

# Activation of CCAAT/Enhancer-binding Protein (C/EBP) $\alpha$ Expression by C/EBP $\beta$ during Adipogenesis Requires a Peroxisome Proliferator-activated Receptor- $\gamma$ -associated Repression of HDAC1 at the *C/ebp $\alpha$* Gene Promoter\*

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Studies have shown that CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) can stimulate adipogenesis in noncommitted fibroblasts by activating expression of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ). Other investigations have established a role for C/EBP $\alpha$  as well as PPAR $\gamma$  in orchestrating the complex program of adipogenic gene expression during terminal preadipocyte differentiation. Consequently, it is important to identify factors regulating transcription of the *C/ebp $\alpha$*  gene. In this study, we demonstrated that inhibition of PPAR $\gamma$  activity by exposure of 3T3-L1 preadipocytes to a potent and selective PPAR $\gamma$  antagonist inhibits adipogenesis but also blocks the activation of C/EBP $\alpha$  expression at the onset of differentiation. Ectopic expression of C/EBP $\beta$  in Swiss 3T3 mouse fibroblasts (Swiss-LAP cells) induces PPAR $\gamma$  expression without any significant enhancement of C/EBP $\alpha$  expression. Treatment of Swiss-LAP cells with a PPAR $\gamma$  agonist induces adipogenesis, which includes activation of C/EBP $\alpha$  expression. To further establish a role for PPAR $\gamma$  in regulating C/EBP $\alpha$  expression, we expressed C/EBP $\beta$  in PPAR $\gamma$ -deficient mouse embryo fibroblasts (MEFs). The data show that C/EBP $\beta$  is capable of inducing PPAR $\gamma$  in *Ppar $\gamma$ +/–* MEFs, which leads to activation of adipogenesis, including C/EBP $\alpha$  expression following exposure to a PPAR $\gamma$  ligand. In contrast, C/EBP $\beta$  is not able to induce C/EBP $\alpha$  expression or adipogenesis in *Ppar $\gamma$ –/–* MEFs. Chromatin immunoprecipitation analysis reveals that C/EBP $\beta$  is bound to the minimal promoter of the *C/ebp $\alpha$*  gene in association with HDAC1 in unstimulated Swiss-LAP cells. Exposure of the cells to a PPAR $\gamma$  ligand dislodges HDAC1 from the proximal promoter of the *C/ebp $\alpha$*  gene, which involves degradation of HDAC1 in the 26 S proteasome. These data suggest that C/EBP $\beta$  activates a single unified pathway of adipogenesis involving its stimulation of PPAR $\gamma$  expression, which then activates C/EBP $\alpha$  expression by dislodging HDAC1 from the promoter for degradation in the proteasome.

The differentiation of preadipocytes into adipocytes is regulated by an elaborate network of transcription factors that control expression of many hundreds of proteins responsible for establishing the mature fat cell phenotype (1). The most notable among these factors

are members of the C/EBP<sup>2</sup> and PPAR families of transcription factors. In fact, it is now well accepted that both PPAR $\gamma$  and C/EBP $\alpha$  function as critical regulators of adipogenesis because deficiency of either of these proteins prevents the development of white adipose tissue in the mouse (2–4). Studies performed in cell culture have positioned PPAR $\gamma$  and C/EBP $\alpha$  in the center of this network of factors where they orchestrate the many functions associated with the mature adipocyte (1, 5). Some functions appear to be governed exclusively by PPAR $\gamma$  such as lipogenesis, whereas others such as insulin-dependent glucose transport and adiponectin expression are dependent on simultaneous expression of both proteins (6–8). Consequently, in our efforts to gain a complete understanding of the processes regulating the function of adipocytes, it is important to identify the mechanisms regulating transcription of both PPAR $\gamma$  and C/EBP $\alpha$ . Several investigators have demonstrated a direct role for C/EBP $\beta$  along with C/EBP $\delta$  in inducing expression of PPAR $\gamma$ 2 through association with C/EBP regulatory elements in the *Ppar $\gamma$ 2* gene promoter (9–12). A similar role for these C/EBPs in inducing transcription of the *C/ebp $\alpha$*  gene has not been established. It is generally assumed, however, that C/EBP $\beta$  does induce C/EBP $\alpha$  based on data from *in vitro* assays showing transactivation of a *C/ebp $\alpha$*  minimal promoter reporter gene in different cell types by C/EBP $\beta$  (13–15). Earlier studies aimed at characterizing the importance of C/EBP $\beta$  in inducing adipogenesis failed to demonstrate induction of C/EBP $\alpha$  expression because the NIH-3T3 fibroblasts used in the experiments do not express C/EBP $\alpha$  (10, 16). In those studies, however, ectopic expression of C/EBP $\beta$  was capable of inducing PPAR $\gamma$ 2 expression, and following exposure to appropriate ligands the NIH-3T3 cells underwent conversion into lipid-laden adipocytes that were unresponsive to insulin because they lacked C/EBP $\alpha$  (6). Other studies performed in cells capable of expressing C/EBP $\alpha$  have demonstrated that PPAR $\gamma$  can activate transcription of the *C/ebp $\alpha$*  gene in the absence of ongoing protein synthesis (17), suggesting that the transcriptional cascade responsible for initiating terminal adipogenesis involves induction of PPAR $\gamma$ 2 by C/EBP $\beta$ , and PPAR $\gamma$ 2 then is responsible for inducing *C/ebp $\alpha$*  along with other adipogenic genes.

Activation of gene expression involves a complex multistep process that includes docking of select transcription factors on regulatory elements within the promoter and/or enhancers of the target genes, which initiates recruitment of a variety of nuclear factors involved in reorganization of surrounding chromatin as well as assembly of the transcrip-

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<sup>2</sup> The abbreviations used are: C/EBP, CCAAT/enhancer-binding protein; PPAR, peroxisome proliferator-activated receptor; LAP, liver-enriched transcriptional activator protein; MIX, isobutylmethylxanthine; DEX, dexamethasone; PBS, phosphate-buffered saline; HDAC, histone deacetylase; MEF, mouse embryo fibroblast; ChIP, chromatin immunoprecipitation; TET, tetracycline; FBS, fetal bovine serum; pol, polymerase.

tional machinery at the promoter. It also appears that in some cases transcription factors can dock on various genes without initiating transcription. These factors appear to recruit corepressors and associated proteins to the gene that suppress transcription until an effector(s) dislodges the repressors to facilitate recruitment of appropriate coactivators. In the case of C/EBP $\beta$ , there is evidence to suggest that it can dock on the promoters of *C/ebp $\alpha$*  and *Ppar $\gamma$*  genes prior to their activation during the early phase of adipogenesis. Adipogenic effectors then facilitate association of the chromatin remodeling complex SWI/SNF with C/EBP $\beta$  on the *Ppar $\gamma$*  gene (18), whereas glucocorticoids are responsible for dislodging an mSin3a-HDAC1 complex from the C/EBP $\beta$  site on the *C/ebp $\alpha$*  gene (15). In an attempt to define the role of PPAR $\gamma$  along with C/EBP $\beta$  in regulating C/EBP $\alpha$  expression during adipogenesis, we ectopically expressed each of the proteins in nonadipogenic fibroblasts and analyzed adipogenic gene expression. The data demonstrate that C/EBP $\beta$  is capable of docking on the *C/ebp $\alpha$*  gene promoter but is incapable of inducing C/EBP $\alpha$  transcription in the absence of PPAR $\gamma$ . Furthermore, treatment of cells expressing C/EBP $\beta$  with glucocorticoids is not capable of inhibiting HDAC1 activity at the *C/ebp $\alpha$*  gene, whereas activation of PPAR $\gamma$  facilitates targeting of the HDAC1 that is associated with the *C/ebp $\alpha$*  gene to the proteasome and thereby inducing transcription.

## EXPERIMENTAL PROCEDURES

**Expression Vectors and Cell Lines**—The p34C/EBP $\beta$ (LAP)-pBI-G plasmid and pBabe-Puro-LAP and pRevTRE-C/EBP $\alpha$ -Myc-His retroviral vectors were produced as described previously (8, 17, 19). The LAP-pBI-G vector was transfected into Swiss mouse 3T3 fibroblasts constitutively expressing the Tet-Off activator protein (Swiss Tet-Off cells; Clontech) along with a puromycin selection plasmid (pBabe-puro). Colonies of cells resistant to 3  $\mu$ g/ml puromycin were selected and analyzed for expression of the pBI-G vector on the basis of tetracycline-responsive  $\beta$ -galactosidase production. The initial selection gave rise to several nonhomogeneous colonies because only 10–20% of the cells expressed  $\beta$ -galactosidase activity. Therefore, one of these colonies was subjected to serial dilution single cell cloning. A colony (LAP-A cells) was selected in which almost the entire population of cells expressed  $\beta$ -galactosidase activity in a tetracycline-responsive manner. To establish retrovirus-producing cell lines, human embryonic kidney 293T cells were seeded at 80% confluence in a 60-mm diameter dish on the day of transfection. Individual cultures of cells were transfected with FuGENE 6 (Roche Applied Science) and 2  $\mu$ g of either the pRevTRE-C/EBP $\alpha$ -Myc-His or pBabe-Puro-LAP vector along with 2  $\mu$ g each of vesicular stomatitis virus G and GP expression plasmids (pVpack; Stratagene). Two days after transfection, culture medium containing high titer virus was harvested and filtered through a 0.45- $\mu$ m pore size filter. The viral filtrate supernatant was used to infect *Ppar $\gamma$* <sup>+/–</sup> or *Ppar $\gamma$* <sup>–/–</sup> mouse embryo fibroblasts (20). Cells were seeded in a 60-mm dish at 25% confluence on the day of infection and incubated with retrovirus overnight in the presence of 4  $\mu$ g of Polybrene/ml (Sigma), and the infection was allowed to proceed for an additional 2 days in fresh growth medium. At this stage, 100  $\mu$ g of hygromycin and/or 2  $\mu$ g/ml puromycin was added to facilitate selection of stable cell lines, which required culture for 2–3 weeks in the presence of the antibiotic.

**Cell Culture**—For experiments, cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum (FBS). Induction of differentiation was achieved by treatment of post-confluent cells with dexamethasone (DEX, 1  $\mu$ M), 3-isobutyl-1-methylxanthine (MIX, 0.5 mM), and insulin (1.67  $\mu$ M). The Swiss-LAP A cells and the other cell lines were differentiated by the same method as for 3T3-L1 preadipocytes (8) and maintained in the presence or absence of

5  $\mu$ M troglitazone (Parke-Davis) or 1  $\mu$ M GW7845 (a highly selective PPAR $\gamma$  agonist) obtained from GlaxoSmithKline. To block PPAR $\gamma$  activity, cells were exposed to T0070907 (a selective PPAR $\gamma$  antagonist) as described previously (21) and obtained from AdipoGenix (Boston). Cells were refed every 2 days.

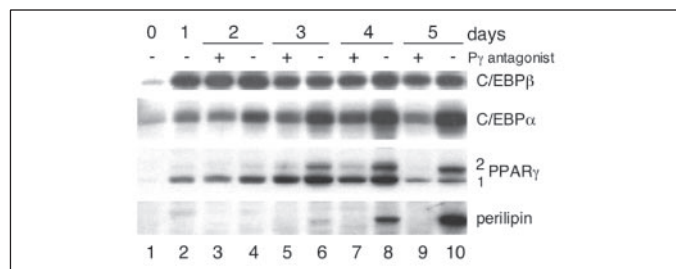
**Cell Extracts and Western Blot Analysis of Proteins**—Isolation and Western blot analysis of whole-cell proteins was performed as described previously (8). Antibodies employed in the analysis were as follows: anti-C/EBP $\alpha$ , anti-C/EBP $\beta$ , anti-PPAR $\gamma$  (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HDAC1–3 (Upstate Biotechnology, Inc., Lake Placid, NY); anti- $\beta$ -catenin (BD Transduction Laboratories); anti-Acrp30/adiponectin (Affinity BioReagents, Golden, CO); and anti-perilipin (Dr. Andy Greenberg, New England Medical Center, Tufts University, Boston).

**Immunoprecipitation**—Cell extracts prepared as described previously (8) were incubated overnight at 4 °C with 3–5  $\mu$ g of the antibody or the same amount of a nonspecific mouse or rabbit IgG. The following day, 30- $\mu$ l protein-G beads were added, and extracts were incubated with rotation for 3 h at 4 °C. The beads were then washed three times with lysis buffer, and antigens were eluted by incubation with 30  $\mu$ l of Laemmli sample buffer with or without 50 mM dithiothreitol.

**RNA Analysis**—Total RNA was extracted with TRIzol® (Invitrogen) according to the manufacturer's instructions. After quantification, 20–25  $\mu$ g from each RNA sample was subjected to Northern blot analysis as described previously (17).

**Chromatin Immunoprecipitation Assays**—Cells were fixed by addition of 37% formaldehyde to a final concentration of 1% formaldehyde and incubation at room temperature for 10 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M. Cells were then trypsinized, scraped, washed with phosphate-buffered saline (PBS), and swelled in hypotonic buffer (10 mM Hepes, pH 7.6, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride). Nuclei were pelleted by microcentrifugation and lysed by incubation in nuclear lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS) on ice for 10 min. The resulting chromatin solution was sonicated with three 30-s pulses at maximum power. After microcentrifugation, the supernatant was pre-cleared with blocked protein A-agarose beads. The chromatin fractions were then immunoprecipitated with 1–2  $\mu$ g of the following antibodies: anti-C/EBP $\beta$ , anti-C/EBP $\alpha$ , anti-acetylated histone H3, anti-acetylated histone H4, anti-HDAC1, anti-RNA polymerase II, or IgG (anti-acetyl-histone H3, anti-acetyl-histone H4, anti-RNA polymerase II, and normal rabbit IgG were purchased from Upstate Biotechnology, Inc.). After incubation at 4 °C overnight, the DNA-protein complexes were immunoprecipitated with protein A-agarose. After washing the DNA-protein complexes, DNA was extracted with phenol/chloroform, precipitated, redissolved, and used as templates for PCR. Different PCR cycles (ranging from 24 to 32) were used to evaluate each assay, and the lowest possible cycle was chosen for presentation. Input and antibody controls were performed at the same number of PCR cycles as the immunoprecipitated complexes. The primers used for the PCR correspond to regions flanking the C/EBP-binding site within the *C/ebp $\alpha$*  gene promoter and are as follows: sense, 5'-CTG GAA GTG GGT GAC TTA GAG G-3'; antisense, 5'-GAG TGG GGA GCA TAG TGC TAG-3'.

**Oil Red O Staining**—The cells were seeded in 35-mm plates, and at the specified stage of differentiation they were rinsed with PBS and fixed with 10% formalin in PBS for 15 min. After two washes in PBS, cells were stained for at least 1 h in freshly diluted Oil Red O solution (6 parts Oil Red O stock solution and 4 parts H<sub>2</sub>O; Oil Red O stock solution is 0.5% Oil Red O in isopropyl alcohol). The stain was then removed, and cells



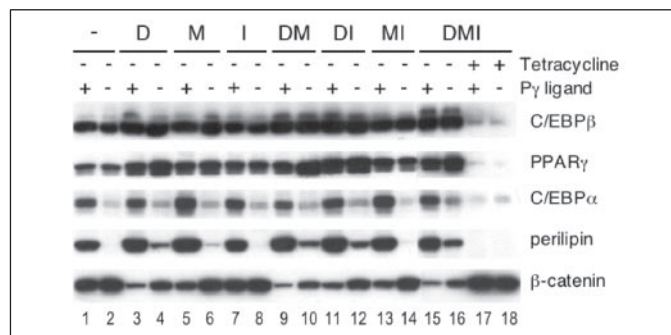
**FIGURE 1. Inhibition of PPAR $\gamma$  activity blocks the induction of C/EBP $\alpha$  expression during the differentiation of 3T3-L1 preadipocytes.** Proliferating 3T3-L1 cells were cultured in 10% calf serum until they reached confluence. After 2 days post-confluence, cells were induced to differentiate by exposure to DEX, MIX, insulin, and 10% FBS. At day 1 of differentiation, cells were treated with or without 10  $\mu$ M T0070907 (PPAR $\gamma$  antagonist) and harvested at the indicated times. Equal amounts of each protein sample were subjected to Western blot analysis using antibodies specific for C/EBP $\beta$ , C/EBP $\alpha$ , PPAR $\gamma$ , and perilipin.

were washed twice with water, with or without counterstain (0.25% Giemsa for 15 min), and then photographed.

## RESULTS

To determine whether activation of C/EBP $\alpha$  expression during the early phase of adipogenesis requires PPAR $\gamma$  activity, we treated 3T3-L1 preadipocytes with T0070907 (a selective and potent antagonist of PPAR $\gamma$  activity) at day 1 following exposure of the confluent cells to the adipogenic inducers DEX, MIX, insulin, and 10% FBS. Fig. 1 shows a Western blot analysis of total proteins extracted from the cells at the indicated days following exposure to the antagonist. It is quite apparent that the PPAR $\gamma$  antagonist completely blocks expression of perilipin (Fig. 1, compare lane 9 with lane 10), a known downstream target of PPAR $\gamma$  (22–24), and also inhibits differentiation into mature adipocytes (data not shown). More important is the observation that blocking PPAR $\gamma$  activity also leads to a significant attenuation of C/EBP $\alpha$  expression. In fact, the antagonist appears to prevent the activation of C/EBP $\alpha$  during the initial 3 days of adipogenesis (Fig. 1, compare lane 5 with lane 6) prior to the expression of the terminal genes such as perilipin. It is interesting that the antagonist also attenuates expression of PPAR $\gamma$ 2. This is likely because of the fact that C/EBP $\alpha$  normally feeds back on the *Ppar $\gamma$ 2* gene (7). There appears to be minimal effect of the PPAR $\gamma$  antagonist on C/EBP $\beta$  expression.

The fact that T0070907 is a specific inhibitor of PPAR $\gamma$  activity suggests that PPAR $\gamma$  contributes to the initial activation of C/EBP $\alpha$  during the differentiation of 3T3-L1 preadipocytes. Because our previous studies had shown that inhibition of C/EBP $\beta$  activity by ectopic expression of a dominant negative C/EBP $\beta$  protein (LIP) blocks both C/EBP $\alpha$  and PPAR $\gamma$  expression (17), we next addressed the role of C/EBP $\beta$  in regulating C/EBP $\alpha$  expression. Earlier studies have demonstrated that C/EBP $\beta$  is capable of inducing expression of PPAR $\gamma$ 2 in NIH-3T3 fibroblasts (9, 16). Unfortunately, it was not possible to assess the role of C/EBP $\beta$  in activating C/EBP $\alpha$  expression because the NIH-3T3 cells do not transcribe the *C/ebp $\alpha$*  gene (10, 16). To assess the contribution of both C/EBP $\beta$  and PPAR $\gamma$  to the induction of C/EBP $\alpha$  during adipogenesis, we ectopically expressed C/EBP $\beta$  in Swiss mouse fibroblasts using the Tet-Off conditional expression system (Swiss-LAP A cells). The Swiss-LAP A cells were cultured for several days in the absence of tetracycline in order to stimulate expression of the ectopic C/EBP $\beta$  protein. At confluence, the cells were exposed to medium containing 10% FBS and various combinations of the adipogenic inducers, DEX, MIX, or insulin, in the presence or absence of a potent PPAR $\gamma$  agonist (GW7845) for 2 days at which time the cells were maintained in medium containing 10% FBS, insulin, and the corresponding quantity of the



**FIGURE 2. Activation of C/EBP $\alpha$  expression in Swiss fibroblasts expressing an ectopic C/EBP $\beta$  requires exposure of cells to a potent PPAR $\gamma$  ligand.** Swiss-LAP A cells were cultured in growth medium with or without tetracycline until confluent. Two days later, the cells were exposed to various combinations of DEX (D), MIX (M), and insulin (I) in medium containing 10% fetal bovine serum for 2 days. Cells were cultured for an additional 4 days in 1.67  $\mu$ M insulin with or without GW7845 (PPAR $\gamma$  agonist) and harvested for Western blot analysis using antibodies against PPAR $\gamma$ , C/EBP $\beta$ ,  $\beta$ -catenin, C/EBP $\alpha$ , and perilipin.

PPAR $\gamma$  agonist for an additional 3 days. Fig. 2 shows abundant expression of the ectopic C/EBP $\beta$  protein, which results in activation of PPAR $\gamma$ 2 expression under all conditions except when cells were cultured in tetracycline to suppress the *C/ebp $\beta$*  gene (Fig. 2, lanes 17 and 18). The ectopic C/EBP $\beta$ , in addition to activating PPAR $\gamma$ 2, was also capable of inducing perilipin expression (marker of adipogenesis) in response to exposure of these Swiss fibroblasts to DEX (Fig. 2, lanes 4, 10, 12, and 16). Exposure to insulin and/or MIX failed to activate the perilipin gene to any significant extent (Fig. 2, lanes 6, 8, and 14). Of interest is the observation that C/EBP $\beta$  did not stimulate expression of C/EBP $\alpha$  in the presence of any of these inducers (Fig. 2, lanes 4, 6, 8, 10, 12, 14, and 16). Importantly, activation of PPAR $\gamma$ 2 activity by treatment with the PPAR $\gamma$  agonist (GW7468) induces expression of C/EBP $\alpha$  as well as perilipin to levels normally expressed in mature adipocytes (data not shown) regardless of the presence of the other effectors (DEX, MIX, or insulin). Fig. 2 also demonstrates the reciprocal relationship between  $\beta$ -catenin and adipogenesis in these fibroblasts as observed previously (19, 25, 26). Taken together, these data suggest that activation of PPAR $\gamma$ 2 expression only requires C/EBP $\beta$ , whereas expression of C/EBP $\alpha$  requires ligand-dependent activation of PPAR $\gamma$  along with C/EBP $\beta$ .

To substantiate further the importance of PPAR $\gamma$  in regulating C/EBP $\alpha$  expression, we stably expressed the p34 LAP isoform of C/EBP $\beta$  in mouse embryo fibroblasts lacking a functional *Ppar $\gamma$*  gene ( $P\gamma^{-/-}$  MEFs) and in a corresponding population of heterozygous ( $P\gamma^{+/-}$ ) MEFs. The cell lines ( $P\gamma^{+/-}$  and  $P\gamma^{-/-}$ ) were exposed to DEX, MIX, and insulin in the presence or absence of troglitazone for 2 days and were then cultured for an additional 3 days in the presence or absence of troglitazone. The cells were either stained with Oil Red O for morphological analysis or harvested for Western blot analysis of total cellular proteins. Fig. 3, A and B, demonstrates that ectopic expression of C/EBP $\beta$  induces some lipid accumulation in the MEFs that contain one functional allele of PPAR $\gamma$  ( $P\gamma^{+/-}$  cells) following exposure to DEX, MIX, and insulin, and this effect is enhanced manyfold in the presence of the troglitazone. As expected, C/EBP $\beta$  failed to induce any lipid accumulation in the PPAR $\gamma$ -deficient cells ( $P\gamma^{-/-}$ ) with or without troglitazone. The absence of adipogenesis in the  $P\gamma^{-/-}$  cells did not necessarily mean that C/EBP $\beta$  was not capable of inducing expression of C/EBP $\alpha$  in these cells because other studies have shown that C/EBP $\alpha$  is incapable of inducing lipid accumulation in the absence of PPAR $\gamma$  (20). Consequently, we analyzed expression of C/EBP $\alpha$  in response to C/EBP $\beta$  in both  $P\gamma^{+/-}$  and  $P\gamma^{-/-}$  cells. Fig. 4A shows

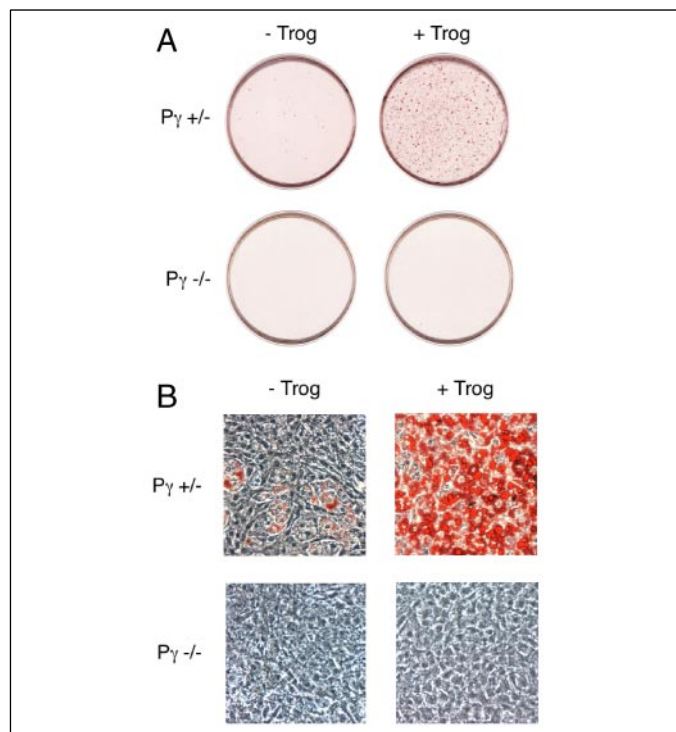


abundant expression of the ectopic C/EBP $\beta$  in both cell lines. Treatment of the P $\gamma$ <sup>+/−</sup> cells with DEX, MIX, and insulin induces a low but detectable level of PPAR $\gamma$ , C/EBP $\alpha$ , perilipin, and adiponectin expression (Fig. 4A, lane 3), and exposure to the PPAR $\gamma$  agonist enhances expression of all four proteins to the abundant levels (lane 4) normally expressed in mature adipocytes. Most importantly, exposure of the PPAR $\gamma$ -deficient cells to DEX, MIX, and insulin with or without PPAR $\gamma$  ligand was incapable of stimulating C/EBP $\alpha$ , perilipin, or adiponectin even though the cells produced abundant quantities of the ectopic C/EBP $\beta$  protein (Fig. 4A, lanes 1 and 2). The Northern blot analysis of

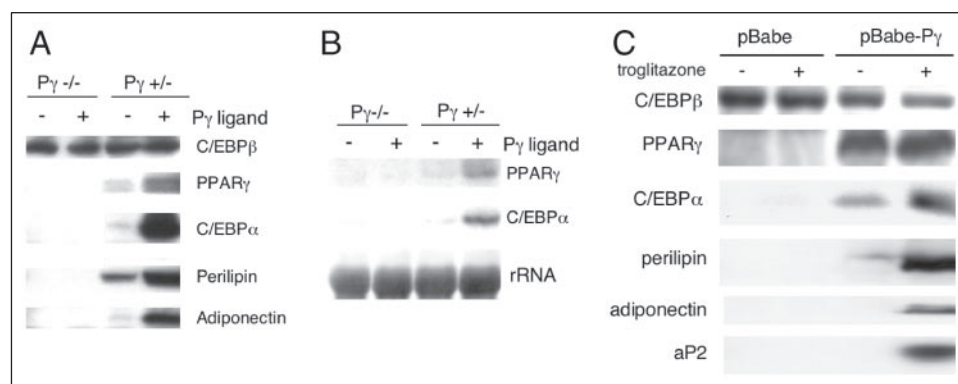
these cells demonstrate that the absence of C/EBP $\alpha$  expression in Ppar $\gamma$ <sup>−/−</sup> MEFs is because of a lack of expression of the corresponding mRNA (Fig. 4B, compare lane 2 with lane 4). These data support the notion that C/EBP $\beta$  activates a single unified pathway of adipogenesis (20) involving its induction of PPAR $\gamma$ , which then activates C/EBP $\alpha$  expression along with other terminal adipogenic programs. To confirm that the only defect in the Ppar $\gamma$ <sup>−/−</sup> MEFs was the lack of PPAR $\gamma$ , we retrovirally expressed PPAR $\gamma$ 2 in these cells that already expressed the ectopic C/EBP $\beta$  and showed that its expression facilitated induction of C/EBP $\alpha$  along with the other markers of the adipogenic program (Fig. 4C).

Other studies have shown that C/EBP $\beta$  as well as C/EBP $\alpha$  can transactivate an ectopic C/ebp $\alpha$  promoter/reporter gene in cells lacking PPAR $\gamma$  activity by binding to a C/EBP-regulatory element in the proximal 5' upstream region (13, 14). We considered it important, therefore, to determine the transcriptional status of the C/ebp $\alpha$  gene promoter with respect to association with C/EBPs as well as other factors in PPAR $\gamma$ -deficient cells. Ppar $\gamma$ <sup>+/−</sup> or Ppar $\gamma$ <sup>−/−</sup> MEFs ectopically expressing either C/EBP $\beta$  or C/EBP $\alpha$  were exposed to DEX, MIX, insulin, and the PPAR $\gamma$  ligand, GW7468, for 5 days, and ChIP analyses were performed using antibodies against C/EBP $\beta$ , C/EBP $\alpha$ , acetylated histone H3 and H4, and a nonspecific preimmune antiserum as a control and PCR primers flanking the C/EBP regulatory element within the C/ebp $\alpha$  gene promoter. Somewhat surprising was the observation that ectopic C/EBP $\beta$  and C/EBP $\alpha$  bound to the C/EBP $\alpha$  promoter in Ppar $\gamma$ <sup>−/−</sup> cells as well as the Ppar $\gamma$ <sup>+/−</sup> controls even though the gene appears to be transcriptionally inactive in the <sup>−/−</sup> cells (Fig. 5, A and B). Fig. 5, A and B, demonstrates further that acetylation of histones H3 and H4 within chromatin associated with the C/ebp $\alpha$  gene only occurs in the Ppar $\gamma$ <sup>+/−</sup> cells and is absent or below detection in cells lacking PPAR $\gamma$  activity (Ppar $\gamma$ <sup>−/−</sup>).

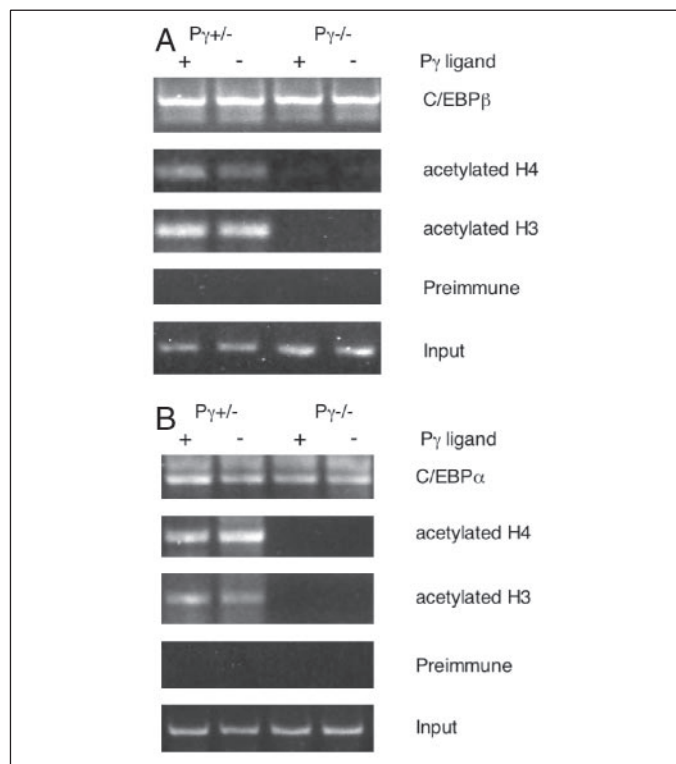
This PPAR $\gamma$ -associated acetylation of the C/EBP $\alpha$  minimal promoter could result from a decrease in the activity of histone deacetylases (HDACs) in the Swiss fibroblasts. To determine the potential involvement of HDACs in regulating adipogenic gene expression, we first analyzed expression of HDAC1–3 in response to exposure of the Swiss-LAP A cells to troglitazone in the presence and absence of tetracycline. Fig. 6 demonstrates that the fibroblasts express abundant amounts of HDAC1 and HDAC3 but no detectable level of HDAC2 when compared with the level present in HeLa cell extracts. Furthermore, activation of PPAR $\gamma$  by exposure to troglitazone has no significant effect on total cell HDAC1 or HDAC3.



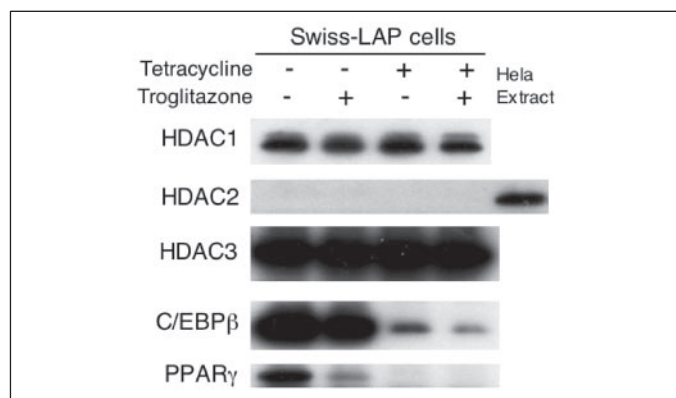
**FIGURE 3. C/EBP $\beta$  induces lipid accumulation in P $\gamma$ <sup>+/−</sup> MEFs but not in P $\gamma$ <sup>−/−</sup> MEFs.** Mouse embryo fibroblasts lacking a functional Ppar $\gamma$  gene (P $\gamma$ <sup>−/−</sup>) and a corresponding population of P $\gamma$ <sup>+/−</sup> MEFs were infected with a retrovirus expressing the 34-kDa (LAP) isoform of C/EBP $\beta$ . The resulting cell lines were cultured in growth medium until confluent. After 2 days, the cells were induced to differentiate as described under "Experimental Procedures" in the presence or absence of troglitazone (Trog). At day 6, cells were fixed and stained with Oil Red O, and either the dishes (A) or a microscopic view of the dishes (B) was photographed.



**FIGURE 4. C/EBP $\beta$  induces C/ebp $\alpha$  gene expression in P $\gamma$ <sup>+/−</sup> MEFs, but not in P $\gamma$ <sup>−/−</sup> MEFs.** P $\gamma$ <sup>+/−</sup> and P $\gamma$ <sup>−/−</sup> MEFs expressing an ectopic C/EBP $\beta$  were induced to differentiate for 6 days as described in Fig. 3. At this time, cells were harvested for either total proteins or RNA as described under "Experimental Procedures." A, equal amounts of total cellular protein were subjected to Western blot analysis of C/EBP $\beta$ , PPAR $\gamma$ , C/EBP $\alpha$ , perilipin, and adiponectin. B, equal amounts of total RNA were analyzed by Northern blot hybridization using cDNA probes corresponding to PPAR $\gamma$  and C/EBP $\alpha$ . The membrane was also stained with methylene blue to visualize the rRNA species and verify the presence of equal amounts of RNA. C, ectopic PPAR $\gamma$ 2 rescues expression of C/EBP $\alpha$  in P $\gamma$ <sup>−/−</sup> cells expressing C/EBP $\beta$ . Ppar $\gamma$ <sup>−/−</sup> MEFs expressing an ectopic C/EBP $\beta$  (LAP) were infected with retrovirus vector containing PPAR $\gamma$ 2 cDNA (pBabe-P $\gamma$ ) or the empty vector (pBabe). The cell lines were cultured in growth medium for 3 days until confluent and were then induced to differentiate as described under "Experimental Procedures." At day 5, cells were harvested for Western blot analysis of C/EBP $\beta$ , PPAR $\gamma$ , C/EBP $\alpha$ , perilipin, aP2, and adiponectin.

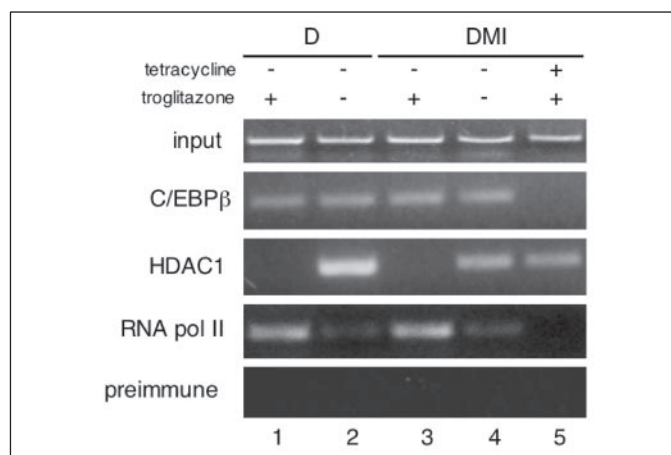


**FIGURE 5. Differences in the acetylation of histones in chromatin associated with the C/EBP $\alpha$  promoter in P $\gamma$ <sup>+/-</sup> MEFs compared with P $\gamma$ <sup>-/-</sup> MEFs.** P $\gamma$ <sup>+/-</sup> and P $\gamma$ <sup>-/-</sup> MEFs constitutively expressing either an ectopic C/EBP $\beta$  (A) or C/EBP $\alpha$  (B) were induced to differentiate for 5 days as described under "Experimental Procedures" in the presence or absence of GW7845 (P $\gamma$  ligand). Cells were harvested and subjected to ChIP analysis using antibodies corresponding to C/EBP $\beta$ , C/EBP $\alpha$ , acetylated histones H3 and H4, and preimmune serum. One percent of input is shown for each experiment.



**FIGURE 6. Level of expression of HDAC1–3 during the differentiation of Swiss-LAP A cells.** The Swiss-LAP A cells were cultured until confluent, at which time the cells were induced to differentiate as described under "Experimental Procedures" with or without troglitazone in the presence or absence of tetracycline. At day 5, the cells were harvested for Western blot analysis of HDAC1, HDAC2, HDAC3, C/EBP $\beta$ , and PPAR $\gamma$ . A sample of HeLa cell extract was also analyzed to confirm expression of HDAC2.

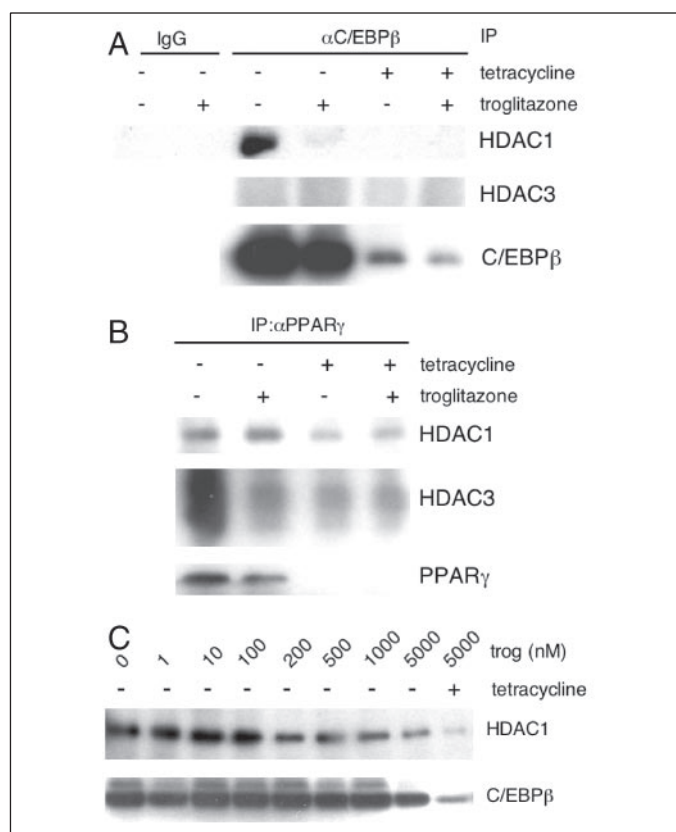
Consequently, the increase in histone acetylation at the *C/ebp $\alpha$*  gene promoter is not because of an overall decrease in the abundance of HDAC1 or HDAC3. To determine whether there is a decrease in HDAC activity at the *C/ebp $\alpha$*  gene promoter, we performed ChIP assays on nuclei isolated from Swiss-LAP A cells following exposure to adipogenic inducers in the presence or absence of the PPAR $\gamma$  ligand. Fig. 7 shows that HDAC1 is associated with the minimal promoter in cells that do not express C/EBP $\alpha$  to any significant extent (*i.e.* in the absence of troglitazone or C/EBP $\beta$ ; Fig. 7, lanes 2, 4, or 5). Interestingly, activation of PPAR $\gamma$  by exposure to troglitazone results in stimulation of this pro-



**FIGURE 7. PPAR $\gamma$ -dependent dislodgment of HDAC1 from the C/EBP $\alpha$  minimal promoter.** Swiss-LAP A cells were differentiated for 5 days in either DEX alone (D) or DEX with insulin and MIX (DMI) with or without troglitazone (5  $\mu$ M) in the presence or absence of tetracycline. The cells were then fixed and harvested for ChIP assay using antibodies to C/EBP $\beta$ , HDAC1, RNA polymerase II, or preimmune serum.

motor as indicated by recruitment of RNA polymerase II (RNA pol II) and a corresponding decrease in the bound HDAC1 (Fig. 7, lanes 1 and 3). It is also important to point out that dexamethasone either alone or in the presence of the other adipogenic inducers, MIX and insulin, is incapable of dislodging HDAC1 from the promoter or recruiting significant quantities of RNA pol II unless PPAR $\gamma$  is also activated by its ligand, troglitazone (Fig. 7, compare lane 2 with lane 1 and lane 4 with lane 3). Fig. 7 also shows binding of the ectopic C/EBP $\beta$  to the minimal promoter in cells that do not transcribe the *C/ebp $\alpha$*  gene as observed previously in P $\gamma$ <sup>-/-</sup> MEFs (Fig. 5). These data are consistent with other studies that suggest that C/EBP $\beta$  acts as a repressor of gene expression prior to its stimulation by select effectors (27). To determine whether the ectopic C/EBP $\beta$  exists within a repressed state prior to activation of adipogenic gene expression in Swiss-LAP A cells, we performed a series of immunoprecipitation assays using anti-C/EBP $\beta$  as well as anti-PPAR $\gamma$  antibodies. Fig. 8, A and C, shows association of HDAC1 with complexes containing C/EBP $\beta$ , whereas HDAC3 appears to associate with PPAR $\gamma$  (Fig. 8B). Furthermore, exposure of the cells to troglitazone causes a decrease in the association of HDAC1 with C/EBP $\beta$  (Fig. 8, A and C) as well as HDAC3 with PPAR $\gamma$  (Fig. 8B). It is therefore conceivable that C/EBP $\beta$  exists in the nucleus in association with HDAC1 and can repress select target genes by docking on corresponding C/EBP-binding sites. Activation of PPAR $\gamma$  with a ligand dislodges its associated HDAC3 and leads to stimulation of C/EBP $\beta$  activity on the *C/EBP $\alpha$*  gene by removing the bound HDAC1.

Several investigations have suggested that the proteasome is intimately involved in regulating transcription of particular genes by controlling the degradation of associated coactivators and/or corepressors (28). Therefore, to assess what effect inhibiting the proteasome might have on C/EBP $\alpha$  as well as adipogenic gene expression, we treated differentiating Swiss-LAP A cells with an inhibitor of the proteasome (MG132) or a vehicle control for 24 h. As expected, Fig. 9A demonstrates that the ectopic C/EBP $\beta$  induces PPAR $\gamma$  expression (lane 1) and that exposure to troglitazone activates C/EBP $\alpha$  expression as well as other adipogenic genes (lane 3). Treatment of cells with MG132 has no apparent effect on expression of the ectopic C/EBP $\beta$  but significantly attenuates its ability to induce PPAR $\gamma$  expression and almost completely eliminates C/EBP $\alpha$  expression. These data are consistent with the notion that targeted degradation of corepressors through the proteasome is required to facilitate tran-

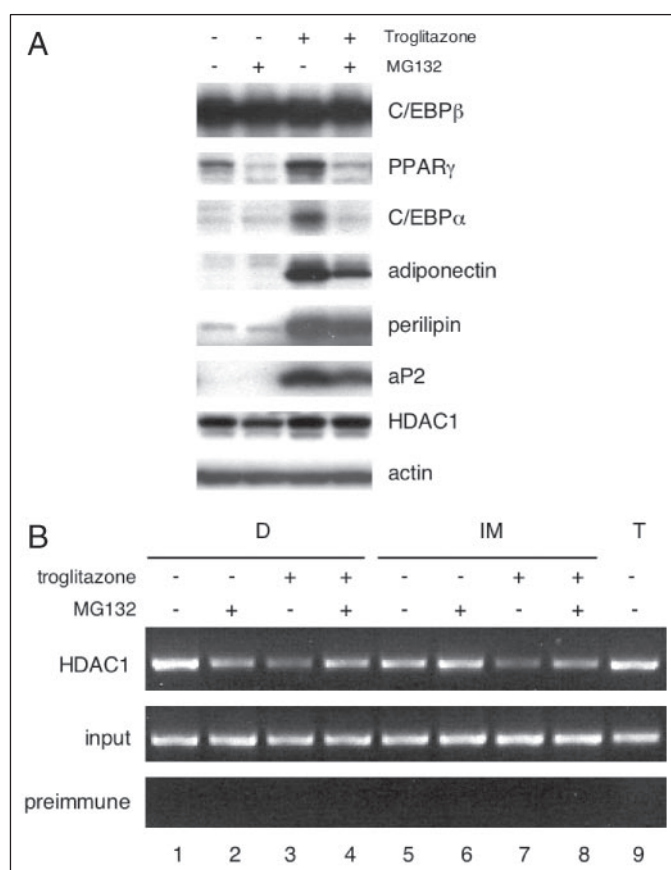


**FIGURE 8. Activation of PPAR $\gamma$  with troglitazone causes the disassociation of HDAC1 with C/EBP $\beta$ .** Confluent LAP A cells were cultured in DEX, MIX, insulin with 5  $\mu$ M troglitazone (A and B) or increasing concentrations of troglitazone (troglitazone) as indicated (C) in the presence or absence of tetracycline. At day 5, cells were harvested, and proteins were immunoprecipitated from the cell extracts using anti-C/EBP $\beta$  (A and C), anti-IgG (A), or anti-PPAR $\gamma$  (B) antibodies as described under "Experimental Procedures." The immunoprecipitates (IP) were then subjected to Western blot analysis using anti-HDAC1, anti-HDAC3, anti-C/EBP $\beta$ , or anti-PPAR $\gamma$  antibodies.

scription of the *C/ebp $\alpha$*  gene and adipogenesis. It is noteworthy that inhibition of the proteasome did not significantly alter the levels of HDAC1 present within the total cellular extracts. The most likely mechanism operating to regulate C/EBP $\alpha$  expression is proteasomal degradation of the HDAC1 associated with the *C/ebp $\alpha$*  gene promoter. To test this notion, we performed ChIP assays on differentiating Swiss-LAP A cells following exposure to MG132 in the presence or absence of troglitazone. As expected, Fig. 9B demonstrates a decrease in the bound HDAC1 when cells are exposed to troglitazone (compare lanes 3 and 7 with lanes 1 and 5), whereas treatment with dexamethasone alone was not capable of dislodging HDAC1 (compare lane 1 (plus dexamethasone) with lane 5 (minus dexamethasone)) from the C/EBP $\alpha$  minimal promoter. Interestingly, inhibition of the proteasome with MG132 significantly attenuates the ability of an activated PPAR $\gamma$  (plus troglitazone) to decrease the level of the bound HDAC1 (Fig. 9B, compare lane 4 with lane 3 and lane 8 with lane 7).

## DISCUSSION

In this study, we demonstrate that inhibition of PPAR $\gamma$  activity by exposure of 3T3-L1 preadipocytes to a potent and selective PPAR $\gamma$  antagonist not only blocks terminal adipogenesis but also inhibits the induction of C/EBP $\alpha$  expression at the onset of the adipogenic process. These data suggest that activation of C/EBP $\alpha$  expression requires PPAR $\gamma$  activity. In support of this notion, we show that ectopic expression of C/EBP $\beta$  in Swiss mouse fibroblasts leads to the



**FIGURE 9. A, induction of *Ppar $\gamma$*  and *C/ebp $\alpha$*  gene expression by C/EBP $\beta$  during adipogenesis in Swiss fibroblasts is dependent on the 26 S proteasome.** Confluent Swiss-LAP A cells were induced to differentiate as described under "Experimental Procedures" with or without troglitazone (5  $\mu$ M) in the absence of tetracycline. At day 4, 20  $\mu$ M MG132 was added for 24 h, and cells were then harvested for Western blot analysis of the indicated proteins. **B, PPAR $\gamma$  ligand-associated dislodgment of HDAC1 from the minimal promoter of the *C/ebp $\alpha$*  gene is dependent on the 26 S proteasome.** Confluent Swiss-LAP A cells were cultured in Dulbecco's modified Eagle's medium containing either DEX (D) or MIX and insulin (IM) with or without troglitazone (5  $\mu$ M) in the absence or presence (T) of tetracycline. At day 4, MG132 (20  $\mu$ M) was added for 24 h, and cells were then harvested for analysis of proteins associating with the minimal promoter of the *C/ebp $\alpha$*  gene using ChIP assay.

activation of PPAR $\gamma$ 2 expression without any significant enhancement of C/EBP $\alpha$  expression. Induction of C/EBP $\alpha$  expression in these cells only occurs following exposure to a PPAR $\gamma$  ligand, which also leads to expression of other adipogenic genes, including perilipin. To further establish the need for PPAR $\gamma$  in activating C/EBP $\alpha$  expression, we employed PPAR $\gamma$ -deficient mouse embryo fibroblasts. The data show that ectopic expression of C/EBP $\beta$  in PPAR $\gamma$ <sup>+/−</sup> MEFs is capable of activating PPAR $\gamma$ 2 expression and, following exposure to a PPAR $\gamma$  ligand, is also capable of inducing C/EBP $\alpha$  expression and terminal adipogenesis. In contrast, C/EBP $\beta$  is not capable of inducing C/EBP $\alpha$  expression or adipogenesis in PPAR $\gamma$ -deficient MEFs. Furthermore, it appears that C/EBP $\beta$  can associate with C/EBP regulatory elements within the promoter of the *C/ebp $\alpha$*  gene in PPAR $\gamma$ -deficient cells even though the acetylation of histones H3 and H4 at this locus is below detectable levels. These data are consistent with the unified theory of adipogenesis, which proposes that PPAR $\gamma$  is the principal regulator of terminal adipogenesis and, as such, is responsible for inducing expression of C/EBP $\alpha$  (20). The mechanism responsible for activating C/EBP $\alpha$  transcription involves a PPAR $\gamma$ -associated dislodgment of HDAC1 from the C/EBP $\alpha$  minimal promoter and a corresponding degradation of the HDAC1 by the proteasome.



The fact that PPAR $\gamma$  is a principal regulator of C/EBP $\alpha$  expression does not exclude a role for C/EBP $\beta$ . In fact, it is likely that C/EBP $\beta$  is required but is not sufficient to initiate C/EBP $\alpha$  expression during the early phase of adipogenesis. Investigators have shown that ectopic expression of PPAR $\gamma$  in nonadipogenic fibroblasts induces adipogenesis, which includes expression of C/EBP $\alpha$  (6, 7, 20). It might be concluded from this that PPAR $\gamma$  activates C/EBP $\alpha$  transcription in the absence of C/EBP $\beta$ . However, in nearly all these previous studies it was difficult to exclude some level of expression of C/EBP $\beta$ , particularly because the differentiation mixture used contained effectors that also elevate the level of expression of the endogenous C/EBP $\beta$ . In fact, recent studies performed in C/EBP $\beta$ -deficient MEFs support a role for C/EBP $\beta$  in facilitating C/EBP $\alpha$  transcription through mechanisms beyond simply inducing expression of PPAR $\gamma$  (29).

What are the mechanisms by which PPAR $\gamma$  regulates C/EBP $\alpha$  expression? The data in Figs. 5 and 7 demonstrate that C/EBP $\beta$  can bind to a C/EBP-regulatory element within the promoter of the *C/ebp $\alpha$*  gene even when the gene is transcriptionally inactive. It appears, however, that activation of transcription only occurs in response to expression of PPAR $\gamma$ . The most likely mechanism would involve association of PPAR $\gamma$  with an upstream enhancer element, which facilitates assembly of the transcriptional machinery at the promoter. A detailed analysis of the upstream regions of the *C/ebp $\alpha$*  gene has not, as yet, identified a functional PPAR-response element. The possibility that PPAR $\gamma$  induces expression of other factors that interact with the upstream region of the gene are discounted by earlier studies showing that activation of C/EBP $\alpha$  expression in CH310T1/2 mouse fibroblasts by PPAR $\gamma$  occurs in the absence of ongoing protein synthesis (17). It is possible that PPAR $\gamma$  alters the structural state of the chromatin associated with the *C/ebp $\alpha$*  gene by mechanisms that do not require protein synthesis or direct binding of PPAR $\gamma$  to corresponding DNA elements. For instance, PPAR $\gamma$  might direct HDAC1 for degradation in the proteasome by associating with a complex at the minimal promoter through protein-protein interactions. In this regard, recent studies have shown that PPAR $\gamma$  can regulate transcription of some genes through direct protein-protein interactions without binding to PPAR-response elements within the corresponding promoter (30). Additionally, other studies have demonstrated that other nuclear hormone receptors could induce a specific depletion of corepressors associated with inactive C/EBP $\beta$  by targeting HDAC1 within the corepressor complex for degradation through the 26 S proteasome (15). In fact, the authors of this study propose that glucocorticoids promote adipogenesis in 3T3-L1 preadipocytes through a nontranscriptional mechanism mediated through the ligand-binding domain of the glucocorticoid receptor, which involves displacement of the mSin3A repressor and HDAC1 from C/EBP $\beta$ . They also suggest that this event contributes to the activation of *C/ebp $\alpha$*  gene expression by C/EBP $\beta$  during the differentiation of 3T3-L1 cells. Our studies argue that such a glucocorticoid activation of C/EBP $\beta$  may be sufficient to induce PPAR $\gamma$  expression (9), but is insufficient for C/EBP $\alpha$  mRNA transcription in which case the 3T3-L1 cells require PPAR $\gamma$  (see Fig. 1).

Our data suggest, however, that the precise mechanisms regulating C/EBP $\alpha$  transcription might be quite complex. Specifically, Fig. 5 demonstrates that acetylation of histones within the core promoter depends on PPAR $\gamma$  but is independent of its association with ligand. In contrast, dislodgment of HDAC1 from the promoter is dependent on exposure of cells to ligand (Fig. 7). One explanation for this apparent discrepancy is that there are several events involved in activating the *C/ebp $\alpha$*  gene

promoter in addition to the PPAR $\gamma$  ligand-associated dislodgment of HDAC1 from C/EBP $\beta$ . For instance, acetylation of the histones may be regulated by PPAR $\gamma$  through association with histone acetyltransferases (*i.e.* p300/CBP) at its N-terminal ligand-independent AF-1 transactivation domain (31). To achieve full activation of the C/EBP $\alpha$  promoter, PPAR $\gamma$  ligand-dependent dislodgment of HDAC1 (and possibly other repressors) and a corresponding recruitment of pol II and its associated transcriptional machinery are likely to be required. The HDACs may be regulating the acetylation of several proteins, including C/EBP $\beta$  as well as the histones; therefore, there may not necessarily be a direct association between acetylated histone and the presence or absence of HDAC1.

In conclusion, these studies complement those of Rosen *et al.* (20) by showing that C/EBP $\beta$  activates a single unified pathway of adipogenesis that involves induction of PPAR $\gamma$  leading to expression of C/EBP $\alpha$  as well as other adipogenic genes. Furthermore, they identify a mechanism by which PPAR $\gamma$  induces C/EBP $\alpha$  expression that involves dislodgment of HDAC1 from C/EBP $\beta$ -associated complexes bound to the C/EBP $\alpha$  minimal promoter. This process appears to require a PPAR $\gamma$ -dependent degradation of HDAC1 by the 26 S proteasome.

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