C/EBPα and aP2 expression. Taken together, the studies shown above demonstrate that culturing LIP cells in troglitazone for 6 days, along with an initial priming with DEX, MIX, and insulin, results in their conversion into adipocytes based on accumulation of lipid droplets in >95% of the cells (Fig. 4B) and the abundant expression of PPARγ2, C/EBPα, and aP2 (Fig. 6). To establish the troglitazone dose dependence of LIP cells, both L1-LIP and L1-V cells were exposed to differentiation medium containing DEX, MIX, insulin, and increasing concentrations of troglitazone. Total protein samples were harvested 6 days later and subjected to Western blot analysis of the indicated proteins. Fig. 7 demonstrates that expression of both C/EBPα and PPARγ2 increased substantially with increasing doses of troglitazone. Expression of aP2 also seemed proportionate to tro-
glitazone concentration, correlative to the number of cells accumulating lipid droplets (data not shown). LIP expression was unaffected by the PPAR\(\gamma\) ligand.

The LIP polypeptide retains the C-terminal basic leucine zipper region of the full-length C/EBP\(\beta\) protein, and therefore, it can dimerize with other C/EBP\(\beta\) isoforms and bind to DNA. It was important, therefore, to determine what effect LIP and/or troglitazone may have on the DNA binding activity of the different C/EBPs during adipogenesis. Consequently, L1-V and L1-LIP cells were treated to differentiate in the presence or absence of troglitazone for 6 days after induction, whole cell proteins were harvested and subjected to Western blot analysis for expression of the indicated proteins. L1-V controls (0 and 10 \(\mu\)M troglitazone) are included.

FIG. 6. Time course of adipogenic gene expression following exposure of L1-LIP cells to troglitazone. L1-LIP cells were induced to differentiate with DEX, MIX, insulin, and FBS in the presence or absence of 10 \(\mu\)M troglitazone (Trog). Total protein extracts were harvested on the indicated days after induction. Western blot analysis was performed as described using antibodies for the indicated proteins.

FIG. 7. Troglitazone dose-dependent rescue of adipogenic gene expression in L1-LIP cells. L1-LIP cells and L1-V cells (L1) were induced to differentiate as in Fig. 6 in the presence of varying concentrations of troglitazone (Trog). Six days after induction, whole cell proteins were harvested and subjected to Western blot analysis for expression of the indicated proteins. L1-V controls (0 and 10 \(\mu\)M troglitazone) are included.

FIG. 8. Changes in C/EBP DNA binding activity during adipogenesis in L1-V and L1-LIP cells. L1-V and L1-LIP cells were treated to differentiate in the presence or absence of 10 \(\mu\)M troglitazone (Trog). A, at 1 and 6 days after induction (d1 and d6, respectively), nuclear proteins were harvested and subjected to electrophoretic mobility shift assay, as described under “Experimental Procedures.” B, supershift analysis of C/EBP DNA binding activity in L1-V and L1-LIP cells induced in the presence or absence of troglitazone. Day 6 nuclear protein samples from L1-V and L1-LIP cells induced to differentiate in the standard inducers, with or without troglitazone, were analyzed by supershift analysis using antibodies to C/EBP\(\beta\) (C\(\beta\)), C/EBP\(\delta\) (C\(\delta\)), C/EBP\(\alpha\) (C\(\alpha\)), or IgG (–).

C/EBP\(\beta\) Regulation of PPAR\(\gamma\) Activity

Exposure to troglitazone for 6 days. To examine the composition of these DNA-protein complexes, a series of supershift assays were performed using antibodies corresponding to C/EBP\(\alpha\), C/EBP\(\beta\), and C/EBP\(\delta\). Fig. 8B, lane 4, demonstrates that a proportion of the complexes expressed at day 6 in L1-V cells consists of C/EBP\(\alpha\) homodimers. As expected, there is a significant increase in C/EBP\(\alpha\) binding activity in LIP cells following exposure to troglitazone for 6 days (Fig. 8B, compare lane 12 with lane 8). In fact, the C/EBP\(\alpha\) binding activity is slightly higher in the LIP cells plus troglitazone compared with that expressed in the vector cells. Furthermore, ectopic expression of LIP has not affected the ability of C/EBP\(\alpha\) to bind to the C/EBP regulatory element. This figure also shows the existence of LIP-LIP homodimers binding to the C/EBP oligonucleotide since the faster migrating complexes present in the LIP cells can be supershifted selectively with an anti-C/EBP\(\delta\) antibody (lanes 6 and 10). C/EBP\(\delta\) is minimally expressed at 6 days under all conditions since there is no detectable supershift with an anti-C/EBP\(\delta\) antibody.
Our observation that LIP cells require an exogenous PPARγ ligand such as troglitazone for their complete conversion into adipocytes suggests that they are not capable of producing the endogenous ligand(s) or activators. Alternatively, it is possible that they express PPARγ at levels below a threshold required for activation by the endogenous activator(s). To determine the reason for the ligand dependence of the LIP cells, we induced PPARγ and C/EBPα to fully differentiated levels by exposing LIP cells to DEX, MIX, insulin, and 10 μM troglitazone for 6 days. We then questioned whether these LIP adipocytes still require the exogenous PPARγ ligand to maintain normal adipocyte gene expression. This was achieved by withdrawing troglitazone from half the cultures at day 6 and measuring expression of PPARγ, C/EBPα, and aP2 in these and a control set of cultures that were maintained in troglitazone for the entire experiment. The Western blot in Fig. 9 shows abundant levels of PPARγ, C/EBPα, and aP2 following 7 days of exposure of LIP cells to troglitazone. Withdrawal of the exogenous ligand at day 6, however, results in an extensive dedifferentiation as indicated by a drop in expression of C/EBPα and aP2 to virtually undetectable levels by day 10 (4 days of withdrawal). Interestingly, the abundance of both PPARγ1 and -γ2 remains constant throughout this period even in the absence of troglitazone. Notably, expression of LIP does not increase when troglitazone is withdrawn; on the contrary, it appears to decrease (Fig. 9).

**DISCUSSION**

The differentiation of 3T3-L1 cells into mature adipocytes requires their exposure to a mixture of hormonal inducers including DEX, MIX, insulin, and FBS. These effectors have been shown to activate a cascade of transcriptional events that culminate in expression of the mature adipocytic phenotype. Most notably, they facilitate the induction of C/EBPβ and C/EBPδ, which together activate expression of PPARγ and C/EBPα (10, 20, 21, 29). The data presented in this study suggest an additional role for C/EBPβ, and the effectors that control its expression, in regulating the production of PPARγ and C/EBPδ ligands. These studies further show that adipogenesis can be induced in 3T3-L1 preadipocytes in the absence of C/EBPβ by exposing the cells to an exogenous PPARγ ligand. This alternative mechanism appears to depend on the ability of insulin and DEX to induce PPARγ1 expression, which is then capable of inducing C/EBPα and PPARγ2 expression following exposure to troglitazone. Induction of C/EBPα gene expression in the absence of exogenous PPARγ ligand depends on MIX and/or C/EBPβ expression. It appears, therefore, that expression of C/EBPα during adipogenesis can be regulated by at least two independent pathways. One pathway involves a MIX-associated induction of C/EBPγ, which transactivates a C/EBP regulatory element within the promoter of the C/EBPα gene (32). The other mechanism can occur in the absence of C/EBPβ due to a DEX-associated induction of PPARγ1, which is also capable of transactivating the C/EBPα gene in the presence of troglitazone.

Previous studies have shown that an important role for MIX and DEX is to induce expression of C/EBPβ and C/EBPδ, respectively, which in turn activate C/EBPα and PPARγ2 expression through C/EBP regulatory elements in the promoters of the corresponding genes (10, 31–34). Our data are consistent with these observations since inhibition of C/EBPβ activity by either omitting MIX or expressing LIP blocks the entire adipogenic gene program.

The absence of an exogenous PPARγ ligand depends on MIX and/or C/EBPβ expression. It appears, therefore, that expression of C/EBPα during adipogenesis can be regulated by at least two independent pathways. One pathway involves a MIX-associated induction of C/EBPγ, which transactivates a C/EBP regulatory element within the promoter of the C/EBPα gene (32). The other mechanism can occur in the absence of C/EBPβ due to a DEX-associated induction of PPARγ1, which is also capable of transactivating the C/EBPα gene in the presence of troglitazone.

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**Mechanisms by Which C/EBPβ May Regulate PPARγ Activity—**The most likely determinant of PPARγ activity is the availability of ligands within the preadipocyte. Even though the natural cellular ligand for PPARγ has not been identified, evidence suggests that derivatives of polyunsaturated fatty acids are potent activators of PPARγ both in vitro assays as
well as in vivo (13, 16) Mechanisms that control the cellular production of polyunsaturated fatty acids or their derivatives may play an important role in regulating adipogenesis. In this regard, studies have shown that ADD1/SREBP-1, a transcription factor that is linked to processes controlling fatty acid production, appears to be involved in the production of endogenous PPARγ ligands (26). In fact, ADD1/SREBP-1 is induced early during adipogenesis, and its ectopic expression in non-adipogenic cells can enhance fat cell formation by directly activating the PPARγ2 gene as well as stimulating production of PPARγ ligands (26, 35, 36). It is conceivable, therefore, that a role for C/EBPβ in regulating PPARγ activity may involve induction and/or activation of ADD1/SREBP-1.

PPARγ Ligand-dependent Induction of C/EBPα Expression—Several investigations have demonstrated that activation of PPARγ in a variety of different fibroblast lines results in expression of many adipogenenic genes including C/EBPα (19, 22–25, 37). Similarly, ectopic expression of C/EBPα in non-adipogenic cells can induce PPARγ expression (22, 23). In fact, Spiegelman and co-workers (22) have suggested that cross-regulatory process are not known. It is containing the differentiated state. The molecular mechanisms however, PPARγ and C/EBPα expression, which may involve production of PPARγ ligands.

In summary, we propose an alternative model for the transcriptional control of adipogenesis (Fig. 10) that incorporates the conclusions drawn from these studies with those already presented by others (10, 21, 22). In this model, C/EBPβ and C/EBPδ regulate production of PPARγ ligands as well as PPARγ2 and C/EBPα expression. Additionally, we suggest that physiological effectors can induce expression of PPARγ1 in the absence of C/EBPβ and C/EBPδ as part of a default pathway. This event can then initiate a cascade of transcription factor expression, commencing with C/EBPα, which in turn induces expression of the entire adipogenic program providing the preadipocyte is exposed to PPARγ ligands. Further dissection of the transcriptional events that regulate production of PPARγ ligands should provide a greater understanding of the processes controlling adipogenesis.

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