Activation of CCAAT/Enhancer-binding Protein (C/EBP) α Expression by C/EBPβ during Adipogenesis Requires a Peroxisome Proliferator-activated Receptor-γ-associated Repression of HDAC1 at the C/ebpα Gene Promoter*

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Studies have shown that CCAAT/enhancer-binding protein β (C/EBPβ) can stimulate adipogenesis in noncommitted fibroblasts by activating expression of peroxisome proliferator-activated receptor-γ (PPARγ). Other investigations have established a role for C/EBPα as well as PPARγ 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/ebpα gene. In this study, we demonstrated that inhibition of PPARγ activity by exposure of 3T3-L1 preadipocytes to a potent and selective PPARγ antagonist inhibits adipogenesis but also blocks the activation of C/EBPα expression at the onset of differentiation. Ectopic expression of C/EBPβ in Swiss 3T3 mouse fibroblasts (Swiss-LAP cells) induces PPARγ expression without any significant enhancement of C/EBPα expression. Treatment of Swiss-LAP cells with a PPARγ agonist induces adipogenesis, which includes activation of C/EBPα expression. To further establish a role for PPARγ in regulating C/EBPα expression, we expressed C/EBPβ in PPARγ-deficient mouse embryo fibroblasts (MEFs). The data show that C/EBPβ is capable of inducing PPARγ in Pparγ+−/− MEFs, which leads to activation of adipogenesis, including C/EBPα expression following exposure to a PPARγ ligand. In contrast, C/EBPβ is not able to induce C/EBPα expression or adipogenesis in Pparγ−−/− MEFs. Chromatin immunoprecipitation analysis reveals that C/EBPβ is bound to the minimal promoter of the C/ebpα gene in association with HDAC1 in unstimulated Swiss-LAP cells. Exposure of the cells to a PPARγ ligand dislodges HDAC1 from the proximal promoter of the C/ebpα gene, which involves degradation of HDAC1 in the 26 S proteasome. These data suggest that C/EBPβ activates a single unified pathway of adipogenesis involving its stimulation of PPARγ expression, which then activates C/EBPα 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/EBPγ and PPAR families of transcription factors. In fact, it is now well accepted that both PPARγ and C/EBPα 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 PPARγ and C/EBPα 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 PPARγ 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 PPARγ and C/EBPα. Several investigators have demonstrated a direct role for C/EBPβ along with C/EBPα in inducing expression of PPARγ through association with C/EBP regulatory elements in the Pparγ gene promoter (9–12). A similar role for these C/EBPs in inducing transcription of the C/ebpα gene has not been established. It is generally assumed, however, that C/EBPβ does induce C/EBPα based on data from in vitro assays showing transactivation of a C/ebpα minimal promoter reporter gene in different cell types by C/EBPβ (13–15). Earlier studies aimed at characterizing the importance of C/EBPβ in inducing adipogenesis failed to demonstrate induction of C/EBPα expression because the NIH-3T3 fibroblasts used in the experiments do not express C/EBPα (10, 16). In those studies, however, ectopic expression of C/EBPβ was capable of inducing PPARγ2 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/EBPα (6). Other studies performed in cells capable of expressing C/EBPα have demonstrated that PPARγ can activate transcription of the C/ebpα gene in the absence of ongoing protein synthesis (17), suggesting that the transcriptional cascade responsible for initiating terminal adipogenesis involves induction of PPARγ2 by C/EBPβ, and PPARγ2 then is responsible for inducing C/ebpα 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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1 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 effect(s) disrupts the repressors to facilitate recruitment of appropriate coreactivators. In the case of C/EBPβ, there is evidence to suggest that it can dock on the promoters of C/ebpα and Pparg 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/EBPβ on the Pparg gene (18), whereas glucocorticoids are responsible for dislodging an mSin3a-HDAC1 complex from the C/EBPβ site on the C/ebpα gene (15). In an attempt to define the role of PPARγ along with C/EBPβ in regulating C/EBPα expression during adipogenesis, we ectopically expressed each of the proteins in nonadipogenic fibroblasts and analyzed adipogenic gene expression. The data demonstrate that C/EBPβ is capable of docking on the C/ebpα gene promoter but is incapable of inducing C/EBPα transcription in the absence of PPARγ. Furthermore, treatment of cells expressing C/EBPβ with glucocorticoids is not capable of inhibiting HDAC1 activity at the C/ebpα gene, whereas activation of PPARγ facilitates targeting of the HDAC1 that is associated with the C/ebpα gene to the transesome and thereby inducing transcription.

**EXPERIMENTAL PROCEDURES**

**Expression Vectors and Cell Lines**—The p34C/EBPβ(LAP)-pBI-G plasmid and pBabe-Puro-LAP and pRevTRE-C/EBPα-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) vector was transfected into Swiss mouse 3T3 fibroblasts constitutively resistant to 3 μg/ml puromycin (Swiss-LAP A cells; Clontech). The LAP-LAP-pBI-G, pBabe-Puro-LAP, and pRevTRE-C/EBPα 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 μg/ml puromycin were selected and analyzed for expression of the pBI-G vector on the basis of tetracycline-responsive β-galactosidase production. The initial selection gave rise to several nonhomogeneous colonies because only 10–20% of the cells expressed β-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 β-galactosidase activity. To establish retinovirus-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 μg of either the pRevTRE-C/EBPα-Myc-His or pBabe-Puro-LAP vector along with 2 μg each of vesicular stomatitis virus G and Gp expression plasmids (pVpack; Stratagene). Two days after transfection, culture medium containing high

**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 μM), 3-isobutyl-1-methylxanthine (MIX, 0.5 mM), and insulin (1.67 μM). 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 μM troglitazone (Parke-Davis) or 1 μM GW7845 (a highly selective PPARγ agonist) obtained from GlaxoSmithKline. To block PPARγ activity, cells were exposed to T0070907 (a selective PPARγ 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/EBPα, anti-C/EBPβ, anti-PPARγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); anti-HDAC1–3 (Upstate Biotechnology, Inc., Lake Placid, NY); anti-β-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 μg of the antibody or the same amount of a nonspecific mouse or rabbit IgG. The following day, 30-μl 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 μl 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 μg 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 MgCl₂, 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 μg of the following antibodies: anti-C/EBPβ, anti-C/EBPα, anti-acetylated histone H3, anti-acetylated histone H4, anti-HDAC1, anti-γIgG, and IgG (anti-acetylated histone H4, anti-HDAC1, anti-γIgG, 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/ebpα gene promoter and are as follows: sense, 5’-CTG GAA GTG GGT-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 H₂O; Oil Red O stock solution is 0.5% Oil Red O in isopropyl alcohol). The stain was then removed, and cells...
C/EBPα Expression Requires PPARγ

FIGURE 1. Inhibition of PPARγ activity blocks the induction of C/EBPα 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, the cells were exposed to DEX, MIX, insulin, and 10% FBS. At day 1 of differentiation, cells were treated with or without 10μM T0070907 (PPARγ antagonist) and harvested at the indicated times. Equal amounts of each protein sample were subjected to Western blot analysis using antibodies specific for C/EBPα, C/EBPβ, PPARγ, and perilipin.

RESULTS

To determine whether activation of C/EBPα expression during the early phase of adipogenesis requires PPARγ activity, we treated 3T3-L1 preadipocytes with T0070907 (a selective and potent antagonist of PPARγ activity) at day 1 following exposure of the confluent cells to the aponeurotic 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 PPARγ antagonist completely blocks expression of perilipin (Fig. 1, compare lane 9 with lane 10), a known downstream target of PPARγ (22–24), and also inhibits differentiation into mature adipocytes (data not shown). More important is the observation that blocking PPARγ activity also leads to a significant attenuation of C/EBPα expression. In fact, the antagonist appears to prevent the activation of C/EBPα 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 PPARγ2. This is likely because of the fact that C/EBPα normally feeds back on the Pparγ2 gene (7). There appears to be minimal effect of the PPARγ antagonist on C/EBPβ expression.

The fact that T0070907 is a specific inhibitor of PPARγ activity suggests that PPARγ contributes to the initial activation of C/EBPα during the differentiation of 3T3-L1 preadipocytes. Because our previous studies had shown that inhibition of C/EBPβ activity by ectopic expression of a dominant negative C/EBPβ protein (LIP) blocks both C/EBPα and PPARγ expression (17), we next addressed the role of C/EBPβ in regulating C/EBPα expression. Earlier studies have demonstrated that C/EBPβ is capable of inducing expression of PPARγ2 in NIH-3T3 fibroblasts (9, 16). Unfortunately, it was not possible to assess the role of C/EBPβ in activating C/EBPα expression because the NIH-3T3 cells do not transcribe the Cebpα gene (10, 16). To assess the contribution of both C/EBPβ and PPARγ to the induction of C/EBPα during adipogenesis, we ectopically expressed C/EBPβ 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/EBPβ 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 PPARγ agonist (GW7845) for 2 days at which time the cells were maintained in medium containing 10% FBS, insulin, and the corresponding quantity of the PPARγ agonist for an additional 3 days. Fig. 2 shows abundant expression of the ectopic C/EBPβ protein, which results in activation of PPARγ2 expression under all conditions except when cells were cultured in tetracycline to suppress the Cebpβ gene (Fig. 2, lanes 17 and 18). The ectopic C/EBPβ, in addition to activating PPARγ2, 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/EBPβ did not stimulate expression of C/EBPα in the absence of any of these inducers (Fig. 2, lanes 4, 6, 8, 10, 12, 14, and 16). Importantly, activation of PPARγ2 activity by treatment with the PPARγ agonist (GW7468) induces expression of C/EBPα 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 β-catenin and adipogenesis in these fibroblasts as observed previously (19, 25, 26). Taken together, these data suggest that activation of PPARγ2 expression only requires C/EBPβ, whereas expression of C/EBPα requires ligand-dependent activation of PPARγ along with C/EBPβ.

To substantiate further the importance of PPARγ in regulating C/EBPα expression, we stably expressed the p34 LAP isoform of C/EBPβ in mouse embryo fibroblasts lacking a functional Pparγ gene (Pγ−/− MEFs) and in a corresponding population of heterozygous (Pγ+/−) MEFs. The cell lines (Pγ+/− and Pγ−/−) 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/EBPβ induces some lipid accumulation in the MEFs that contain one functional allele of PPARγ (Pγ+/− cells) following exposure to DEX, MIX, and insulin, and this effect is enhanced manifold in the presence of the troglitazone. As expected, C/EBPβ failed to induce any lipid accumulation in the PPARγ-deficient cells (Pγ−/−) with or without troglitazone. The absence of adipogenesis in the Pγ−/− cells did not necessarily mean that C/EBPβ was not capable of inducing expression of C/EBPα in these cells because other studies have shown that C/EBPα is incapable of inducing lipid accumulation in the absence of PPARγ (20). Consequently, we analyzed expression of C/EBPα in response to C/EBPβ in both Pγ+/− and Pγ−/− cells. Fig. 4A shows...
abundant expression of the ectopic C/EBPβ in both cell lines. Treatment of the Pγ+/− cells with DEX, MIX, and insulin induces a low but detectable level of PPARγ, C/EBPα, perilipin, and adiponectin expression (Fig. 3), and exposure to the PPARγ agonist enhances expression of all four proteins to the abundant levels (lane 4) normally expressed in mature adipocytes. Most importantly, exposure of the PPARγ-deficient cells to DEX, MIX, and insulin with or without PPARγ ligand was incapable of stimulating C/EBPα, perilipin, or adiponectin even though the cells produced abundant quantities of the ectopic C/EBPβ protein (Fig. 4A, lanes 1 and 2). The Northern blot analysis of these cells demonstrate that the absence of C/EBPα expression in Pparγ−/− 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/EBPβ activates a single unified pathway of adipogenesis (20) involving its induction of PPARγ, which then activates C/EBPα expression along with other terminal adipogenic programs. To confirm that the only defect in the Pparγ−/− MEFs was the lack of PPARγ, we retrovirally expressed PPARγ2 in these cells that already expressed the ectopic C/EBPβ and showed that its expression facilitated induction of C/EBPα along with the other markers of the adipogenic program (Fig. 4C).

Other studies have shown that C/EBPβ as well as C/EBPα can transactivate an ectopic C/ebpα promoter/reporter gene in cells lacking PPARγ 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/ebpα gene promoter with respect to association with C/EBPs as well as other factors in PPARγ-deficient cells. Pparγ+/− or Pparγ−/− MEFs ectopically expressing either C/EBPβ or C/EBPα were exposed to DEX, MIX, insulin, and the PPARγ ligand, GW7468, for 5 days, and ChIP analyses were performed using antibodies against C/EBPβ, C/EBPα, 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/ebpα gene promoter. Somewhat surprising was the observation that ectopic C/EBPβ and C/EBPα bound to the C/EBPα promoter in Pparγ−/− cells as well as the Pparγ+/− controls even though the gene appears to be transcriptionally inactive in the −/− 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/ebpα gene only occurs in the Pparγ−/− cells and is absent or below detection in cells lacking PPARγ activity (Pparγ−/−).

This PPARγ-associated acetylation of the C/EBPα 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 PPARγ by exposure to troglitazone has no significant effect on total cell HDAC1 or HDAC3.
C/EBPα Expression Requires PPARγ

Consequently, the increase in histone acetylation at the C/EBPα 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/ebpα 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 PPARγ ligand. Fig. 7 shows that HDAC1 is associated with the minimal promoter in cells that do not express C/EBPα to any significant extent (i.e. in the absence of troglitazone or C/EBPβ, Fig. 7, lanes 2, 4, or 5). Interestingly, activation of PPARγ by exposure to troglitazone results in stimulation of this promoter 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 PPARγ 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/EBPβ to the minimal promoter in cells that do not transcribe the C/ebpα gene as observed previously in Pγ−/− MEFs (Fig. 5). These data are consistent with other studies that suggest that C/EBPβ acts as a repressor of gene expression prior to its stimulation by select effectors (27). To determine whether the ectopic C/EBPβ 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/EBPβ as well as anti-PPARγ antibodies. Fig. 8, A and C, shows association of HDAC1 with complexes containing C/EBPβ, whereas HDAC3 appears to associate with PPARγ (Fig. 8B). Furthermore, exposure of the cells to troglitazone causes a decrease in the association of HDAC1 with C/EBPβ (Fig. 8, A and C) as well as HDAC3 with PPARγ (Fig. 8B). It is therefore conceivable that C/EBPβ exists in the nucleus in association with HDAC1 and can repress select target genes by docking on corresponding C/EBP-binding sites. Activation of PPARγ with a ligand dislodges its associated HDAC3 and leads to stimulation of C/EBPβ activity on the C/ebpα 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/EBPα 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/EBPβ induces PPARγ expression (lane 1) and that exposure to troglitazone activates C/EBPα expression as well as other adipogenic genes (lane 3). Treatment of cells with MG132 has no apparent effect on expression of the ectopic C/EBPβ but significantly attenuates its ability to induce PPARγ expression and almost completely eliminates C/EBPα expression. These data are consistent with the notion that targeted degradation of corepressors through the proteasome is required to facilitate tran-
description of the *C/ebpα* 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/ebpα* expression is proteasomal degradation of the HDAC1 associated with the *C/ebpα* 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/EBPα minimal promoter. Interestingly, inhibition of the proteasome with MG132 significantly attenuates the ability of an activated PPARγ (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 PPARγ activity by exposure of 3T3-L1 preadipocytes to a potent and selective PPARγ antagonist not only blocks terminal adipogenesis but also inhibits the induction of *C/EBPα* expression at the onset of the adipogenic process. These data suggest that activation of *C/EBPα* expression requires PPARγ activity. In support of this notion, we show that ectopic expression of *C/EBPβ* in Swiss mouse fibroblasts leads to the activation of PPARγ2 expression without any significant enhancement of *C/EBPα* expression. Induction of *C/EBPα* in these cells only occurs following exposure to a PPARγ ligand, which also leads to expression of other adipogenic genes, including perilipin. To further establish the need for PPARγ in activating *C/EBPα* expression, we employed PPARγ-deficient mouse embryo fibroblasts. The data show that ectopic expression of *C/EBPβ* in Pparγ+/- MEFs is capable of activating PPARγ2 expression and, following exposure to a PPARγ ligand, is also capable of inducing *C/EBPα* expression and terminal adipogenesis. In contrast, *C/EBPβ* is not capable of inducing *C/EBPα* expression or adipogenesis in PPARγ-deficient MEFs. Furthermore, it appears that *C/EBPβ* can associate with *C/EBP* regulatory elements within the promoter of the *C/ebpα* gene in PPARγ-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 PPARγ is the principal regulator of terminal adipogenesis and, as such, is responsible for inducing expression of *C/EBPα* (20). The mechanism responsible for activating *C/EBPα* transcription involves a PPARγ-associated dislodgment of HDAC1 from the *C/EBPα* minimal promoter and a corresponding degradation of the HDAC1 by the proteasome.
C/EBPα Expression Requires PPARγ

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

What are the mechanisms by which PPARγ regulates C/EBPα expression? The data in Figs. 5 and 7 demonstrate that C/EBPβ can bind to a C/EBP-regulatory element within the promoter of the C/ebp gene even when the gene is transcriptionally inactive. It appears, however, that activation of transcription only occurs in response to expression of PPARγ. The most likely mechanism would involve association of PPARγ 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/ebpα gene has not, as yet, identified a functional PPAR-response element. The possibility that PPARγ induces expression of other factors that interact with the upstream region of the gene are discounted by earlier studies showing that activation of C/EBPα expression in CH310T1/2 mouse fibroblasts by PPARγ occurs in the absence of ongoing protein synthesis (17). It is possible that PPARγ alters the structural state of the chromatin associated with the C/ebpα gene by mechanisms that do not require protein synthesis or direct binding of PPARγ to corresponding DNA elements. For instance, PPARγ 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 PPARγ 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/EBPβ by targeting HDAC1 within the corepressor complex for degradation through the 26S 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/EBPα. They also suggest that this event contributes to the activation of C/EBPα gene expression by C/EBPβ during the differentiation of 3T3-L1 cells. Our studies argue that such a glucocorticoid activation of C/EBPβ may be sufficient to induce PPARγ expression (9), but is insufficient for C/EBPα mRNA transcription in which case the 3T3-L1 cells require PPARγ (see Fig. 1).

Our data suggest, however, that the precise mechanisms regulating C/EBPα transcription might be quite complex. Specifically, Fig. 5 demonstrates that acetylation of histones within the core promoter depends on PPARγ 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/ebpα gene promoter in addition to the PPARγ ligand-associated dislodgment of HDAC1 from C/EBPα. For instance, acetylation of the histones may be regulated by PPARγ 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/EBPβ promoter, PPARγ 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/EBPβ 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/EBPα activates a single unified pathway of adipogenesis that involves induction of PPARγ leading to expression of C/EBPα as well as other adipogenic genes. Furthermore, they identify a mechanism by which PPARγ induces C/EBPα expression that involves dislodgment of HDAC1 from C/EBPα-associated complexes bound to the C/EBPα minimal promoter. This process appears to require a PPARγ-dependent degradation of HDAC1 by the 26S proteasome.

Acknowledgment—We are grateful to Dr Evan Rosen for generously providing the Pparγ+/− and Pparγ−/− mouse embryo fibroblasts.

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