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Induction of Peroxisome Proliferator-Activated Receptor γ during the Conversion of 3T3 Fibroblasts into Adipocytes Is Mediated by C/EBPβ, C/EBPδ, and Glucocorticoids

ZHIDAN WU,[1,2] NANCY L. R. BUCHER,[3] AND STEPHEN R. FARMER[1]*

Departments of Biochemistry[1] and Pathology,[3] Boston University School of Medicine, Boston, Massachusetts 02118, and Department of Chemistry, Boston University, Boston, Massachusetts 02215[2]

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The differentiation of 3T3 preadipocytes into adipocytes is accompanied by a transient induction of C/EBPβ and C/EBPδ expression in response to treatment of the cells with methylisobutylxanthine (MIX) and dexamethasone (DEX), respectively. In this report, we demonstrate that peroxisome proliferator-activated receptor γ (PPARγ) expression in 3T3-L1 preadipocytes is induced by MIX and DEX, suggesting that C/EBPβ and C/EBPδ may be involved in this process. Using a tetracycline-responsive expression system, we have recently shown that the conditional ectopic expression of C/EBPβ in NIH 3T3 fibroblasts (β2 cells) in the presence of DEX activates the synthesis of peroxisome PPARγ mRNA. Subsequent exposure of these cells to PPAR activators stimulates their conversion into adipocytes; however, neither the expression of C/EBPβ nor exposure to DEX alone is capable of inducing PPARγ expression in the β2 cell line. We find that unlike the case for 3T3 preadipocytes, C/EBPδ is not induced by DEX in these 3T3 fibroblasts and therefore is not relaying the effect of this glucocorticoid to the PPARγ gene. To define the role of glucocorticoids in regulating PPARγ expression and the possible involvement of C/EBPδ, we have established an additional set of NIH 3T3 cell lines expressing either C/EBPδ alone (δ23 cells) or C/EBPδ and C/EBPβ together (β/δ39 cells), using the tetracycline-responsive system. Culture of these cells in tetracycline-deficient medium containing DEX, MIX, insulin, and fetal bovine serum shows that the β/δ39 cells express PPARγ and aP2 mRNAs at levels that are almost equivalent to those observed in fully differentiated 3T3-L1 adipocytes. These levels are approximately threefold higher than their levels of expression in the β2 cells. Despite the fact that these β/δ39 cells produce abundant amounts of C/EBPβ and C/EBPδ (in the absence of tetracycline), they still require glucocorticoids to attain maximum expression of PPARγ mRNA. Furthermore, the induction of PPARγ mRNA by exposure of these cells to DEX occurs in the absence of ongoing protein synthesis. The δ23 cells, on the other hand, are not capable of activating PPARγ gene expression when exposed to the same adipogenic inducers. Finally, attenuation of ectopic C/EBPβ production at various stages during the differentiation process results in a concomitant inhibition of PPARγ and the adipogenic program. These data strongly suggest that the induction of PPARγ gene expression in multipotential mesenchymal stem cells (NIH 3T3 fibroblasts) is dependent on elevated levels of C/EBPβ throughout the differentiation process, as well as an initial exposure to glucocorticoids. C/EBPδ may function by synergizing with C/EBPβ to enhance the level of PPARγ expression.

The differentiation of preadipocytes into adipocytes is regulated by at least two families of transcription factors, the CCAAT/enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptors (PPARs), that are responsive to various adipogenic inducers, including fetal bovine serum (FBS), insulin, dexamethasone (DEX), methylisobutylxanthine (MIX), long-chain fatty acids, retinoids, and prostanoids (3–5, 10, 27, 32).

Three members of the C/EBP family, α, β, and δ, are expressed at specific times during adipogenesis in a manner that is consistent with a regulatory role for each protein during the differentiation process (3). C/EBPα appears to perform an important function in the activation and maintenance of the terminally differentiated phenotype (17, 22, 28). C/EBPβ and C/EBPδ are expressed at the onset of differentiation and are considered to regulate C/EBPα production (32).

The PPARs (PPARα, PPARγ, and PPARδ/Nuc1) are members of the nuclear hormone receptor superfamily, which includes receptors for the retinoid, thyroid, and steroid hormones (9, 12, 16). These PPAR proteins are expressed to various levels in many tissues, where they regulate the metabolism of lipids by activating the expression of key enzymes (15). For instance, activation of PPARα in the liver induces the proliferation of peroxisomes and associated enzymes, resulting in long-chain fatty acid metabolism via the β-oxidation cycle (8, 14). PPARγ is considered to play an important role in regulating adipogenesis, since it is expressed most abundantly in adipose tissue and is induced during the early phase of differentiation of both 3T3-L1 and 3T3-F442A preadipocytes (5, 26).

The most conclusive evidence that both C/EBPα and PPARγ play a critical role in regulating adipogenesis has come from studies that demonstrate the conversion of multipotential mesenchymal stem cells (NIH 3T3 fibroblasts) into adipocytes in response to the ectopic expression of each of these proteins (11, 27). Furthermore, when the two proteins are expressed together, they act synergistically to promote a level of adipogenesis in these fibroblasts equivalent to that observed in the well-established 3T3 preadipocyte cell lines (27). In this regard, it is important to note that two genes associated with the terminally differentiated state of adipocytes, the aP2 and phosphoenolpyruvate carboxykinase genes, contain regulatory ele-

* Corresponding author. Mailing address: Department of Biochemistry, Boston University School of Medicine, 80 E. Concord St., Boston MA 02118. Phone: (617) 638-4186. Fax: (617) 638-5339.

ments within their promoters and/or enhancers which can be transactivated by C/EBPs and PPARs (7, 20, 25, 26). It is possible, therefore, that C/EBPα and PPARγ represent two arms of a network of transcription factors which are capable of establishing the terminally differentiated state of adipocytes.

Recent studies by Yeh et al. suggest that the early induction of C/EBPβ and C/EBPδ provides an important catalytic function during the differentiation process by relaying the effects of MIX and DEX in a cascade-like fashion to activate terminal differentiation (32). Consistent with this notion, we have recently shown that the conditional ectopic expression of C/EBPβ in NIH 3T3 cells exposed to DEX activates the synthesis of PPARγ mRNA, and subsequent exposure of these cells to PPAR activators stimulates their conversion into adipocytes (31). Neither the overexpression of C/EBPβ nor treatment with DEX alone is sufficient to induce the expression of PPARγ, though when present together, these effectors act synergistically. Cao et al. have shown that treatment of 3T3-L1 preadipocytes with DEX induces the expression of C/EBPδ (3). It is possible, therefore, that the ability of DEX to enhance PPARγ expression in the NIH 3T3/C/EBPβ cells is due to its activation of C/EBPδ production, thereby facilitating the formation of C/EBPβ-C/EBPδ heterodimers. These heterodimeric complexes may be the prerequisite adipogenic transcriptional activators that initiate PPARγ and C/EBPα expression.

In these investigations, we demonstrate that the induction of PPARγ mRNA in 3T3-L1 preadipocytes is enhanced manyfold by exposure of the cells to DEX. To define the role that glucocorticoids play in regulating PPARγ expression and the possible involvement of C/EBPδ, we have established a set of NIH 3T3 cell lines expressing either C/EBPδ alone or C/EBPδ and C/EBPβ together, using the tetracycline-responsive expression system. We show that the simultaneous overexpression of both C/EBPδ and C/EBPβ in cells exposed to DEX results in an extensive induction of PPARγ mRNA production which is several times greater than that induced by C/EBPβ alone. In contrast, ectopic expression of C/EBPδ alone is incapable of activating PPARγ mRNA expression, even in the presence of a cocktail of adipogenic inducers including DEX, MIX, insulin, and FBS. We further show that the induction of PPARγ in the cells expressing both C/EBPβ and C/EBPδ is enhanced manyfold by glucocorticoids and occurs in the absence of ongoing protein synthesis. Our observations suggest that C/EBPβ, C/EBPδ, and glucocorticoids play a direct role in initiating the early phase of adipogenesis through the induction of PPARγ.

MATERIALS AND METHODS

Plasmid constructions and stable transfections. Tetracycline transactivator (tTA) (pUHD15-1) and tetracycline operator-cytomegalovirus promoter (pUHD10-3) expression plasmids were obtained from H. Bujard, ZFMB, University of Heidelberg, Heidelberg, Germany (13). The tTA-dependent C/EBPδ expression vector was generated by ligating 1 kb of C/EBPδ cDNA from an *Eco*RI-*Bam*HI double digest of the MSV-C/EBPδ plasmid (provided by S. McKnight, Tularik, Inc., South San Francisco, Calif.) to the *Eco*RI-*Bam*HI sites within the polylinker region of pUHD10-3. The tTA-dependent C/EBPβ expression vector was constructed as described previously (31). Transcription of C/EBPβ and C/EBPδ mRNAs from the resultant plasmids, Tet-O-C/EBPβ and Tet-O-C/EBPδ, is driven by the cytomegalovirus promoter containing seven repeats of the tetracycline operator upstream of the C/EBPβ or C/EBPδ cDNA in response to coexpression of tTA from the pUHD15-1 plasmid.

The NIH 3T3 β2 cell line, which expresses C/EBPβ in a tetracycline-dependent manner, was described previously (31). The inducible C/EBPδ and C/EBPβ/δ cell lines were generated by transfecting the Tet-O-C/EBPδ expression vector either alone or in combination with the Tet-O-C/EBPβ vector, along with a pBabe-puromycin plasmid, into the tTA NIH 3T3 cell line (transfected with the tTA vector alone). Transfection was performed with LipofectAMINE as outlined by the supplier (GIBCO BRL, Life Technologies, Inc., Gaithersburg, Md.). The transfected cells were selected in Dulbecco's modified Eagle medium (DMEM; GIBCO BRL) containing 10% FBS, 0.4 mg of G418 (GIBCO BRL) per ml, 1.5 μg of puromycin (Sigma) per ml, and 1 μg of tetracycline per ml for 10 to 14 days. The resistant clones were subsequently isolated and propagated in cell lines. These stable cell lines were screened by Northern (RNA) blot analysis to identify clones that expressed abundant quantities of C/EBPδ mRNA (δ23) or C/EBPδ and C/EBPβ mRNAs (β/δ39) in response to the withdrawal of tetracycline.

Cell culture. 3T3-L1 preadipocytes were maintained in growth medium consisting of DMEM containing 10% normal calf serum (Intergen, Co., Purchase, N.Y.) and were induced to differentiate as described previously (24). The NIH 3T3 stable cell lines (β2, δ23, and β/δ39) were induced to undergo adipogenesis by being grown to ~50% confluence in DMEM containing 10% FBS and 1 μg of tetracycline per ml. Tetracycline was then withdrawn from the medium (to induce C/EBPβ and C/EBPδ expression), and the cells were allowed to grow for an additional 48 to 72 h in the absence of tetracycline, during which time they reached confluence. At this stage, designated day 0, adipogenesis was induced by refeeding with fresh medium containing 10% FBS, 1 μM DEX, 0.5 mM MIX, and 5 μg of insulin per ml for 48 h. The cells were subsequently maintained in 10% FBS–5 μg of insulin per ml–50 μM 5,8,11,14-eicosatetraynoic acid (ETYA; Sigma), or as indicated, and were refed every 2 days.

RNA analysis. Total RNA was isolated from the NIH 3T3 cell lines and the 3T3-L1 cells as described by Chomczynski and Sacchi (6). Cells were washed twice with ice-cold phosphate-buffered saline and lysed with solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol). The lysate was extracted with acidic phenol-chloroform, and then subjected to an overnight isopropanol precipitation at −20°C. RNA samples were analyzed by Northern blot hybridization as described previously (21). Probes were labeled by random priming using the Klenow fragment of DNA polymerase I (Promega) and [α-^{32}P]dCTP (DuPont, NEN Research Products, Boston, Mass.). cDNAs used as probes were as follows: C/EBPα, C/EBPβ, and C/EBPδ (3), 422/aP2 (2, 23), and PPARγ (1).

Electrophoretic mobility shift assays. DNA binding assays were performed as described previously (21). Ten micrograms of nuclear extract was incubated with 3 μg of poly(dI-dC), 2 μl of carrier mix (50 mM $MgCl_2$, 340 mM KCl), and delta buffer (0.1 mM EDTA, 40 mM KCl, 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6], 8% Ficoll, 1 mM dithiothreitol) at 4°C for 15 min. Then [γ-^{32}P]dATP-radiolabeled double-stranded oligonucleotide (50,000 cpm) was added to the reaction mixture, which was incubated for 30 min at 4°C. For supershift assays, the appropriate antibody was added to the nuclear extract and incubated at room temperature for 1.5 h before addition of the probe. The anti-C/EBPβ and anti-C/EBPδ antibodies were obtained from Santa Cruz Biotechnology. Binding of the oligonucleotide to specific proteins was determined by fractionating the nuclear proteins through a nondenaturing 6% polyacrylamide gel at 200 V for 2.5 h at 4°C in TBE buffer (80 mM Tris-borate, 2 mM EDTA [pH 8.0]). The gel was dried at 80°C for 1 h before exposure to X-Omat autoradiography film. The sequence of the C/EBP oligonucleotide used in this study is 5′-gatccGCGTTGCGCCACGATG-3′ (21). The double-stranded oligonucleotide was end labeled by T4 DNA polynucleotide kinase (New England Biolabs) and [γ-^{32}P]dATP (DuPont).

RESULTS

Effect of adipogenic inducers on PPARγ mRNA expression during the conversion of 3T3-L1 preadipocytes into adipocytes. Confluent 3T3-L1 preadipocytes cultured in medium containing 10% FBS were exposed to various effectors for 48 h and then maintained in medium containing only FBS and insulin for an additional 48 h. Total RNA was isolated and subjected to Northern blot analysis as shown in Fig. 1A. Cells maintained in insulin for the entire 4-day culture period express a moderate level of PPARγ mRNA but virtually undetectable levels of C/EBPα and aP2 mRNAs (lane 1). Exposure of the cells to MIX in the presence or absence of insulin during the initial 48 h has little effect on the basal level (insulin alone) of PPARγ expression, although this treatment does induce aP2 mRNA (lanes 2 and 4). In contrast, culture of the preadipocytes in the presence of DEX results in a significant induction of PPARγ and aP2 expression (lanes 3 and 5), which is further enhanced when DEX and MIX are present together (lanes 6 and 7).

To assess the specificity of the DEX effect, preadipocytes were also exposed to triamcinolone acetonide (a glucocorticoid agonist) and estradiol-17β (a nonglucocorticoid steroid) in the presence of insulin and MIX. Exposure to triamcinolone acetonide results in an extensive induction of PPARγ, C/EBPα, and aP2 mRNA expression (Fig. 1A, lane 8) compared with

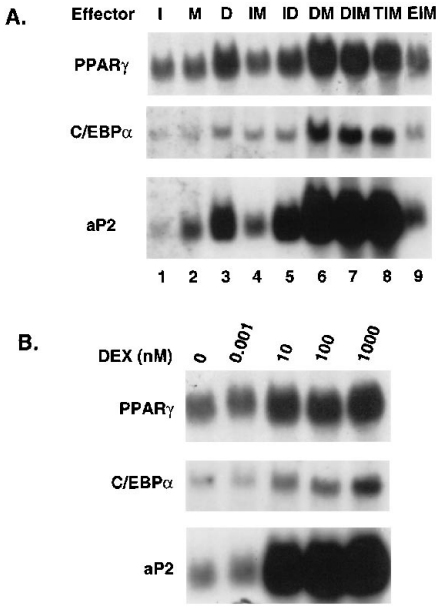

FIG. 1. (A) Induction of adipogenic genes by different effectors during the differentiation of 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were exposed to various combinations of effectors for 48 h as indicated in DMEM containing 10% FBS. The cells were then maintained in medium containing 10% FBS and 2.5 μg of insulin per ml for an additional 48 h. Twenty micrograms of total RNA isolated from the individual cultures was analyzed by Northern blot hybridization using the following ^{32}P-labeled cDNA probes: C/EBPα, PPARγ, and aP2. The effectors used were insulin (I; 10 μg/ml), DEX (D; 1 μM); MIX (M; 0.5 mM); triamcinolone acetonide (T; 1 μM); and β-estradiol (E; 1 μM). (B) DEX dose-dependent induction of PPARγ mRNA in 3T3-L1 preadipocytes. Confluent 3T3-L1 preadipocytes were treated with various concentrations of DEX as indicated in DMEM containing 10% FBS and insulin (10 μg/ml) for 48 h. The cells were then maintained in DMEM with 10% FBS and insulin (2.5 μg/ml) for an additional 48 h. Total RNA (20 μg) was subjected to Northern blot analysis as described in Materials and Methods.

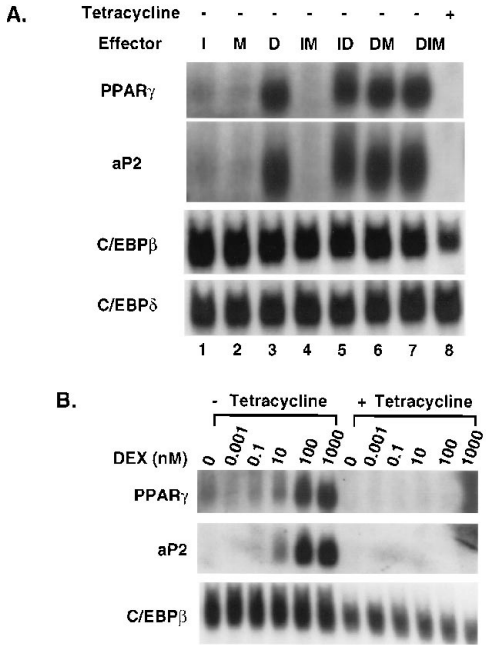

FIG. 2. (A) Induction of PPARγ and aP2 mRNAs by ectopic expression of C/EBPβ in NIH 3T3 β2 cells exposed to DEX and other adipogenic inducers. Cells were grown to confluence in DMEM containing 10% FBS, exposed to a combination of different effectors for 48 h, and then maintained in medium containing 10% FBS, ETYA (50 μM), and insulin (5 μg/ml) for an additional 4 days. Tetracycline was present in the control culture as indicated. Twenty micrograms of total RNA extracted from the different cultures was subjected to Northern blot analysis using ^{32}P-labeled cDNA probes corresponding to C/EBPβ, C/EBPδ, PPARγ, and aP2 mRNAs. The effectors were insulin (I; 5 μg/ml); DEX (D; 1 μM); and MIX (M; 0.5 mM). (B) DEX dose-dependent induction of PPARγ in NIH 3T3 cells (β2 cells) ectopically expressing C/EBPβ. Confluent β2 cells grown in tetracycline-deficient medium with 10% FBS were exposed to insulin (5 μg/ml) and the indicated concentrations of DEX for 48 h; they were then maintained for an additional 4 days in medium containing only insulin and FBS. Control cells were exposed to tetracycline as indicated. Twenty micrograms of total RNA isolated from each culture was analyzed by Northern blot hybridization using the cDNA probes corresponding to PPARγ, aP2, and C/EBPβ mRNAs.

the level of expression observed in cells treated with insulin and MIX alone (lane 4). Treatment with estradiol-17β, on the other hand, has no effect on the adipogenic gene program (lane 9).

To determine whether the glucocorticoid-induced changes in PPARγ mRNA are consistent with the involvement of a glucocorticoid receptor, we cultured cells in insulin and FBS with various concentrations of DEX for 48 h and then cultured them for an additional 2 days in medium containing only insulin and FBS. Figure 1B shows that the dose of DEX required to promote maximal expression of PPARγ mRNA is approximately 10 nM, which is within the range of the K_d for the association of DEX with the glucocorticoid receptor in 3T3-L1 cells (19).

Effects of glucocorticoids on the adipogenic gene program in NIH 3T3 cells that ectopically express C/EBPβ. In 3T3-L1 preadipocytes, the synergistic effect of glucocorticoids (DEX or triamcinolone acetonide) and MIX on PPARγ mRNA expression may be due to the effects of these agonists on C/EBPδ and C/EBPβ, respectively (3). To investigate this possibility, we have used lines of undifferentiated NIH 3T3 cells which do not express PPARγ but which we have modified to ectopically express various C/EBPs, using a tightly controlled tetracycline-responsive expression system. In a selected line that ectopically produces C/EBPβ (β2 cells), we have recently shown that induction of C/EBPβ to a required threshold level by culture in tetracycline-depleted medium activates PPARγ expression and that subsequent addition of a PPAR activator (in this instance ETYA, a synthetic analog of arachidonic acid) results in further differentiation of these β2 cells into adipocytes (31). To examine more critically the effect of various adipogenic inducers on the differentiation of these cell lines, we cultured the β2 cells in medium deprived of tetracycline in order to activate C/EBPβ and analyzed the expression of selected mRNAs by Northern blot hybridization. Treatment of the cells with insulin and/or MIX (Fig. 2A, lanes 1, 2, and 4) has no effect on either PPARγ or aP2 mRNA expression. In contrast, exposure of the cells to DEX alone or in the presence of insulin and/or MIX (lanes 3, 5, 6, and 7) results in an extensive induction of PPARγ mRNA expression. The observation that exposure to DEX and MIX appears to have no additional effect on PPARγ mRNA expression compared with exposure of these β2 cells to DEX alone contrasts with the additive effect of these inducers on PPARγ expression seen in 3T3-L1 cells (Fig. 1A). This difference is probably due to the fact that since the β2 cells overexpress exogenous C/EBPβ, they may not require MIX to enhance the expression of the endogenous C/EBPβ gene as would be the case in the 3T3-L1 preadipocytes. The conditions that enhance PPARγ mRNA production also result in the activation of the adipogenic program, as illustrated here by the expression of aP2 mRNA. Figure 2B shows that the concentration of DEX required to induce PPARγ and aP2 expression

FIG. 3. The level of C/EBPδ expression is not altered by the treatment of β2 cells with DEX. β2 cells were grown to confluence in the presence or absence of tetracycline, at which time they were treated with DEX and insulin in DMEM containing 10% FBS for 48 h and then maintained in DMEM with insulin and 10% FBS. Total RNA and nuclear proteins were isolated from each indicated time point as described in the Materials and Methods. (A) Twenty micrograms of RNA was analyzed by Northern blot hybridization as described in the legend to Fig. 1. Ten micrograms of nuclear extract from each time point was subjected to supershift electrophoretic mobility shift assay using a radiolabeled C/EBP oligonucleotide probe (B). Anti-C/EBPβ and anti-C/EBPδ antibodies were used in this supershift assay.

in the presence of elevated levels of C/EBPβ is 100 nM. This dose is somewhat higher than that required to initiate adipogenesis in 3T3-L1 cells.

As noted earlier, the function of DEX in regulating adipogenesis in both the β2 fibroblasts and the 3T3-L1 preadipocytes may be to increase the abundance of C/EBPδ, which is then capable of forming heterodimeric complexes with the overexpressed C/EBPβ. These heterodimers may be the principal activators of PPARγ transcription as well as other adipogenic genes, such as the aP2 gene, which contain C/EBP regulatory elements within their promoters. To investigate this possibility, we analyzed C/EBPδ mRNA expression and DNA binding activity at the indicated times following exposure of the β2 cells to DEX in tetracycline-depleted medium. As shown in Fig. 3A, however, we find no change in the abundance of C/EBPδ mRNA throughout the 96-h period following treatment with the glucocorticoid. To test the possibility that DEX enhances the synthesis of functional C/EBPδ protein without inducing mRNA production, we also performed gel mobility supershift assays on nuclear extracts isolated from the β2 cells at times following exposure to the glucocorticoid. Figure 3B shows that all of the C/EBP complexes that are capable of binding to a consensus C/EBP oligonucleotide contain C/EBPβ, since all of the C/EBP binding activity can be supershifted with the anti-C/EBPβ antibody. Furthermore, the abundance of these C/EBP complexes remains fairly constant throughout the 96-h treatment period. In contrast, there is a very low level of C/EBPδ binding activity in the untreated cells (Fig. 3B, lane 7), which increases only modestly at 4 h following exposure of the cells to DEX (the pertinent controls of the supershift assays with anti-C/EBPβ and anti-C/EBPδ antibodies are shown in Fig. 4B). These data suggest that C/EBPδ is not directly relaying the effect of DEX in the β2 cell line.

Roles of C/EBPδ and C/EBPβ in activating PPARγ gene expression in NIH 3T3 cells. Although DEX fails to activate the C/EBPδ gene in NIH 3T3 cells, it nevertheless seems likely that expression of this gene does provide a necessary function during the differentiation of preadipocytes. To assess the importance of C/EBPδ in activating PPARγ expression, we established an additional set of NIH 3T3 cell lines expressing C/EBPδ alone (δ23 cells), or C/EBPβ and C/EBPδ together (β/δ39 cells), under the control of the tetracycline-responsive system. Figure 4A shows a Northern blot analysis of RNA isolated from the δ23 and β/δ39 cells cultured in the presence or absence of tetracycline. Analysis of the original β2 cell line is also shown for comparison. All three cell lines express abundant amounts of the respective mRNAs from the exogenous cDNA vector in response to tetracycline withdrawal from the culture medium. The level of induction of C/EBPδ in the δ23 and β/δ39 cells and C/EBPβ in the β/δ39 cells is comparable to that of C/EBPβ mRNA expression in the β2 cells, which is approximately three- to fivefold above the endogenous level. To determine whether these ectopically expressed mRNAs produce functional proteins, we performed a series of gel mobility supershift assays using nuclear proteins isolated from each cell line maintained in tetracycline-depleted medium. Figure 4B shows that control fibroblasts which do not produce any exogenous C/EBP proteins (i.e., tTA cells, which are transfected with the tTA vector alone) express a low but measurable level of C/EBP DNA binding activity that may be accounted for by the presence of endogenous complexes containing both C/EBPβ and C/EBPδ, as indicated by the anti-C/EBPβ and anti-C/EBPδ antibody supershifts. As shown previously, the β2 cells express predominantly C/EBPβ homodimers, with very little C/EBPδ complexes present. In contrast, the δ23 cells show significantly higher levels of C/EBPδ DNA binding activity, as expected for cells expressing abundant amounts of C/EBPδ mRNA (Fig. 4A). It is interesting that these fibroblasts, which were transfected with only the C/EBPδ expression vector, also demonstrate significant C/EBPβ DNA binding activity compared with the control tTA cells. This finding suggests that the ectopically expressed C/EBPδ protein is capable of transactivating the endogenous C/EBPβ gene to some extent. The β/δ39 cells express abundant amounts of both C/EBPβ and C/EBPδ DNA binding activity, which is consistent with the dual overexpression of the corresponding mRNAs. It appears that while all of the C/EBP dimers produced by these β/δ39 cells contain C/EBPβ, as shown in the supershift of the entire C/EBP DNA binding activity by the anti-C/EBPβ antibody, not all of them contain C/EBPδ, since the anti-C/EBPδ antibody is able to supershift only a fraction of the binding activity. Nevertheless, these cells display significantly more C/EBPδ DNA binding activity than either the control tTA cells or the β2 cells, which indicates that the exogenous cDNA is functional in these cells. It appears, therefore, that the β2 cells contain predominantly C/EBPβ homodimers, the δ23 cells contain C/EBPδ homodimers as well as C/EBPβ/C/EBPδ heterodimers, and the β/δ39 cells contain abundant amounts of both of these homodimers and heterodimers.

We have previously shown that the level of PPARγ mRNA expression is directly related to the level of C/EBPβ DNA binding activity in the β2 cells, which produce predominantly C/EBPβ homodimers (31). To determine whether other C/EBP complexes can activate PPARγ mRNA expression, we induced C/EBP expression in the cell lines by growing the cells to confluence in medium lacking tetracycline. These cells were then exposed to DEX, MIX, insulin, and FBS for 48 h and

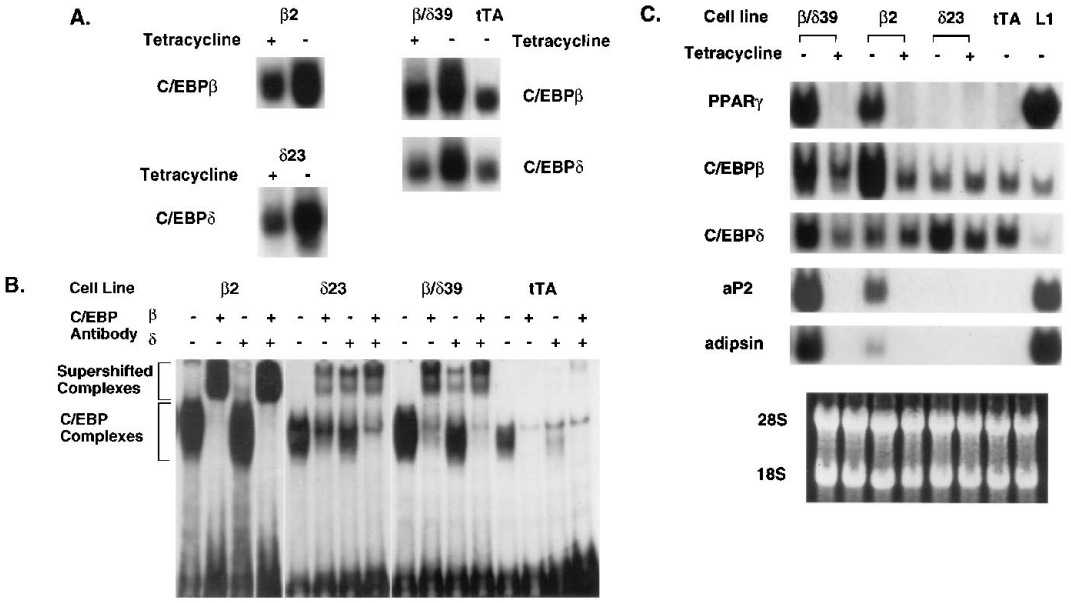

FIG. 4. Ectopic expression of C/EBPβ or of C/EBPδ and C/EBPβ together can induce PPARγ in NIH 3T3 cells, whereas C/EBPδ alone is incapable of activating PPARγ expression. (A) Tetracycline-dependent expression of C/EBPβ, C/EBPδ, and C/EBPβ/δ mRNAs in NIH 3T3 β2, δ23, and β/δ 39 stable cell lines, respectively. Cells were grown in the presence or absence of tetracycline as indicated for 48 h. Total RNA was isolated from each culture, and 20 μg was subjected to Northern blot hybridization. (B) Analysis of C/EBP binding activities in the stable NIH 3T3 fibroblast cell lines. Cells were cultured in the absence of tetracycline. Nuclear proteins were extracted, and 10 μg of each extract was subjected to electrophoretic mobility shift assay using a radiolabeled C/EBP oligonucleotide probe. For the supershift assay, each extract was incubated with the appropriate anti-C/EBP antibody prior to addition of the probe. (C) Initiation of PPARγ, aP2, and adipsin mRNAs in response to overexpression of particular C/EBPs in the NIH 3T3 cell lines. Postconfluent β2, δ23, β/δ 39, and tTA cells were cultured in the presence or absence of tetracycline prior to being exposed to DEX, MIX, and insulin for 48 h. This initial stimulation was followed by maintenance in medium containing insulin, ETYA, and FBS for an additional 4 days, again in the presence or absence of tetracycline. Total RNA was extracted, and 25 μg of each RNA was subjected to Northern blot analysis as described in Materials and Methods. L1, 3T3-L1 adipocytes. The RNA gel stained with ethidium bromide was included as a loading control.

subsequently maintained in medium containing FBS, insulin, and ETYA for an additional 4 days. Total RNA was extracted at this stage and analyzed on Northern blots as shown in Fig. 4C. The β/δ39 and β2 cells express abundant amounts of PPARγ and aP2 mRNAs in the absence of tetracycline, whereas the δ23 and tTA cells do not express either of these adipocyte-specific genes. Indeed, the level of expression of these mRNAs in the β/δ39 cells is almost equivalent to that expressed in fully differentiated 3T3-L1 cells, which is approximately threefold greater than the level measured in the β2 cells. These data suggest that together, the C/EBPβ-C/EBPδ heterodimers and C/EBPβ homodimers which form in the β/δ39 cells are significantly more potent activators of PPARγ transcription than the C/EBPβ homodimers alone which are present in the β2 cells. Furthermore, it is interesting that the level of expression of the C/EBPβ-C/EBPδ complexes observed in the δ23 cells is incapable of activating PPARγ, aP2, and adipsin mRNA expression even though the abundance of C/EBPδ mRNA in these cells is as high as it is in the β/δ39 cells when cultured in tetracycline-deprived medium. Accordingly, it appears that overexpression of C/EBPβ, either alone or in combination with C/EBPδ, is required for the induction of PPARγ mRNA expression.

NIH 3T3 cells that express abundant amounts of both C/EBPβ and C/EBPδ still require DEX to induce PPARγ mRNA expression. The abundant expression of PPARγ mRNA observed in the β/δ39 cells compared with the level seen in the β2 cells suggests that C/EBPδ contributes to the activation of the PPARγ gene. It is possible, therefore, that the effect of DEX in promoting the differentiation of 3T3-L1 cells depends primarily on the induction of C/EBPδ. It is also possible, however, that DEX activates transcription of the PPARγ gene in synergy with C/EBPβ by interacting with a glucocorticoid receptor. If the only role of this glucocorticoid is to enhance C/EBPδ production, then maximum expression of PPARγ mRNA in the β/δ39 cells should occur in the absence of DEX. The Northern blot analysis in Fig. 5 shows that overexpression of both C/EBPβ and C/EBPδ in the β/δ39 cells cultured in tetracycline-deficient medium containing FBS and insulin only modestly enhances PPARγ mRNA expression (compare lanes 1 and 8). Exposure of these cells to DEX (lane 3), however, results in an extensive induction of PPARγ mRNA to levels that are almost equivalent to those measured in fully differentiated 3T3-L1 adipocytes (data not shown). It is interesting that treatment with MIX and insulin can also activate PPARγ mRNA production, though to a level significantly lower than that observed in the DEX-treated cells. In addition, triamcinolone acetonide is also a potent activator of PPARγ, aP2, and adipsin expression in these cells (lane 9), whereas estradiol-17β is incapable of inducing the adipogenic program (lane 10). As discussed previously regarding 3T3-L1 cells, the effect of DEX on the differentiation of the β/δ39 cells can also be attributed solely to its glucocorticoid activity rather than to some nonspecific steroidal property.

Induction of PPARγ mRNA by DEX in β/δ39 cells is independent of ongoing protein synthesis. The data presented in Fig. 5 suggest that DEX enhances PPARγ mRNA expression in the β/δ39 cells by a mechanism separate from that involving the activation of C/EBPδ. It is possible that DEX induces the production of another transcription factor that cooperates with the C/EBPβ-C/EBPδ heterodimers to initiate PPARγ expression, rather than itself acting on the PPARγ gene. To address whether the action of DEX is dependent on the synthesis of an additional regulatory protein, we treated the β/δ39 cells with

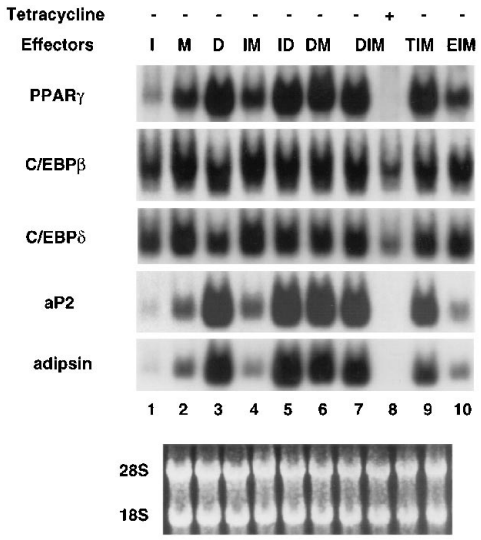

FIG. 5. Maximum expression of PPARγ in NIH 3T3 cells expressing C/EBPβ and C/EBPδ (β/δ39) is still dependent on DEX. Confluent β/δ39 cells were exposed to different effectors for 48 h and subsequently maintained in medium containing FBS, insulin, and ETYA for an additional 4 days. Tetracycline (1 μg/ml) was present in the control culture as indicated. Total RNA was isolated at day 6 from each culture, and 20 μg was analyzed by Northern blot hybridization using ^{32}P-labeled cDNA probes for PPARγ, aP2, adipsin, C/EBPβ, and C/EBPδ. I, insulin (5 μg/ml); D, DEX (1 μM); M, MIX (0.5 mM); T, triamcinolone acetonide (1 μM); E, β-estradiol (1 μM). The RNA gel stained with ethidium bromide was included as a loading control.

DEX in the presence or absence of cycloheximide, an inhibitor of protein synthesis, and measured the production of PPARγ mRNA by Northern blotting. In the experiment shown in Fig. 6, confluent β/δ39 cells cultured in FBS were treated with 5 μg of cycloheximide per ml for 1 h and then exposed to either insulin and cycloheximide or insulin, DEX, and cycloheximide for the indicated times; control cells were treated with insulin and DEX in the absence of cycloheximide. Exposure of confluent β/δ39 cells to DEX and insulin in the presence of ongoing protein synthesis induces PPARγ mRNA within the initial 4 h. Maximum levels do not accumulate until 24 h posttreatment, although a transient burst of C/EBPδ mRNA

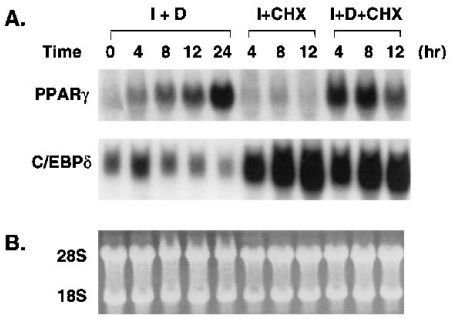

FIG. 6. Induction of PPARγ by DEX in NIH 3T3 (β/δ39) cells expressing both C/EBPβ and C/EBPδ is independent of ongoing protein synthesis. (A) Confluent β/δ39 cells cultured in the absence of tetracycline were treated with 5 μg of cycloheximide per ml for 1 h and then exposed to either insulin and cycloheximide (I+CHX) or insulin, DEX, and cycloheximide (I+D+CHX) for the indicated times. Control cells were treated with insulin and dexamethasone in the absence of cycloheximide (I+D). Total RNA was extracted from each culture, and 25 μg of each sample was subjected to Northern blot analysis. (B) The RNA gel stained with ethidium bromide.

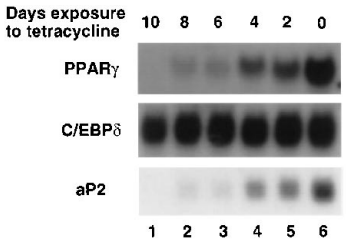

FIG. 7. Induction of PPARγ is dependent on the expression of C/EBPβ through the entire differentiation process. β2 cells were grown in tetracycline-free medium to activate C/EBPβ expression and initiate differentiation. When the cells reached confluence, PPARγ expression was induced by exposing the cells to DEX, MIX, and insulin in DMEM with 10% FBS for 48 h. The cells were then maintained in medium containing FBS, insulin, and ETYA for an additional 6 days. Tetracycline was added back to the cultures at the indicated times prior to the termination of the experiment on day 10. Twenty micrograms of total RNA from each culture was analyzed by Northern blot hybridization using ^{32}P-labeled cDNA probes for PPARγ, aP2, and C/EBPδ.

expression is observed 4 h posttreatment in these cells, which already overexpress exogenous C/EBPδ mRNA. Treatment of these β/δ39 cells with DEX and insulin in the presence of cycloheximide results in a rapid, robust activation of PPARγ mRNA expression that reaches a maximal level within 4 to 8 h posttreatment, whereas treatment with only insulin and cycloheximide has no effect on PPARγ mRNA production. The expression of C/EBPδ mRNA, however, is superinduced manyfold by exposure of the cells to cycloheximide in the presence or absence of DEX. This superinduction may be due to the loss of a labile repressor that regulates the expression of either the exogenous or the endogenous C/EBPδ gene.

Expression of PPARγ and aP2 mRNAs in NIH 3T3 cells is dependent on elevated levels of C/EBPβ DNA binding activity through the entire differentiation process. The data presented above demonstrate that both the enhanced expression of C/EBPβ DNA binding activity and exposure of NIH 3T3 cells to DEX are required to initiate PPARγ mRNA expression during the early phase of adipogenesis. Following the initial stimulus, however, DEX can be removed and the cells continue to undergo differentiation in the presence of FBS, insulin, and an appropriate PPAR activator. To determine whether elevated levels of C/EBPβ DNA binding activity are required throughout the differentiation process, we inhibited expression of the exogenous C/EBPβ gene at different stages of adipogenesis by reexposing the cells to tetracycline. In this experiment (Fig. 7), differentiation was initiated in subconfluent β2 cells by removing tetracycline from the growth medium. The cells were grown for an additional 2 days, during which time they reached confluence. At this stage, the cultures were exposed for 48 h to the adipogenic inducers DEX, MIX, and insulin in medium containing FBS in order to activate PPARγ gene expression. These activated cells were then cultured in medium containing 10% FBS, insulin, and ETYA for an additional 6 days to promote expression of the adipogenic gene program (as demonstrated by the expression of aP2 mRNA). To determine the effect of suppressing exogenous C/EBPβ production on the adipogenic gene program, tetracycline was restored to the culture medium at different times during this differentiation process. All the cultures were harvested on day 10 and analyzed for PPARγ and aP2 expression, as well as C/EBPβ DNA binding activity (data not shown). As demonstrated previously (31), β2 cells that are exposed to tetracycline for the entire 10-day period express minimal C/EBPβ DNA binding activity (31) and undetectable levels of the adipocyte-specific mRNAs (Fig. 7, lane 1). In contrast, when these cells

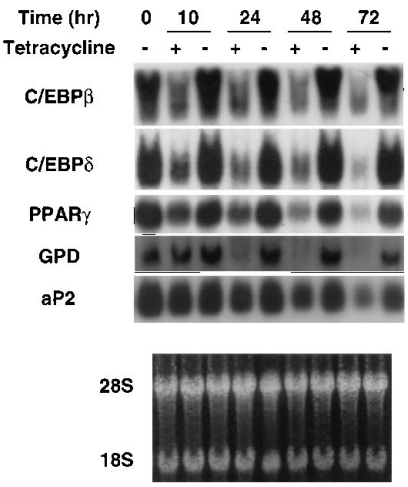

FIG. 8. The differentiation program is reversed by tetracycline, which inhibits the expression of C/EBPβ and C/EBPδ in β/δ39 cells. Postconfluent β/δ39 cells were induced to differentiate as described in Materials and Methods. At day 6 of differentiation (referred as 0 h), tetracycline (1 μg/ml) was added to half of the cultures, while the other half were maintained in tetracycline-free medium as a control. Total RNA was isolated from each culture at the indicated times after the addition of tetracycline. Twenty-five micrograms of total RNA from each sample was subjected to Northern blot analysis using ^{32}P-labeled cDNA probes for PPARγ, C/EBPβ, C/EBPδ, GPD, and aP2. The RNA gel stained with ethidium bromide is included as a loading control.

are cultured in the absence of the antibiotic for the entire time course, they express the exogenous C/EBPβ protein (31) and abundant quantities of PPARγ and aP2 mRNAs (lane 6). When these cells are exposed to tetracycline for progressively longer periods of time (2 to 8 days), there is a corresponding decrease in the abundance of the adipocyte-specific mRNAs (lanes 5 through 1, respectively). We have also found (data not shown) that the rate of turnover of the C/EBPβ mRNA and protein is extremely rapid such that the exogenous C/EBPβ present in tetracycline-deficient cells can be completely degraded within 48 h following reexposure to the antibiotic. Taken together, these data suggest that elevated levels of the exogenous C/EBPβ DNA binding activity are required throughout the differentiation of NIH 3T3 cells in order to attain maximal expression of the adipogenic genes and thereby express the fully differentiated phenotype.

Inhibition of the ectopically expressed C/EBPβ and C/EBPδ in fully differentiated β/δ39 cells results in a significant down-regulation of the adipogenic gene program. It is possible that upon reaching their fully differentiated state, NIH 3T3 cells no longer require a constant elevated expression of C/EBPβ to maintain the adipogenic gene program. To assess the role of the C/EBPs in regulating the terminal stages of adipogenesis in these fibroblasts, the expression of both C/EBPβ and C/EBPδ in the β/δ39 cells which had been induced to differentiate for 6 days was inhibited by restoring tetracycline to the culture medium. The fact that following stimulation to differentiate, the β/δ39 cells express maximum levels of PPARγ, aP2, and adipsin by day 6 (unpublished data) indicates that they have reached their fully differentiated state. In the experiment shown in Fig. 8, tetracycline (1 μg/ml) was added to half of the cultures at day 6 (referred as 0 h), while the other half were maintained in tetracycline-free medium. Total RNA was isolated from the cultures at the indicated times and subjected to Northern blot analysis. Addition of tetracycline results in an extensive decrease in the abundance of the ectopically expressed C/EBPβ and C/EBPδ mRNAs within the initial 10 h compared with their abundance in the tetracycline-free control cultures. By 72 h, the abundance of each of these mRNAs has returned to the levels expressed by the endogenous genes (data not shown), indicating that expression of the ectopic genes has been totally inhibited and the preexisting mRNA has turned over. The rate of decay of the PPARγ mRNA is almost identical to that of the C/EBP mRNAs, suggesting that the PPARγ gene is completely dependent on the elevated expression of C/EBPs and that the PPARγ mRNA has a short half-life. This extensive decrease in C/EBPβ, C/EBPδ, and PPARγ expression eventually results in the down-regulation of the adipogenic gene program, as indicated here by a decrease in the abundance of GPD and aP2 mRNAs within the 72-h period following the restoration of tetracycline to the culture medium. It is important to note that the drop in the levels of these two adipogenic mRNAs is not apparent until 24 h, at which time the expression of GPD decays at a much faster rate. These data are consistent with the model that the addition of tetracycline causes an inhibition of C/EBP expression which effectively shuts off the PPARγ gene within the first 10 h. PPARγ protein then decays during the following 14-h period, resulting in a suppression of the adipogenic genes, including the GPD and aP2 genes. Finally, these data show that expression of the adipogenic gene program in fully differentiated β/δ39 cells is completely dependent on the ectopic expression of C/EBPδ and/or C/EBPβ.

DISCUSSION

Activation of adipogenesis in 3T3-L1 preadipocytes is initiated by treating confluent cells with DEX and MIX in medium containing FBS and insulin. This process involves the sequential expression of transcription factors that play a role in regulating the adipogenic gene program (18, 30). The earliest known factors to be induced are C/EBPβ and C/EBPδ, which are observed during the initial 24 to 48 h, followed shortly thereafter by PPARγ. Studies by Yeh et al. indicate that both C/EBPβ and C/EBPδ are required to activate C/EBPα expression 3 to 4 days after stimulation of the cells to differentiate (32). The results reported here show that the induction of PPARγ gene expression during the conversion of multipotential mesenchymal stem cells (NIH 3T3) into adipocytes is dependent on both elevated levels of C/EBPβ and exposure of the cells to glucocorticoids. The role of C/EBPβ in this process is most likely facilitated by the presence of at least two C/EBP consensus binding sites in the proximal promoter region of the mouse PPARγ gene (33). The effects of the glucocorticoid appear to be mediated through a constitutively expressed glucocorticoid receptor, since they occur in the absence of ongoing protein synthesis (Fig. 6) and are specific to the glucocorticoid family of steroid hormone receptors (Fig. 5). Furthermore, the concentration dependence of these responses is within the range of that expected for the glucocorticoid receptor (10 nM). Since the limited sequence analysis of the mouse PPARγ gene has not yet identified a consensus sequence corresponding to the glucocorticoid response element, it is premature to discuss the precise molecular mechanisms responsible for the activation of PPARγ gene expression.

That glucocorticoids induce the expression of C/EBPδ during the differentiation of 3T3-L1 preadipocytes suggests that the primary role of these steroid hormones in committed adipoblasts is to enhance the levels of C/EBPδ in order for C/EBPβ-C/EBPδ heterodimers to form and subsequently induce PPARγ expression. The results presented in Fig. 1, however, do not distinguish a direct involvement of glucocorticoids in activating PPARγ mRNA production in these preadipocytes

from an indirect mechanism involving an increase in C/EBPδ. The higher level of PPARγ gene expression in NIH 3T3 β/δ39 cells than in NIH 3T3 β2 cells strongly suggests that C/EBPδ plays a mechanistic role in regulating PPARγ transcription during the conversion of these mesenchymal stem cells, but Fig. 4 demonstrates that the enhanced expression of C/EBPδ alone, without a corresponding enrichment in C/EBPβ, is an ineffective activator of PPARγ and the adipogenic program. Taken together, these data suggest that critical threshold levels of these C/EBP complexes need to be attained in order for PPARγ transcription to be activated. In addition, it seems likely that the preferred complexes contain a combination of C/EBPβ homodimers and C/EBPβ-C/EBPδ heterodimers.

The activation of C/EBPβ and C/EBPδ in response to DEX and MIX during the early phase of adipogenesis in 3T3-L1 preadipocytes is transient: both C/EBP isoforms reach maximum levels of expression 5- to 10-fold greater than that observed in proliferating cells 24 to 48 h following exposure to the adipogenic inducers, and these levels decrease thereafter. C/EBPδ expression declines precipitously to virtually undetectable levels, whereas C/EBPβ decreases at a much slower rate to a level that is approximately 50% of that found in preadipocytes. It is possible, therefore, that once these C/EBPs have initiated PPARγ transcription in the uncommitted mesenchymal cells (NIH 3T3 fibroblasts), they are no longer required for the terminal stages of the differentiation process. The data in Fig. 7 suggest that this is not the case, however, since exposure of activated β2 cells, expressing abundant quantities of both PPARγ and aP2, to tetracycline results in the rapid loss of their ability to express the adipogenic phenotype as the abundance of C/EBPβ declines. In addition, the data presented in Fig. 8 demonstrate that exposure of fully differentiated β/δ39 cells to tetracycline results in both an inhibition of ectopically expressed C/EBPβ and C/EBPδ and a significant down-regulation of PPARγ, GPD, and aP2. It appears, therefore, that maintenance of the adipogenic gene program in NIH 3T3 fibroblasts is dependent on a sustained elevated expression of at least C/EBPβ. Such an observation is consistent with the notion that many of the downstream adipogenic genes, which contain regulatory elements corresponding to both PPARγ and C/EBPs, require the presence of these transcription factors to facilitate their expression during the terminal stages of differentiation. Presumably, the induction of C/EBPα transcription 3 to 4 days into the differentiation of 3T3-L1 preadipocytes provides this important function of activating and maintaining terminal differentiation. In this regard, it is interesting that the overexpression of either C/EBPβ alone or C/EBPβ and C/EBPδ together is unable to initiate C/EBPα expression in NIH 3T3 fibroblasts (data not shown). Inhibition of the ectopically expressed C/EBPs by reexposure of the activated fibroblasts to tetracycline results, therefore, in a cell with small amounts of endogenous C/EBPβ and C/EBPδ and no C/EBPα. Consequently, it follows that under these circumstances, adipogenic genes that depend on C/EBPs for their expression, such as the PPARγ and aP2 genes, are no longer transcribed, and adipogenesis is attenuated.

Recent investigations have provided convincing evidence that both C/EBPβ and C/EBPδ are involved in mechanisms that activate C/EBPα transcription in 3T3-L1 preadipocytes (32). Results of a series of transfection studies have shown that the promoter for the C/EBPα gene can be transactivated by C/EBPβ (21) and C/EBPδ (31a) through a C/EBP regulatory element at positions −175 to −190 upstream of the transcriptional start site (21). The lack of C/EBPα expression in the NIH 3T3/C/EBP cell lines suggests, therefore, that C/EBPβ and C/EBPδ expression is necessary but not sufficient to initiate C/EBPα transcription. What the other inducers are is not known. Apparently, regulation of C/EBPα gene expression differs somewhat from regulation of expression of other adipogenic genes which facilitate the synthesis and deposition of fat droplets in that these other genes are activated by PPARγ and C/EBPβ. Induction of C/EBPα transcription may involve a set of transcription factors that represent a separate pathway of adipogenic regulators. It is also conceivable that the C/EBPα gene is repressed in mesenchymal stem cells, and this repression may not be relieved simply by the overexpression of C/EBPβ and C/EBPδ. A possible candidate responsible for such a repression is the protein complex referred to as C/EBPα undifferentiated protein (CUP), which is present in proliferating undifferentiated cells (29). CUP has been shown to bind strongly to the C/EBP regulatory element within the C/EBPα promoter.

The mechanisms that activate C/EBPβ and C/EBPδ gene transcription in 3T3-L1 preadipocytes appear not to be operating to the same extent in NIH 3T3 cells, since exposure of the latter cells to a full complement of adipogenic inducers containing DEX, MIX, insulin, and FBS does not induce their expression. It is quite possible, therefore, that commitment of mesenchymal stem cells to the adipogenic lineage involves acquisition of signal transduction pathways that induce C/EBPβ and C/EBPδ expression in response to the adipogenic inducers. These regulatory processes may include autoregulatory circuits in which C/EBPs are capable of cross-activating each other's expression. In this regard, the data in Fig. 4B show that the ectopic production of C/EBPδ in NIH 3T3 cells enhances C/EBPβ expression; however, the levels reached are still not high enough to induce PPARγ or to stimulate adipogenesis. It is possible that the mechanisms responsible for activating either C/EBPβ or C/EBPδ gene expression require a minimal threshold level of C/EBP expression as well as the appropriate direct inducer for each gene. For instance, induction of C/EBPβ requires enhanced amounts of C/EBPδ as well as stimulation of cyclic AMP (cAMP) signalling pathways, suggesting a synergy between C/EBP and cAMP response proteins. Similarly, induction of C/EBPδ requires C/EBPβ in addition to glucocorticoids. An understanding of the interactions of these C/EBP transcription factors and extracellular inducers should provide insight into the mechanisms that control the conversion of mesenchymal stem cells into adipocytes.

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