Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis

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Activation of adipogenesis in 3T3 preadipocytes by exposure to the adipogenic inducers dexamethasone, methylisobutylxanthine, insulin, and fetal bovine serum is accompanied by a transient burst of C/EBPβ protein expression that precedes the induction of the fat gene program. In this study we have investigated the role of C/EBPβ in initiating the adipogenic program by overexpressing C/EBPβ in multipotential NIH-3T3 fibroblasts. Conditional ectopic expression of C/EBPβ was accomplished by using an artificial transcriptional regulatory system based on the Escherichia coli tetracycline repressor to generate a stable cell line, β2, that expresses C/EBPβ mRNA and protein in a tightly controlled tetracycline dose-dependent manner. Induction of C/EBPβ DNA-binding activity in NIH-3T3 β2 cells exposed to dexamethasone in the presence of insulin and fetal bovine serum activates the expression of an adipocyte-specific nuclear hormone receptor, PPARγ, that stimulates the conversion of these fibroblasts into committed preadipocytes. Either ectopic expression of C/EBPβ or treatment with dexamethasone alone is incapable of inducing PPARγ expression, but when present together, they have a synergistic effect on the adipogenic program. Exposure of these stimulated cells to a PPAR activator 5,8,11,14-eicosatetraynoic acid (ETYA) results in the accumulation of fat droplets and expression of the adipocyte-enriched genes aP2 and glycerol phosphate dehydrogenase (GPD). The number of β2 cells that can differentiate into adipocytes is related to the concentration of tetracycline and, therefore, the amount of the exogenous C/EBPβ protein expressed. C/EBPβ can induce PPARγ mRNA in the absence of ETYA; however, expression of aP2 mRNA and maximum fat deposition is dependent on the PPAR activator. Our results suggest that enhanced expression of C/EBPβ converts multipotential mesenchymal precursor cells into preadipocytes that respond to adipogenic inducers, including dexamethasone and PPAR activators to differentiate into adipocytes.

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The differentiation of multipotential stem cells into functionally distinct cell types within different tissues involves the activation of a regulatory network of nuclear factors and the cessation of cell proliferation. Significant progress toward an understanding of these elaborate processes has been made by investigators studying the in vitro differentiation of mesenchymal cells into fat and muscle. In both cases, families of transcription factors have been identified that play a direct role in regulating the expression of tissue-restricted genes in the different cell types [Tapscott and Weintraub 1991, Vasseur-Cognet and Lane 1993]. The advances made in understanding adipogenesis have stemmed from the isolation and characterization of the 3T3-L1 and 3T3-F442A preadipocyte cell lines that were originally derived from mouse fibroblasts [Green and Kehinde 1974, 1975, 1976]. In the case of 3T3-L1 cells, differentiation is induced upon exposure of a confluent population of cells to fetal bovine serum (FBS), dexamethasone (DEX), methylisobutylxanthine (MIX), and insulin. Differentiation of 3T3-F442A cells, however, requires only exposure of confluent cells to FBS and insulin. In both cases, the differentiation is marked by a major change in cell morphology that includes the accumulation of large lipid droplets and the induction of a program of adipocyte-specific genes.

Regulation of this program of gene expression has recently been shown to involve two families of transcription factors, the CCAAT/enhancer-binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs). C/EBPs are members of a diverse group of nuclear factors that contain a leucine zipper domain required for dimer formation and a basic DNA-binding do-
main that binds to regulatory elements in the promoters and/or enhancers of target genes. Expression of the C/EBP genes is regulated in such a way that three of the proteins (α, β, and γ) are restricted to a limited number of tissues, most notably fat and liver [Cao et al. 1991; Williams et al. 1991]. All three C/EBPs readily form heterodimers with one another, exhibit similar DNA-binding activities, and are capable of trans-activating reporter genes that contain C/EBP regulatory elements within their promoters [Cao et al. 1991]. The pattern of expression of these genes during in vitro adipogenesis is consistent with a role for each protein in controlling the sequential activation of the adipogenic gene program [Cao et al. 1991]. Recent studies suggest that C/EBPα has a direct role to play in establishing the quiescent, terminally differentiated state of adipocytes (Samuelsson et al. 1991; Umek et al. 1991, Lin and Lane 1994), and it can also stimulate adipogenesis when overexpressed in various mouse fibroblast cell lines [Freytag et al. 1994]. Whether C/EBPα is responsible for initiating adipogenesis is questionable, however, because its expression occurs relatively late in the differentiation process [Vasseur-Cognet and Lane 1993].

A search for an adipocyte-specific transcription factor responsible for activating the adipogenic gene program led Spiegelman and co-workers [Tontonoz et al. 1994a] to identify a tissue-restricted member of the PPAR family of nuclear hormone receptors, PPARγ. As is the case with the C/EBPs, the PPAR proteins consist of a family of related proteins that are expressed to varying extents in different tissues [Kliwer et al. 1994]. PPARα and PPARγ are produced along with C/EBPs in tissues involved in energy metabolism. PPARα is expressed abundantly in liver [Isseman and Green 1990, Dreyer et al. 1992, Zhu et al. 1993], whereas PPARγ expression is most prominent in adipose tissue [Tontonoz et al. 1994a]. PPARγ and NUC1 are both expressed at low levels in many tissues [Schmidt et al. 1992, Chen et al. 1993, Kliwer et al. 1994]. PPARs are activated by a structurally diverse group of compounds, the peroxisome proliferators, which include the fibrate class of hypolipidemic drugs, herbicides, phthalate plasticizers, and long-chain fatty acids [Dreyer et al. 1992; Gottlicher et al. 1992; Keller et al. 1993]. Activation of these receptors by specific peroxisome proliferators in target tissues such as liver and fat results in the expression of a variety of genes that encode proteins and enzymes involved in regulating lipid metabolism [Dreyer et al. 1992; Keller et al. 1993; Tontonoz et al. 1994b]. Activation of PPARα in the liver and kidney causes a dramatic increase in the size and number of peroxisomes, thereby enhancing the capacity of these tissues to metabolize long-chain fatty acids through an increased production of enzymes that function in the β-oxidation cycle [Dreyer et al. 1992]. The high level of PPARγ expression in adipose tissue and its early induction during the differentiation of 3T3-L1 and 3T3-F442A preadipocytes suggest that this factor may initiate fat gene expression [Chawla et al. 1994; Tontonoz et al. 1994a]. A recent study demonstrates that retroviral expression of PPARγ2 in NIH-3T3 mouse fibroblasts promotes adipocyte differentiation in a manner that is dependent upon the concentration of PPAR activators in the culture medium [Tontonoz et al. 1994b]. Furthermore, the extent of this differentiation is enhanced severalfold when C/EBPα is expressed together with PPARγ2, suggesting that both factors cooperate to induce the expression of adipocyte-specific genes. In fact, at least two genes expressed in fat, Ap2 and PEPCK, contain regulatory elements within their promoters or enhancers that respond to trans-activation by C/EBPα and PPARγ [Christy et al. 1989; Park et al. 1990, Tontonoz et al. 1994a, 1995].

It appears, therefore, that PPARγ and C/EBPα are both capable of inducing fat-specific gene expression in multipotential precursor cells such as NIH-3T3 fibroblasts, but it is not known whether one of these factors is responsible for inducing the expression of the other. It is unlikely that C/EBPα regulates PPARγ, because it is expressed at least 1 day after the induction of PPARγ during the differentiation of 3T3 preadipocytes [Tontonoz et al. 1994b]. It is more likely that PPARγ regulates C/EBPα. If this is the situation, then an important question is, Which transcription factors activate PPARγ? The early transient activation of C/EBPβ and C/EBPδ in 3T3-L1 cells in response to DEX and MIX [Cao et al. 1991] suggests that these factors may provide this important function of activating PPARγ. To address this question, we have developed an inducible expression system to facilitate the production of different amounts of C/EBPs in NIH-3T3 cells in response to an extracellular regulator. During the final stages of this work, a study by McKnight and co-workers [Yeh et al. 1995] demonstrated that retrovirus-mediated overexpression of C/EBPβ in NIH-3T3 cells can induce adipogenesis in the presence of adipogenic inducers. This observation was in conflict with earlier investigations by Freytag et al. [1994] who reported that C/EBPβ was not capable of converting fibroblasts into fat cells. In the present study we demonstrate that conditional ectopic expression of C/EBPβ in NIH-3T3 cells activates the synthesis of PPARγ mRNA and subsequent exposure of these cells to PPAR activators stimulates their conversion into adipocytes.

Results

Conditional expression of C/EBPβ using the TET activator expression system

Previous investigations have shown that PPARγ and C/EBPβ mRNAs are induced severalfold during the initial period following the differentiation of 3T3 preadipocytes into adipocytes [Cao et al. 1991; Tontonoz et al. 1994a]. It appears, however, that the peak of expression of C/EBPβ protein precedes the activation and accumulation of PPARγ mRNA. These data are consistent with our hypothesis that C/EBPβ is involved in activating PPARγ expression during fat cell differentiation. To test this idea, we established a stable line of NIH-3T3 cells that produce controlled amounts of C/EBPβ protein from an inducible expression vector, and we have deter-
determined that C/EBPβ can stimulate adipogenesis by inducing PPARγ expression. To conditionally express C/EBPβ proteins in NIH-3T3 cells, we employed an artificial transcriptional regulatory system developed by Gossen and Bujard (1992) that uses the bacterial tetracycline-resistant operator/repressor (Materials and methods). C/EBPβ cDNA was subcloned into the expression vector, pUHD10-3, downstream of a synthetic promoter composed of tandem repeats of the tetracycline operator and a CMV promoter. Activation of this minimal promoter is dependent on coexpression of an artificial trans-activator (tTA) composed of the tetracycline repressor fused to the activation domain of the herpes simplex virus transcriptional activator VP16. The presence of tetracycline prevents the binding of this trans-activator to the operator and, therefore, inhibits the expression of C/EBPβ mRNA (Fig. 1A). The plasmid, pUHD15-1, expressing tTA was transfected together with the C/EBPβ expression vector and a neomycin-resistant plasmid. Conditional expression of C/EBPβ mRNA in stably transfected cells was assessed by Northern blot analysis of total RNA isolated from G418-resistant colonies cultured in the presence or absence of tetracycline. One clone, referred to as β2, which produced enhanced levels of C/EBPβ mRNA in response to culture medium lacking tetracycline, was selected for further analysis. Cell lines expressing abundant quantities of tTA trans-activator alone were selected as controls.

The Northern blot analysis in Figure 1B shows a modest level of C/EBPβ mRNA expression in cells grown in the presence of tetracycline (lane 1). Removal of the drug results in a gradual but extensive induction of the exogenous mRNA over a 48-hr period. The size of this mRNA is larger than the endogenous species as shown by its migration to a slightly higher position in the gel. This is attributable to the transcription of part of the expression vector that contains the SV40 polyadenylation element giving rise to an additional ~100 nucleotides in the exogenous mRNA. Addition of tetracycline to these stimulated cells results in a rapid decrease of C/EBPβ mRNA levels within the initial 4 hr after treatment, indicating that the exogenous C/EBPβ mRNA species is very short lived. Western blot analysis of total cell lysates obtained from β2 cells maintained for several days in the presence or absence of tetracycline was performed with an anti-C/EBPβ antibody. Figure 1C shows that β2 cells grown in the presence of tetracycline and the NIH-3T3 cells expressing the tet activator alone (tTA cells) contained detectable quantities of the 32-kD C/EBPβ (LAP) polypeptide but significantly lower amounts of the 21-kD C/EBPβ (LIP) protein. Culture of the β2 cells in the absence of tetracycline resulted in an increase by severalfold of both C/EBPβ polypeptides, indicating that the ectopically expressed C/EBPβ mRNA (see Fig. 1A) is translated correctly in this 3T3 fibroblast clone.
The DNA-binding activity of C/EBP polypeptides in 3T3-L1 preadipocytes and NIH-3T3 fibroblasts

Preadipocytes have been shown to express at least three isoforms of C/EBP, α, β, and δ, at specific times during their differentiation into adipocytes. Each of these C/EBP polypeptides can associate with the others to form homo- and heterodimeric complexes that bind to DNA with different affinities (Cao et al. 1991). To identify the complexes expressed during the early phase of preadipocyte differentiation and to compare them with C/EBP complexes in the β2 cells, we performed a series of gel mobility supershift assays. Figure 2A (lane 1) shows that 3T3-L1 preadipocytes exposed to the adipogenic inducers DEX, MIX, insulin, and FBS for 2 days result in the expression of a heterogeneous group of C/EBP complexes that are capable of binding to a consensus C/EBP binding site. At 4 days following induction of differentiation, the abundance of these complexes decreases quite significantly (lane 7). Supershift analysis with antibodies against the three C/EBP isoforms, α, β, and δ, shows that the major C/EBP binding activity expressed at day 2 consists of complexes containing C/EBPβ (Fig. 2A, lane 3). Other reports have demonstrated that C/EBPβ polypeptides are also abundantly expressed at this stage of the differentiation, but the supershift assay shown in lane 4 suggests that these proteins do not constitute a significant proportion of the total DNA-binding activity of the C/EBP complexes. The data in lane 2 shows a low but detectable level of C/EBPα binding activity at 2 days post induction. It is worth noting that the anti-C/EBPα antibody supershifts almost all of the C/EBP complexes (lane 3) except two low-abundance species that appear to correspond to δδ homodimers (the lower species) and αα homodimers (the upper species). Specifically, the lower species in lane 3 can be supershifted when the 2-day nuclear extracts are treated with anti-C/EBPβ and anti-C/EBPδ antibodies together (lane 5), and the upper species is shifted when anti-C/EBPδ antibody is included with the other two antibodies (lane 6). By day 4 of differentiation, the binding activity of both the C/EBPβ and C/EBPδ complexes drop severalfold (cf. lane 9 with lane 3 and lane 10 with lane 4). In fact, it appears that the total C/EBP binding activity...
activity expressed at this stage of adipogenesis is accounted for by complexes primarily involving C/EBPα (lane 8). Figure 2B shows that nuclear extracts isolated from NIH-3T3 cells, either β2 cells maintained in tetracycline (lane 3) or fibroblasts transfected with the tTA expression vector alone (lane 11), have low but measurable levels of C/EBP binding activity when compared with differentiating preadipocytes (Fig. 2A) or normal rat liver (Fig. 2B, lane 1). Most of this activity in the 3T3 cells is attributable to C/EBPβ polypeptides (Fig. 2B, lanes 5,12). Culture of the β2 cells in the absence of tetracycline for 48 hr results in a severalfold induction of total C/EBP binding activity (Fig. 2B, lane 7), which can be attributed to the overexpressed C/EBPβ proteins because the anti-C/EBPβ antibody supershifts almost all of the C/EBP complexes produced in these cells (lane 9). There is very little C/EBPα DNA-binding activity and no C/EBPα activity.

Conditional expression of C/EBPβ in NIH-3T3 cells stimulates adipogenic conversion

To investigate the ability of C/EBPβ protein to induce PPARγ in NIH-3T3 cells and subsequently to stimulate adipogenesis, we cultured subconfluent β2 cells for 48 hr in the absence of tetracycline to elevate C/EBPβ expression to maximum levels during which time the culture reached confluence. Exposure of these cells to the adipogenic inducers DEX, MIX, insulin, and FBS for 48 hr, followed by maintenance in FBS containing insulin and the PPAR activator 5,8,11,14-eicosatetraynoic acid (ETYA), caused a morphological differentiation within 6–10 days after confluence. At this time, 25%–30% of the fibroblasts had differentiated into large round cells containing several small lipid droplets or a few large droplets (Fig. 3). No evidence of differentiation was observed in the cultures maintained in the presence of tetracycline, suggesting that activation of the adipogenic program is dependent upon the expression of C/EBPβ protein. Similarly, culture of the control tTA cells in medium lacking tetracycline was incapable of stimulating lipid droplet formation even when cells were exposed to the adipogenic inducers [data not shown].

To determine whether the morphological differentiation seen in Figure 3 was attributable to the activation of the adipogenic gene program, we performed a Northern blot analysis of total RNA isolated from β2 cells cultured in the presence or absence of tetracycline for the indicated days following exposure to the adipogenic inducers outlined above including ETYA. These data shown in Figure 4 demonstrate a significant increase in mRNAs coding for PPARγ and the differentiation-linked adipocyte genes aP2 and GPD. The PPARγ mRNA was detected first 24–48 hr after confluence, and its expression continued to rise, reaching an abundant plateau between 4 and 10 days. Induction of aP2 and GPD mRNAs lagged behind PPARγ mRNA by 2 days because they were not detected until 4 days after treatment. PPARγ and aP2 mRNAs reached a level of expression ~20%–30% of that measured in fully differentiated 3T3-L1 cells; this level is consistent with the extent of conversion of these NIH-3T3 (β2) fibroblasts into adipocytes. GPD mRNA, however, reached significantly lower levels of expression than expected, and C/EBPα mRNA was not detected during the 10-day culture period. Culture of β2 cells in tetracycline prevents the activation of the adipogenic genes as indicated by a complete absence of PPARγ, aP2, and GPD mRNAs throughout the differentiation period. C/EBPβ mRNA expression is transiently induced in β2 cells maintained in tetracycline in response to the adipogenic inducers. In fact, overexpression of C/EBPβ appears to prevent this induction and results in a gradual decrease of C/EBPβ mRNA as observed during the differentiation of 3T3-L1 cells. As expected, the tTA control cells failed to express the adipose genes following exposure to the adipogenic inducers in the absence of tetracycline (lanes 15,16).

During the initial isolation of the β2 cells, we identified other clones that expressed C/EBPβ mRNA at levels significantly higher than those present in nontransfected NIH-3T3 cells but lower than levels expressed by the β2 cells. In addition, some clones that were transfected with...
C/EBPβ induces adipogenesis in mouse fibroblasts

Figure 4. Ectopic induction of C/EBPβ in NIH-3T3 β2 cells activates the adipogenic gene program. β2 cells were cultured in growth medium containing tetracycline (1 μg/ml). Two days before the cells reached confluence, fresh medium was added so that one-half of the population was maintained in tetracycline (β2 + tetracycline), whereas the other half was deprived of the antibiotic (β2 - tetracycline). tTA (tTA-T) and 3T3-L1 (L1) cells were maintained in tetracycline-free media. At day 0 (confluence), all four populations of cells (β2 + / - tetracycline; tTA-T and L1) were exposed to the inducers of adipogenesis as described in Materials and methods. Twenty micrograms of total RNA was isolated from the various cells at the indicated days (L1 preadipocytes were differentiated for 10 days) and analyzed on Northern blots using 32P-labeled cDNA probes corresponding to PPARγ, aP2, GPD, C/EBPα, C/EBPβ, and C/EBPβ mRNAs.

Figure 5. Variation in the level of C/EBPβ expression and the extent of adipogenic conversion in additional stable transfectants of NIH-3T3 cells. (A) Analysis of the binding activity of C/EBPβ in G418-resistant cell lines generated by transfection with the tTA and C/EBPβ expression vectors together (β clones) or the tTA vector alone (T1, T2). All the cell lines were cultured in tetracycline-free media to confluence and then induced to differentiate as described in Materials and methods. Nuclear proteins of each clone were extracted 6 days after confluence. Ten micrograms of each extract was subjected to supershift EMSA using 32P-labeled C/EBP oligonucleotide as probe and an anti-C/EBPβ antibody. (B) Expression of PPARγ and aP2 mRNAs in β clones and tTA cell lines. All clones were cultured and differentiated as in A. Twenty micrograms of total RNA was isolated from each clone at 6 days following induction to differentiate and was analyzed by Northern blot using 32P-labeled cDNA probes corresponding to PPARγ, aP2, and C/EBPβ mRNAs.
the adipogenic gene program when exposed to differentiation medium.

The extent of the differentiation of NIH-3T3 cells into adipocytes is dependent on the amount of C/EBPβ protein

The data shown in Figure 5 suggest that the extent of induction of the adipose-specific genes may be related directly to the amount of C/EBPβ protein expressed in these fibroblasts. To test this hypothesis, we propagated the β2 cells in tetracycline, then split the population into fresh medium containing varying concentrations of tetracycline. Nuclear extracts were prepared after a 48-hr culture period and analyzed by both Western blot and supershift electrophoretic mobility-shift assay (EMSA) using an anti-C/EBPβ antibody. Figure 6A shows that there is a dose-dependent increase in both forms of the C/EBPβ protein (LAP-32kD and LIP-21kD) as the concentration of tetracycline is attenuated. At concentrations between 50ng/ml and 1000 ng/ml, there are low amounts of LAP polypeptides but virtually undetectable quantities of the LIP polypeptides as observed previously in Figure 1C. Induction of C/EBPβ protein synthesis occurs when β2 cells are exposed to doses of 10 ng/ml and below, reaching a maximum level of expression at 0–1.0 ng/ml. The anti-C/EBPβ supershift EMSA shown in Figure 6B demonstrates that the increased expression of these C/EBPβ polypeptides results in a corresponding increase in DNA-binding activity. To determine whether this tetracycline dose-dependent induction of the C/EBPβ polypeptides corresponds to an increase in the percentage of differentiated preadipocytes, we cultured β2 cells in different amounts of tetracycline and exposed them to differentiation medium containing 50 μM ETYA. Figure 7A shows a direct relationship between the percentage of cells containing fat droplets and the concentration of tetracycline in the media. Tetracycline at 1000 ng/ml completely inhibited adipogenesis, whereas cells cultured in concentrations lower than 50 ng/ml began to show signs of differentiation as revealed by the presence of Oil Red O-stained fat droplets. The dose dependence of this response is shown in Figure 7B in which the percentage of cells staining positive with Oil Red O at the different concentrations of the antibiotic are graphed. It is interesting that although at 50 ng/ml we were unable to detect an enhanced expression of C/EBPβ proteins (see Fig. 6), the Oil Red O stain was capable of detecting a small percentage of cells that had differentiated into adipocytes. As the concentration of the antibiotic is decreased, there is a corresponding increase in the percentage of cells expressing fat droplets, reaching a maximum of 25%–30% conversion at 0.1 ng/ml. Figure 7C shows that this pattern of morphological differentiation correlates with a tetracycline dose-dependent induction of PPARγ and aP2 mRNAs. It is noteworthy to mention that there is no significant change in the expression of C/EBPβ mRNA at all concentrations of tetracycline at this stage of the differentiation process.

Induction of PPARγ expression in NIH-3T3 cells is dependent on DEX as well as enhanced C/EBPβ DNA-binding activity

During our initial investigations, we observed that the differentiation of tetracycline-deficient β2 cells into adipocytes was dependent on exposure to differentiation medium containing DEX, MIX, insulin, and FBS (data not shown). To determine which of these adipogenic inducers was required to activate the adipose-specific genes, we cultured β2 cells in medium containing calf serum in the presence or absence of tetracycline and allowed them to grow to confluence (~2 days). At this time (day 0), the tetracycline-deficient cells were exposed to FBS containing insulin alone (I), insulin + DEX (ID), insulin + MIX (IM), or all three inducers together (DIM). Cells maintained in tetracycline were also exposed to DIM. After 2 days of treatment, the media of all the cultures was changed to insulin and FBS. Four days later, total RNA was isolated and analyzed by Northern blot hybridization. Figure 8A demonstrates that DEX is the major activator of adipogenesis in these tetracycline-deficient cells because the only inducers that could stimulate PPARγ and aP2 expression was ID and DIM (lanes 2,4). Insulin alone or insulin + MIX had virtually no effect on the expression of these adipose-specific genes. As shown in Figure 4 above, if cells are exposed to DIM in the presence of tetracycline, which prevents the ectopic expression of C/EBPβ protein, the adipogenic program is not activated (Fig. 8A, lane 5). Figure 8B further demonstrates that PPARγ mRNA is induced within 12–24 hr
Figure 7. Differentiation of NIH-3T3 β2 cells into adipocytes is directly related to the amount of the C/EBPβ protein expressed. [A] Tetracycline dose-dependent conversion of β2 cells into Oil Red O positive adipocytes. β2 cells were induced to differentiate as described in Materials and methods in differentiation medium containing different concentrations of tetracycline (as indicated at the top) and 50 μM ETYA. Cells were fixed and stained with Oil Red O at 9 days postconfluence. Magnification, 20×. [B] Quantitation of the percentage of β2 cells that are stained with Oil Red O for each concentration of tetracycline. Two hundred cells in 10 random fields were counted. [C] Tetracycline dose-dependent induction of PPARγ and αP2 mRNAs. Total RNA was isolated from β2 cells cultured for 8 days in differentiation medium containing the indicated concentrations of tetracycline and analyzed by Northern blot as described in Materials and methods.
following exposure of tetracycline-deficient β2 cells to DEX. Maximum levels of PPARγ expression are reached by 72–96 hr after treatment. Cells maintained in tetracycline are incapable of expressing PPARγ throughout this time period. It is worth noting that the β2 cells that have been deprived of tetracycline for 48 hr prior to the start of this experiment (day 0) express detectable amounts of PPARγ even before they are exposed to DEX. In contrast, the β2 cells maintained in tetracycline through the entire experimental period and exposed to the glucocorticoid do not express PPARγ mRNA. These data suggest that C/EBPβ is the principal activator of PPARγ mRNA expression and DEX significantly enhances this response. It is also important to note that there is no significant change in the expression of C/EBPδ mRNA in the presence or absence of tetracycline.

The C/EBPβ-dependent induction of adipogenesis in NIH-3T3 cells is enhanced by a synthetic PPAR activator, ETYA

The early induction of PPARγ mRNA following exposure of the tetracycline-deficient β2 cells to the adipogenic inducers suggests that this fat-specific transcription factor is responsible for inducing the expression of the downstream genes such as aP2 and GPD and stimulating the production of triglycerides. Other studies have shown that adipose differentiation of preadipocyte cell lines and PPARγ-expressing NIH-3T3 cells is enhanced manyfold by lipophilic activators of PPARs. To determine the importance of activating PPARs during the adipogenic conversion of the β2 cells, we initiated differentiation with DEX, MIX, insulin, and FBS as described above [Fig. 4], but during the switch to maintenance medium (FBS, insulin, and ETYA), half the population of cells were deprived of ETYA. Figure 9A shows that culture of tetracycline-deprived β2 cells in medium containing 50 μM ETYA induces 25%–30% of the population to convert to fat cells, whereas in the absence of ETYA the level of conversion is greatly reduced (~5% of the population). The Northern blot analysis shown in Figure 9B demonstrates that activation of PPARγ mRNA by C/EBPβ and the adipogenic inducers [DEX] does not require ETYA because the same level of PPARγ expression occurs in the absence or presence of the synthetic fatty acid [cf. lane 2 with lane 3]. In contrast, aP2 mRNA expression, which has been shown previously to be stimulated by the ectopic production of PPARγ, is enhanced severalfold by culture of the stimulated β2 cells in ETYA [20–50 μM]. Furthermore, exposure of the cells to ETYA in the absence of DEX and MIX is incapable of activating both PPARγ and aP2 mRNA expression [Fig. 9C], demonstrating that ETYA is not an inducer of PPARγ gene expression in cells [NIH-3T3 fibroblasts] that do not express PPARγ.

Discussion

These investigations provide evidence that C/EBPβ plays an important regulatory role during the early stages of adipogenesis by activating the expression of adipogenic...
C/EBPβ induces adipogenesis in mouse fibroblasts

Figure 9. Effects of ETYA on C/EBPβ-dependent induction of adipogenesis in NIH-3T3 β2 cells. (A) The conversion of NIH-3T3 β2 cells into adipocytes by ectopic expression of C/EBPβ is enhanced by ETYA. β2 cells were induced to differentiate by exposure to 1 μM DEX, 0.5 mM MIX, 10% FBS, and 5 μg/ml of insulin for 48 hr and were then maintained in either differentiation medium (DMEM containing 5 μg/ml of insulin and 10% FBS) alone [top] or differentiation medium plus 50 μM of ETYA [bottom] for 9 days. Cells were stained with Oil Red O to indicate the accumulation of fat droplets. (B) Expression of aP2 mRNA in β2 cells that express abundant amounts of PPARγ mRNA is dependent on ETYA. β2 cells cultured in the presence (+) or absence (−) of tetracycline were induced to differentiate as described above and were maintained for 4 days in differentiation medium containing insulin and the indicated amounts of ETYA (0, 20, and 50 μM). Total RNA (20 μg) from each condition was subjected to Northern blot analysis using the 32P-labeled cDNA probes as indicated. (C) ETYA alone is not capable of activating PPARγ expression in tetracycline-deficient β2 cells. β2 cells were cultured in tetracycline-free medium to confluence. Cells were then incubated in medium containing insulin plus ETYA or insulin, DEX, and MIX after 48 hr, both populations of cells were exposed to differentiation medium containing insulin and 50 μM of ETYA and maintained in this medium for an additional 4 days. Twenty micrograms of total RNA from each condition was analyzed by Northern blot.

The earliest response to the enhanced expression of C/EBPβ in NIH-3T3 cells is the increase in PPARγ mRNA. This event precedes the appearance of aP2 and GPD mRNAs and the accumulation of fat droplets. Furthermore, this C/EBPβ-dependent induction of PPARγ gene expression occurs in the absence of ETYA, whereas expression of aP2 mRNA and maximum fat deposition are dependent on an exogenous activator of PPARγ. These data suggest that an important role of C/EBPβ is to induce PPARγ that is then responsible for activating the gene program involved in synthesizing and depositing triglycerides. C/EBPβ may also play an additional role in these β2 cells by synergizing with PPARγ to promote the transcription of the downstream target genes. In fact, many adipocyte-enriched genes contain binding sites for C/EBPβ (Vasseur-Cognet and Lane 1993), and at least two of them, aP2 and PEPCK, also contain DR-1 hormone response elements that can interact with PPARγ (Tontonoz et al. 1994a, 1995). In this regard, our
preliminary data [not shown] suggest that C/EBPβ may function to maintain expression of the adipocyte genes at the later stages of differentiation process because addition of tetracycline to β2 cells (to inhibit C/EBPβ) after PPARγ has reached a maximum level of expression (day 4) reduces aP2 expression.

Enhanced expression of C/EBPβ alone is not sufficient to activate adipogenesis in the NIH-3T3 β2 cell line because exposure to DEX in the presence of insulin and FBS is also required. What role DEX and the other inducers is playing is not known, but information may be gained from investigations on the mechanisms that regulate the early phase of 3T3-L1 preadipocyte differentiation. Studies by Cao et al. [1991] show that DEX enhances C/EBPβ mRNA expression, whereas MIX is responsible for inducing C/EBPβ mRNA production in the preadipocytes. It is unlikely that these effectors are involved in stimulating either C/EBPβ or C/EBPβ expression in the NIH-3T3 β2 cells because C/EBPβ, of course, is activated by removal of tetracycline, whereas C/EBPβ appears to decrease somewhat soon after the differentiation process has commenced (Fig. 4). These observations suggest that DEX must be fulfilling a function other than enhancing expression of the C/EBPβ proteins during the differentiation of the β cells. Other investigations [Lemberger et al. 1994] have demonstrated that glucocorticoids can activate PPARα gene expression in primary cultures of rat hepatocytes, suggesting that the PPAR genes may contain glucocorticoid response elements in their promoters and/or enhancers. The role of insulin and FBS in promoting adipogenesis in both 3T3-L1 and β2 cells is unclear; one possibility is that they promote the translation of the induced adipogenic mRNAs.

The mechanism by which C/EBPβ induces PPARγ mRNA is not known, although, on the basis of studies analyzing the regulation of other C/EBP-responsive genes, it seems reasonable to propose that PPARγ gene transcription is likewise regulated by binding of various C/EBPs to corresponding binding sites within the promoter and/or enhancer of the PPARγ gene. In fact, we have shown that ectopic expression of C/EBPα in NIH-3T3 cells can also induce expression of the PPARγ gene and stimulate adipogenesis (Z. Wu et al., unpubl.). The data shown in Figure 2 suggest that the C/EBP complex responsible for inducing PPARγ expression and stimulating adipogenesis in the 3T3-L1 and β2 cells consist primarily of C/EBPβ homodimers.

It is possible that the forced expression of C/EBPβ homodimers in the NIH-3T3 cells and the hormonal induction of these and other complexes in 3T3-L1 cells (Fig. 2A) result in somewhat different programs of gene expression. In this regard, it is interesting that C/EBPα gene expression is not induced by C/EBPβ in the β2 cells. This observation is consistent with that of McKnight and co-workers [Yeh et al. 1995] who have recently found that constitutive, retrovirus expression of C/EBPβ in NIH-3T3 cells induces fat droplet accumulation and aP2 expression without enhancing C/EBPα expression. The promoter of the mouse C/EBPα gene contains a C/EBP regulatory element that is capable of activating C/EBPα transcription in transfection studies in which C/EBPα and C/EBPβ expression vectors are coexpressed along with C/EBPα promoter/CAT reporter gene constructs [Christy et al. 1991; Rana et al. 1995]. It appears, therefore, that C/EBPβ may be necessary but not sufficient to induce C/EBPα transcription during adipogenesis. Other factors may include C/EBPβ that, as discussed above, appears to contribute only minimally to the formation of functional C/EBP complexes during the early stages of the differentiation of β2 cells into adipocytes. In addition, the sustained high levels of C/EBPβ homodimers expressed in the differentiating β2 cells may in some way prevent the activation of C/EBPα transcription even though other fat-enriched genes that contain C/EBP-binding sites are activated. In fact, during the normal differentiation of 3T3-L1 cells there is a several-fold decrease in the abundance of the C/EBPβ proteins prior to the induction of C/EBPα gene expression [Cao et al. 1991; Fig. 2A]. The C/EBPβ polypeptide referred to as LIP, which is a potent inhibitor of C/EBPα (LAP) activity [Descomes and Schibler 1991], is also abundantly expressed in β2 cells following removal of tetracycline (Fig. 1C). It is possible, therefore, that LIP attenuates the activity of LAP during the induction of adipogenesis in β2 cells by reducing LAP’s ability to trans-activate some of the adipocyte-enriched genes including C/EBPα. This may be one reason why only 25%-30% of the β2 cell population differentiate into adipocytes. C/EBPα may also be regulated by PPARγ, and the C/EBPα gene may resemble other downstream genes such as aP2 and PEPCk by possessing DR-1 hormone response elements. If this is the case, then it is conceivable that activation of C/EBPα by PPARγ may depend on a PPARα activator that is more potent than the ETYA used in these studies.

As stated at the outset, 3T3 preadipocyte cell lines were originally derived from mouse fibroblasts by selecting colonies that could differentiate into fat cells when exposed to the appropriate medium [Green and Kehinde 1974, 1975, 1976]. An important question in light of the present studies is, Which molecular mechanisms are responsible for committing multipotential fibroblast cells to the adipogenic lineage? It is very apparent that NIH-3T3 mouse fibroblasts cannot simply be induced to differentiate into adipocytes by addition of DEX, MIX, insulin, and FBS, suggesting that the signaling pathways that respond to these effectors do not converge on fat-specific genes in these nondifferentiated cells. Our data show that enhancing the production of C/EBPβ polypeptides can convert a fibroblast into a preadipocyte when exposed to these adipogenic inducers and suggests that the mechanism responsible for commitment to the adipogenic lineage involves the induction of C/EBPα gene transcription and translation. It is interesting that elevation of cAMP levels by treatment with MIX in 3T3-L1 preadipocytes causes a severalfold increase in C/EBPβ gene expression and, therefore, facilitates induction of PPARγ mRNA. Similar treatment of normal, untransfected NIH-3T3 cells with MIX has little or no effect on C/EBPβ production, and the cells are incapable of converting into adipocytes. It is important to note that 3T3-
F442A preadipocytes do not require MIX or DEX to differentiate into adipocytes. It is possible that these cells are slightly further along in the differentiation process than 3T3-L1 cells, and, therefore, they may constitutively express abundant amounts of C/EBPβ and C/EBPδ mRNAs. They may then only require insulin and/or FBS to enhance synthesis of the corresponding proteins to activate the fat gene program including PPARγ expression. It is unclear whether the mechanisms responsible for inducing C/EBPβ expression in preadipocytes are specific to the adipogenic lineage. The mechanisms that activate PPARγ expression appear not to be adipocyte specific because they can operate in NIH-3T3 cells, unless induction of C/EBPβ proteins in these fibroblasts converts them to preadipocytes prior to the increase in PPARγ expression. An additional component of the commitment process may include the production and/or utilization of PPAR activators. It is interesting that differentiation of β2 fibroblasts into adipocytes after the induction of PPARγ by C/EBPβ requires stimulation with ETYA to presumably activate transcription of genes containing PPAR response elements. In contrast, 3T3-L1 and 3T3-F442A preadipocytes do not require any additional stimulation from PPAR activators to differentiate into fat cells other than the fatty acids present in the serum (Chawla and Lazar 1994). It is possible that these preadipocytes synthesize a specific ligand for PPARγ and that this event is adipocyte specific and contributes to the acquisition of the adipogenic lineage.

In light of the observations made in this study, it is interesting to compare this regulatory network of adipogenic transcription factors with those expressed in myogenic cells. In the muscle cells, there are several nuclear regulatory proteins including MyoD, Myf5, Myogenin, Mrf4, and myocyte enhancer factor 2 (MEF2) that are all involved in regulating skeletal muscle gene expression. Many of the genes coding for these myogenic factors can be trans-activated by the other family members, giving rise to a complex regulatory circuit that controls the expression of muscle-specific genes during myogenesis (Edmondson et al. 1992; Weintraub 1993; Olson and Klein 1994). A similar situation may exist in adipocytes in which PPARs and C/EBPs may represent two arms of a network of regulatory factors that can trans-activate each other and, in so doing, amplify the expression of the downstream adipocyte-enriched genes.

Materials and methods

Plasmid constructions

tTA (pUHD15-1) and tetracycline operator–CMV promoter expression plasmids were obtained from H. Bujard of ZFMB, University of Heidelberg, Germany (Gossen and Bujard 1992). The MSV–C/EBPβ plasmid was provided by S. McKnight of Tularik, Inc., South San Francisco, California. To construct a tTA-dependent C/EBPβ expression vector, the 1.5-kb C/EBPβ cDNA from an EcoRI–BamHI double digest of the MSV–C/EBPβ plasmid was inserted into the EcoRI–BamHI sites within the polylinker region of pUHD10-3. Transcription of C/EBPβ mRNA from the resultant plasmid, Tet-O–C/EBPβ, is driven by the CMV promoter containing seven repeats of the tet operator upstream of the C/EBPβ cDNA in response to coexpression of tTA from the pUHD15-1 plasmid.

Cell culture and transfections

3T3-L1 preadipocytes were maintained in growth medium consisting of Dulbecco’s modified Eagle medium (DMEM; Gibco BRL, Life Technologies, Inc., Gaithersburg, MD) containing 10% normal calf serum (ICN, Co., Purchase, NY) and were induced to differentiate as described previously by Student et al. (1980). This involved growing cells to confluence that was considered day 0 of the differentiation program. At this stage, cells were exposed to fresh DMEM containing 10% FBS, 1 μM DEX, 0.5 mM MIX, and 10 μg/ml of insulin for 48 hr to initiate adipogenesis. The medium was then replaced with DMEM containing 10% FBS and 2.5 μg/ml of insulin, and cells were refed every other day. The NIH-3T3 mouse fibroblasts were maintained in DMEM with 10% FBS. To establish an inducible C/EBPβ expression cell line, the fibroblasts were transfected with pUHD15-1(tTA), Tet-O–C/EBPβ and pSV2neo with lipofectAMINE following the protocol provided by the supplier (Gibco BRL). Briefly, a mixture of 10 μg of Tet-O–C/EBPβ, 10 μg of pUHD15-1, 1 μg of pSV2neo plasmids, and 40 μl of lipofectAMINE in 1.6 ml of DMEM was incubated at room temperature for 30 min and then diluted to 8 ml with DMEM. Cells [1.5 x 10^5] per 100-mm dish were washed twice with DMEM, and the diluted DNA–lipid mixture was added. After 6 hr of incubation at 37°C, the cells were refed with DMEM supplemented with 10% FBS and 1 μg/ml of tetracycline and were cultured for an additional 36 hr before 0.4 mg/ml of G418 was added. The transfected cells were selected in DMEM containing 0.4 mg/ml of G418 for 10 to 14 days, and the resistant clones were subsequently isolated and propagated into cell lines. The stable cell lines were maintained in DMEM containing 10% FBS, 1 μg/ml of tetracycline, and 0.2 mg/ml of G418 and were screened by Northern blot analysis to identify a clone (β2) that expressed abundant amounts of C/EBPβ mRNA in response to withdrawal of tetracycline.

To differentiate the β series of stable transfectants into adipocytes, cells were grown to ~50% confluence in DMEM/10% FBS containing 1 μg/ml of tetracycline. To activate C/EBPβ expression, tetracycline was removed from the culture media, and the cells were allowed to grow for an additional 48 hr by which time they had reached confluence. At this stage, referred to as day 0, adipogenesis was induced by addition of fresh medium containing 1 μM of DEX, 0.5 mM of MIX, and 5 μg/ml of insulin. After 48 hr, the medium was changed to DMEM containing 10% FBS, 5 μg/ml of insulin, and 50 μM of ETYA or as indicated. The cells were refed every 2 days. To confirm the appearance of adipocyte morphology, especially the accumulated fat droplets in the cytoplasm, the cells were fixed by 10% formaldehyde in phosphate-buffered saline and stained with Oil Red O (Preece 1972).

RNA analysis

Total RNA was isolated from the NIH-3T3 β2 and 3T3-L1 cells by the procedure of Chomczynski and Sacchi (1987). Cells were washed twice with ice-cold phosphate-buffered saline and lysed with Solution D (4M guanidinium thiocyanate, 25 mM sodium citrate at pH 7.0, 0.5% Sarkosyl, 0.1 M 2-mercaptoethanol), and the lysate was extracted with acidic phenol/chloroform, this was followed by isopropanol precipitation at ~20°C overnight. Twenty micrograms of each RNA sample was analyzed by Northern blot hybridization as described previously [Bond and...
Western blot (immunoblot) analysis

Nuclei were isolated from normal liver hepatocytes, 3T3-L1, and NIH-3T3 β2 cells following a procedure described previously [Rana et al. 1994]. The cells were washed twice with ice-cold phosphate-buffered saline and lysed with NP-40 lysis buffer (10 mM Tris-HCl at pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40). The cell lysates were then transferred to a conical tube and incubated on ice for 15 min, and nuclei were pelleted by centrifugation at 1000 g for 5 min at 4°C. The pellets were washed again with lysis buffer, resuspended in nuclear suspension buffer [50 mM Tris-HCl at pH 7.5, 10 mM magnesium acetate, 40% glycerol, 1 mM dithiothreitol (DTT)], and stored at −80°C until ready for use. To extract nuclear proteins, the stored nuclei were thawed on ice and harvested by centrifugation at 1000 g for 10 min; protein extraction buffer (0.4 M NaCl, 5 mM EDTA, 10 mM sodium HEPES at pH 7.5, 0.2 mM PMSF, and 1 mM DTT) was added to the nuclear pellet and incubated on ice for 20 min with occasional mixing. The nuclear protein supernatant was recovered by centrifugation for 15 min and stored at −80°C after addition of glycerol to a final concentration of 15%. The protein concentration was determined using Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Forty micrograms of nuclear protein was fractionated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Membranes were incubated in TBST (25 mM Tris-HCl at pH 8.0, 125 mM NaCl, and 0.1% Tween 20) containing 10% nonfat dry milk [Carnation Co., Los Angeles, CA] to block nonspecific binding. The blots were then incubated with primary antibody, a polyclonal anti-C/EBPβ antibody [1:1000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA] in blocking solution (TBST containing 5% milk) for 1 hr at room temperature. After being washed three times with the blocking solution, the blots were incubated with a secondary antibody, horseradish peroxidase-conjugated rabbit anti-μg [1:1000 dilution, Sigma Chemical Co., St Louis, MO] for 1 hr. After a final wash with TBST, the blots were developed by the enhanced chemiluminescence system [Amersham Life Sciences, Arlington, IL] and visualized by exposure to autoradiography film.

EMSA

DNA-binding assays were performed as described previously [Rana et al. 1994]. Ten micrograms of nuclear extract was incubated with 3 μg of poly(dI-dC), 2 μl of carrier mix [50 mM MgCl₂, and 340 mM KCl], and delta buffer [0.1 mM EDTA, 40 mM KCl, 25 mM HEPES at pH 7.6, 8% Ficoll], and 1 mM DTT at 4°C for 15 min. The [32P]cDNA-control double-stranded oligonucleotide [50,000 cpm] was added to the reaction mixture and incubated for 30 min at 4°C. For the supershift assay, the appropriate antibody was added to the nuclear extract and incubated at room temperature for 1 hr before the probe was added. The anti-C/EBPα, C/EBPβ and C/EBPγ 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 150–200 V for 3–5 hr at 4°C in TBE buffer (0.09 M Tris-borate, 0.002 M EDTA at pH 8.0). The gel was dried at 80°C for 1 hr before exposure to autoradiography. The sequence of the C/EBP oligonucleotide used in this study is 5'-ggatccGCGT-TGCCGCACGATG-3' [Christy et al. 1991]. The double-stranded oligonucleotide was end-labeled by T4 DNA polymerase using cDNA probes and expression vectors. We also thank Deborah Dobson and Jacqueline Stephens for helpful discussions and advice on culturing 3T3 preadipocytes. This work was funded by U.S. Public Health Service grants DK45048 and CA39099 from the National Institutes of Health.

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Note added in proof

Zhu et al. [Proc. Natl. Acad. Sci. 92: 7921–7925, 1995] have recently demonstrated that the promoter of the mouse PPARγ gene contains two C/EBP sites.

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