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Tandem Repeat of C/EBP Binding Sites Mediates PPARγ2 Gene Transcription in Glucocorticoid-Induced Adipocyte Differentiation

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Abstract Bone marrow stromal stem cells differentiate into many different types of cells including osteoblasts and adipocytes. Long-term glucocorticoid treatment decreases osteoblastic activity but increases adipocytes. We investigated the mechanism of glucocorticoid-induced PPARγ2 transcription. Treatment of human bone marrow stromal cells with dexamethasone induced the differentiation of these cells into adipocytes as measured by oil-red O staining, and Northern blot analysis showed that dexamethasone strongly induced PPARγ2 mRNA expression in cells cultured in adipocyte induction medium. Moreover, the mRNA of C/EBPδ, an adipocyte-promoting transcription factor, was also induced by dexamethasone in the presence of induction medium. Gel mobility shift assays using purified GST-C/EBPδ fusion protein showed that C/EBPδ specifically binds to a 40-base pair DNA element from PPARγ2 promoter, which was found to contain a tandem repeat of C/EBP binding sites. Transfection studies in mouse mesenchymal C3H10T1/2 cells showed that it is the tandem repeat of the C/EBP binding site in PPARγ2 promoter region that regulates dexamethasone-mediated PPARγ2 gene activation. We conclude that glucocorticoid-induced adipogenesis from bone marrow stromal cells is mediated through a reaction cascade in which dexamethasone transcriptionally activates C/EBPδ; C/EBPδ then binds to PPARγ2 promoter and transactivates PPARγ2 gene expression. This activated master regulator, in turn, initiates the adipocyte differentiation. J. Cell. Biochem. 76:518–527, 2000.

Key words: bone marrow stromal cell; glucocorticoid; osteoporosis; adipocyte; osteoblast

Mesenchymal cells in higher vertebrates are replaced in response to repair, growth, and senescence. The replacement cells are derived from undifferentiated precursor cells. The bone marrow stromal cell [Pittenger et al., 1999; Prockop, 1997] provides a useful model for this process, being capable of forming osteoblasts, adipocytes, myocytes, and fibroblasts. Imbalanced differentiation between adipocytes and osteoblasts in bone marrow may lead to osteoporosis and bone fractures. Patients under glucocorticoid therapy accumulate large number of fat cells in their bone marrow, suggesting that glucocorticoids cause the conversion of marrow stem cells into adipocytes.

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; GST, glutathione S-transferase; IBMX; 3-isobutyl-1-methylxanthine; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element.

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Adipocyte differentiation is a complex process that requires communication between extracellular stimuli and a coordinated network of receptors and transcription factors in the nucleus. Many such nuclear factors have been identified [for recent reviews, see Gregoire et al., 1998; Darlington et al., 1998], among them the peroxisome proliferator-activated receptor-γ2 (PPARγ2) is a key regulator of adipocyte differentiation [Tontonoz et al., 1994a]. It is a member of the nuclear hormone receptor subfamily of transcription factors, expressed predominantly in fat [Chawla et al., 1994], and capable of activating a battery of genes necessary for lipid metabolism. These include lipoprotein lipase [Schoonjans et al., 1996], phosphoenolpyruvate carboxykinase [Tontonoz et al., 1995], fatty acid-binding and transport proteins [Tontonoz et al., 1994b; Frohnert et al., 1999], and stearoyl-CoA desaturase 1 [Miller et al., 1996]. Functional PPAR response elements (PPREs) have been identified in the regulatory regions of these genes. Activation of PPARγ2 by

its ligand [Forman et al., 1995; Kliewer et al., 1995] induces cell cycle withdrawal and terminal adipocyte differentiation in a variety of plastic mesenchymal cell lines [Lehmann et al., 1995; Gimble et al., 1996; Altiok et al., 1997; Hu et al., 1995]. The pivotal regulatory role that PPARγ2 plays during adipocyte differentiation is recognized by its tissue specific expression, its early expression [Chawla et al., 1994], and its ability to direct fibroblasts and myoblasts to differentiate into adipocytes when ectopically expressed in these cells [Tontonoz et al., 1994a; Hu et al., 1995].

In addition to PPARγ2, CCAAT/enhancer-binding proteins (C/EBPs) are implicated in adipocyte differentiation. C/EBPs are a family of bZIP nuclear transcription factors, these factors posses a leucine zipper and a basic domain at its carboxy-terminus which confer the ability of DNA binding and dimerization, either as homodimer or heterodimer with other family members [Lekstrom-Himes et al., 1998]. Three members of this family transcription factors, C/EBPα, -β, and -δ, have been shown directly involved in the induction of adipocyte differentiation [Darlington et al., 1998; Mandrup et al., 1997]. Of these three, C/EBPβ and C/EBPδ are expressed early and transiently in the process of adipogenesis and are induced respectively by cAMP and glucocorticoids [Cao et al., 1991; Wu et al., 1996]. Together, these two factors can synergistically induce C/EBPα and PPARγ2 expression [Darlington et al., 1998; Mandrup et al., 1997]. C/EBPα, however, is required to terminate mitotic clonal expansion for terminal differentiation [Umek et al., 1991] and cross-regulate PPARγ2 expression [Wu et al., 1999], thus maintaining the differentiated state [Christy et al., 1991].

Although glucocorticoids can induce bone marrow stromal cells to differentiate into adipocytes [Meunier et al., 1971; Lukert et al., 1990], the molecular basis is poorly defined. We studied PPARγ2 and C/EBPδ expression during differentiation of primary stromal cells. We found that glucocorticoids upregulates PPARγ2 expression, and that this upregulation correlates with its role in inducing stromal cells to differentiate into adipocytes. We show that glucocorticoids also upregulate C/EBPδ expression, and characterize the binding of C/EBPδ to a specific PPARγ2 gene promoter region.

MATERIALS AND METHODS

Isolation of Human Bone Marrow Stromal Cells

Human bone marrow stromal cells were isolated from surgical waste ribs or femoral head. Bone was flushed with α-MEM containing heparin (10 units/ml) and DNase I (1 μg/ml). The cells were pelleted at 500*g* for 10 min and the fat containing supernatant was decanted. The cells were resuspended in α-MEM containing 10% heat-inactivated fetal bovine serum and transferred to a 50 ml centrifuge tube containing 15 ml of histopaque-1077 (Sigma, St. Louis, MO). Following centrifugation at 500*g* for 30 min, the erythrocytes and granulocytes were removed, and the mononuclear cells at the interface were harvested, washed three times with α-MEM, and seeded in culture flasks.

Cell Culture and Differentiation

Human bone marrow stromal cells and mouse mesenchymal C3H10T1/2 cells (ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. For standard adipocyte differentiation, 2 days after cells reached confluence (referred as day 0), cells were exposed to differentiation medium, DMEM containing 10% FBS, 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 μM dexamethasone for 2 days. Cells then were maintained in DMEM with 10% FBS for 10 to 14 days with medium changed every third day. Adipocytes were identified by oil red O staining. Cells were washed with phosphate-buffered saline (PBS) and fixed in 10% formaldehyde in PBS for 1 h. After fixation, cells were washed with PBS and stained with 0.5% oil red O (Sigma) in isopropyl alcohol for 5 min. Cells then were washed with PBS and photographed.

Northern Blot Analysis

Total RNA (20 μg/lane) from human bone marrow stromal cells, or C3H10T1/2 cells, was separated by electrophoresis in agarose gel and blotted onto Nylon membranes (rapid downward transfer system, Schleicher & Schuell, Dassel, Germany). The RNA was cross-linked to the membrane with ultraviolet light. Following a 4-h prehybridization at 42°C (5 × Denhardt's solution, 40% formamide, 5 × SSC, 0.2% SDS, 20 mM sodium phosphate, 2 mM EDTA, and 0.1 mg/ml denatured salmon sperm DNA), the membrane was hybridized overnight in the same buffer and temperature to a α-[^{32}P]-dCTP

(NEN Life Science Products Inc., Boston, MA) randomly labeled PPARγ2 or C/EBPδ [Lin et al., 1994] cDNA probes. After hybridization, the membrane was washed twice for 15 min with 2 × SSC, 0.2% SDS at room temperature, and once for 15 min at 65°C. The wash was repeated under high stringency conditions using 0.2 × SSC, 0.2% SDS, and the blot was then exposed to X-ray film at -70°C.

Expression and Purification of GST-C/EBPδ Fusion Protein

To make GST-C/EBPδ fusion protein, the full-length C/EBPδ gene was amplified by polymerase chain reaction (PCR) from expression plasmid MSV-C/EBPδ (kindly provided by Steven L. McKnight, Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX) [Cao et al., 1991], and inserted in frame into pGEX-5X-1 (Amersham Pharmacia Biotech, Piscataway, NJ) between EcoR I and Sal I sites. BL21 bacterial cells were transformed with GST-C/EBPδ fusion plasmid. The fusion protein was purified by glutathione-agarose 4B (Sigma) following induction with isopropyl-β-D-thiogalactopyranoside. The pellet of BL21 cells from the isopropyl-β-D-thiogalactopyranoside induced culture was lysed with NETN buffer (20mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) in the presence of lysozyme and proteinase inhibitors. Following sonication and centrifugation to remove cell debris, the supernatant was collected and incubated at 4°C with glutathione-agarose beads in NETN. After several washes with NETN, the fusion protein was eluted with a buffer containing 20 mM glutathione and 50 mM Tris, pH 8. The protein concentration was quantified using Bradford assay (Bio-Rad Laboratories, Hercules, CA) and analyzed with SDS-polyacrylamide gels.

Electrophoretic Mobility Shift Assays

Three double-stranded oligomers were generated by annealing pairs of oligonucleotides (sense strands are shown). The tandem repeat, 5′-TGTATTTTACTGCAATTTTAAAAAGCAATCAATATTGAAC-3′, corresponding to nucleotide -311 to -351; the distal half site, 5′-TTTTACTGCAATTTTAAAAA-3′, corresponding to -328 to -348; and the proximal half site, 5′-TTTAAAAAGCAATCAATATT-3′, corresponding to -316 to -336 of the PPARγ2 promoter region (GenBank accession: S79043). The C/EBP binding sites are underlined. These DNA fragments were end-labeled with γ-[^{32}P]-ATP using T4 polynucleotide kinase. Unincorporated free nucleotides were removed by gel filtration (Micro-Spin G-25, Amersham Pharmacia Biotech). Binding reactions were carried out at room temperature in a buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 10% glycerol, 10 µg BSA, 3µg polydI/dC, and 1 mM DTT for 20 min. Each 20 µl reaction contained 2 µg of GST-fusion protein and 50,000 cpm of labeled probe. The Protein-DNA complexes were resolved in 4% polyacrylamide gels (acrylamide: bis = 80:1) containing 0.25 × TBE. For supershift assays, anti-C/EBPδ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reaction and incubated for an additional 10 min at room temperature.

Plasmid Constructs

A 615-base pair 5′ flanking region plus 64 base pairs of the 5′ untranslated region of the PPARγ2 gene was amplified by PCR from genomic DNA of the mouse mesenchymal C3H10T1/2 cell line using primers from the published sequence (GenBank accession: S79403). The primer sequences for up-stream are: 5′-ATATGAGCTCTTTATAGAATTTGGATAGCAGTA-3′ for 615Luc; 5′-ATATGAGCTCTTAAAAAGCAATCAATATTG-3′ for 334Luc; 5′-ATATTGAACAATCTCTGCTC-3′ for 320Luc. The down-stream primer common for all three is, 5′-ATATAAGCTTAACAGCATAAAACAGAGATTTGC-3′. Sac I and Hind III restriction sites were added to the up-stream and down-stream primers, respectively, underlined. The PCR products were inserted between Sac I/Hind III sites (615Luc and 334Luc), and Sma I/Hind III sites (320Luc) of pGL3-basic vector (Promega, Madison, WI) to generate reporter plasmid 615Luc, 334Luc, and 320Luc. A synthetic two-time repeat of C/EBP binding sequence, 2 × (5′-TGTATTTTACTGCAATTTTAAAAAGCAATCAATATTGAAC-3′), was inserted between the Kpn I and Xho I sites of a TK-Luc vector [Cao et al., 1993] to generate TK-2 × CAAT.

Transfection and Luciferase Assays

C3H10T1/2 cells were transiently transfected with the indicated constructs and the internal control pRL-SV40 vector using a mixture of cationic and neutral lipids (Tfx-50, Promega) as described [Shi et al., 1999]. When increasing amounts of expression vectors were trans-

fected, total DNA was kept constant by addition of PcDNA3 (Invitrogen, Carlsbad, CA). Luciferase activities were then assayed 48 h post-transfection with separate substrates to detect the luciferase (firefly) in the pGL3-basic plasmid and to the second luciferase (Renilla) encoded by the pRL-SV40 vector (dual luciferase assay kit, Promega) according to the manufacturer's directions. Values were normalized to the renilla luciferase activity expressed from pRL-SV40 reporter plasmid. Luciferase values shown in the figures are representative of transfection experiments performed in triplicate in at least three independent experiments.

RESULTS

Glucocorticoid Induces Adipocyte Differentiation of Human Bone Marrow Stromal Cells

To study the effects of the glucocorticoid dexamethasone on bone marrow stromal cell differentiation, we isolated human bone marrow stromal cells from human ribs or femur heads. The cells were cultured in DMEM or adipocyte induction medium containing insulin, isobutylmethylxanthine, or induction medium with dexamethasone. The dexamethasone-containing medium induced adipocyte differentiation by 14 days as demonstrated with oil red O staining (Fig. 1C). Cells cultured in DMEM (Fig. 1A), or induction medium without dexamethasone (Fig. 1B) had no lipid accumulation, although the cell morphology was altered somewhat in the induction medium (Fig. 1B). Similar results were obtained using the mouse mesenchymal cell line C3H10TI/2 (not illustrated). These results indicate that dexamethasone effectively induces adipocyte differentiation from bone marrow stromal cells cultured in induction medium.

Glucocorticoid Induces PPARγ2 Gene Transcription

Because PPARγ2 is a key regulator that initiates adipogenesis [Tontonoz et al., 1994a], whether dexamethasone can stimulate PPARγ2 mRNA expression in stromal cells was examined. Total RNA was harvested in cells cultured in control media, or treated with dexamethasone. Northern blot analysis showed that PPARγ2 mRNA expression was strongly induced in cells treated with dexamethasone (Fig. 2A, lane 3), while in cells grown in media without dexamethasone (lane 1 and 2) the PPARγ2 mRNA was almost non-detectable. These re-

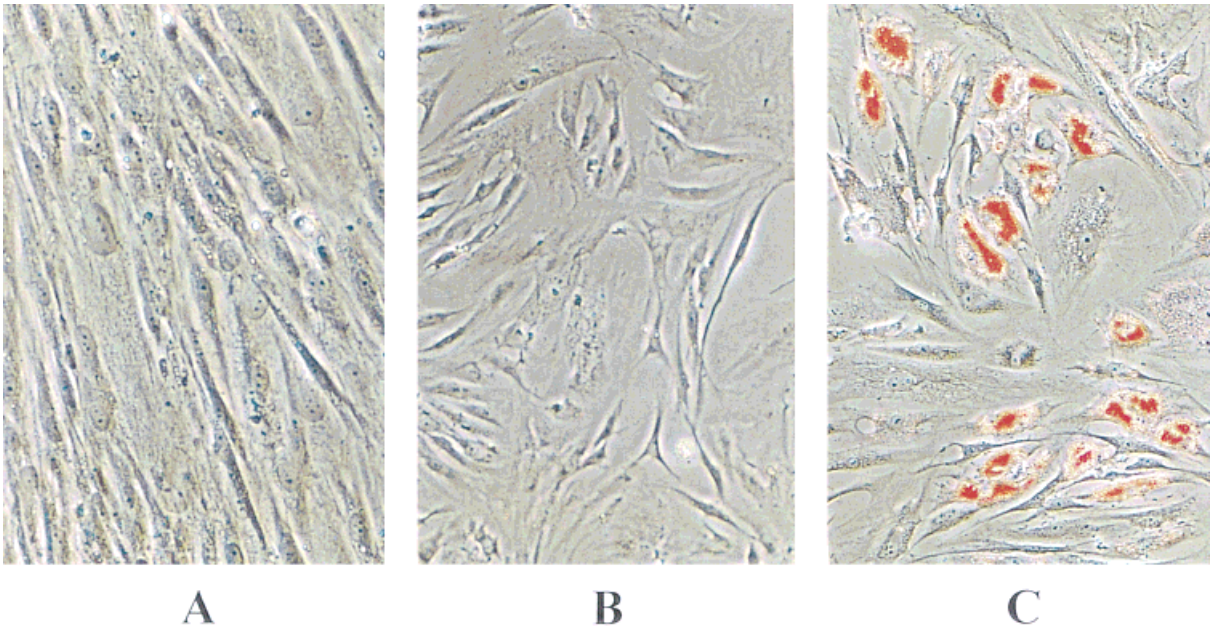

Fig. 1. Effect of dexamethasone on differentiation of human bone marrow stromal cells. Human bone marrow stromal cells were cultured for 14 days. **A**: Cells cultured in regular DMEM media. **B**: Cells cultured in DMEM containing 10 μg/ml insulin, 0.5 mM isobutylmethylxanthine. **C**: Cells cultured in DMEM containing 10 μg/ml insulin, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone. Each culture has been stained with oil-red O to show lipid as red color.

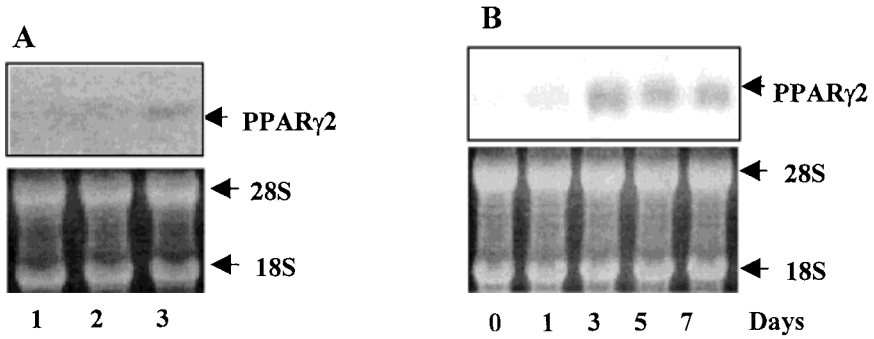

Fig. 2. Effect of dexamethasone on PPARγ2 mRNA expression in human bone marrow stromal cells. Northern blot analysis for PPARγ2 mRNA is shown. **A**: Total RNA from cells cultured in regular DMEM (**lane 1**), DMEM with 10 μg/ml insulin, 0.5 mM isobutylmethylxanthine (**lane 2**), and DMEM with 10 μg/ml insulin, 0.5 mM isobutylmethylxanthine, and 1 μM dexamethasone (**lane 3**), was separated on denaturing agarose gel, blotted onto positively charged Nylon membrane and hybridized with a ^{32}P-labeled PPARγ2 cDNA probe. Only the cells in dexamethasone express significant PPARγ2. **B**: Dexamethasone activates PPARγ2 mRNA expression in a time-dependent manner. Total RNA from cells cultured in differentiation medium in the presence of 1 μM dexamethasone was harvested at the times indicated and analyzed as in (A). Equal loading of lanes is shown by ethidium bromide staining of 28S and 18S ribosomal RNAs in each case.

sults were consistent with a role for dexamethasone-induced PPARγ2 in the observed differentiation (Fig. 1), so further experiments were performed. A time-course experiment showed that level of PPARγ2 mRNA peaked between 24 and 72 h. There was then essentially no change in PPARγ2 mRNA expression level over the times studied (to day 7; Fig. 2B). These results suggested that dexamethasone transcriptionally activated PPARγ2 gene expression in stromal cells cultured in induction medium. Genes regulated by glucocorticoids are classified into three categories: primary response gene, secondary response gene and primary delayed response gene. In primary response genes, glucocorticoid receptors directly interact with the regulatory DNA element of the target gene and stimulate gene transcription in hours. Conversely, secondary and delayed primary response genes respond to glucocorticoids depending on ongoing protein synthesis and the induction was delayed from hours to days. Apparently, this glucocorticoid-induced PPARγ2 gene transcription is not primary response mechanism, and it is likely involved in ongoing protein synthesis.

C/EBPδ Stimulates PPARγ2 Gene Transcription

To gain insight into the mechanism of glucocorticoid-induced PPARγ2 gene expression, we cloned a 615-base pair 5′ flanking region plus 64 base pairs of the 5′ untranslated region of the PPARγ2 gene. Analysis of the cloned promoter sequence revealed a tandem repeat of C/EBP binding sites, separated by eight base-pairs at –351 to –320 bases relative to the transcription start site. To examine whether the tandem repeat of C/EBP binding sites mediated glucocorticoid-induced transcription, we generated three promoter-reporter constructs with different regions of the promoter deleted (Fig. 3A). Cotransfection of these constructs with a C/EBPδ expression plasmid in mouse C3H10T1/2 cells showed that C/EBPδ stimulated the promoter activity of construct which contains the tandem repeat of C/EBP binding sites, 615Luc, more than 80-fold compared to luciferase activity in the absence of cotransfected C/EBPδ (Fig. 3B). Deletion of the distal C/EBP half site (334Luc) reduced C/EBPδ-induced the reporter activity 80%. These results indicated that the tandem repeat of C/EBP binding sites is the element mediating C/EBPδ stimulation. To validate that the tandem repeat of C/EBP binding sites is a functional response element, we inserted two copies of tandem repeat C/EBP binding sequence 5′ to a minimal thymidine kinase (TK) promoter-driving luciferase vector (TK-2 × CAAT construct, Fig. 4A). Cotransfection of TK-2 × CAAT with a C/EBPγ expression plasmid in C3H10T1/2 cells enhanced the luciferase expression 20-fold (Fig. 4B), indicating that the tandem repeat of C/EBP binding sites is a functional response element for C/EBPδ. We also examined whether glucocorticoids stimulate intrinsic C/EBPδ gene expression in C3H10T1/2 mesenchymal cells. The cells were cultured in differentiation medium without dexamethasone for 10 days and then exposed to 1 μM dexamethasone for 0–24 h.

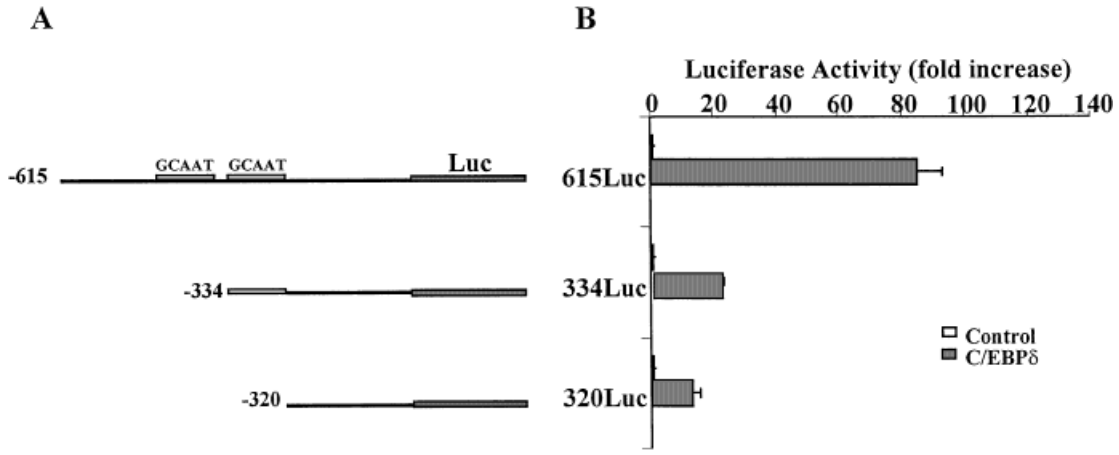

Fig. 3. Effect of C/EBPδ on PPARγ2 gene expression. **A**: Schematic diagram of the PPARγ2 promoter reporter construct. **B**: PPARγ2 promoter reporter construct (A) was cotransfected in C3H10T1/2 cells with or without a C/EBPδ expression plasmid. The cells were then incubated for 48 h and luciferase activity was measured.

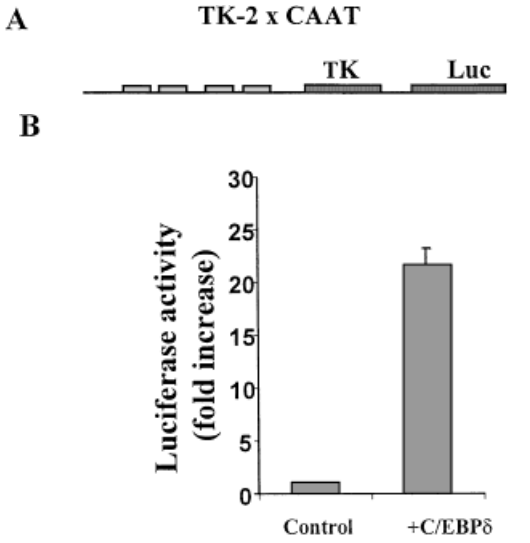

Fig. 4. Activation of the PPARγ2 gene by dexamethasone is mediated through a tandem repeat of C/EBP binding sites. **A**: Diagram of the TK-2 × CAAT luciferase reporter construct. **B**: The TK-2 × CAAT luciferase reporter construct was cotransfected with C/EBPδ expression plasmid into C3H10T1/2 cells. Bar 1: TK-2 × CAAT alone; Bar 2: TK-2 × CAAT cotransfected with C/EBPδ expression vector.

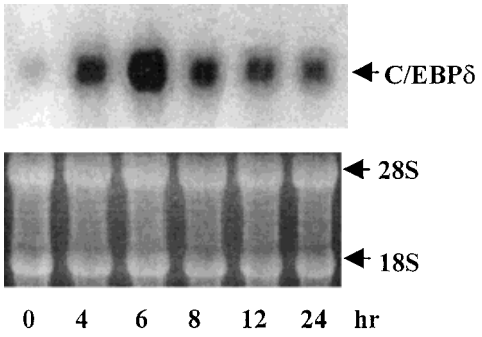

Fig. 5. C/EBPδ gene transcription is induced rapidly by dexamethasone. Northern analysis of C/EBPδ mRNA as a function of time after dexamethasone addition is shown. C3H10T1/2 cells were cultured in adipocyte induction medium for 10 days in the absence of dexamethasone, and then exposed to 1 μM dexamethasone for the indicated time period. Total RNA from these cells was separated on a denaturing agarose gel, transferred to membrane and hybridized with a randomly labeled C/EBPδ cDNA probe. Equal loading of lanes is shown by ethidium bromide staining of 28S and 18S ribosomal RNAs.

Northern blot analysis (Fig. 5) showed that dexamethasone induced C/EBPδ mRNA expression rapidly, reaching the peak level in 6 h and then declining.

C/EBPδ Binds to the PPARγ2 Promoter

Having demonstrated that dexamethasone induces C/EBPδ expression and that C/EBPδ transactivates PPARγ2 gene transcription, how C/EBPδ recognizes and binds to the tandem repeat of the C/EBP binding sites was studied. Gel mobility shift assays were performed using three double-stranded oligonucleotide probes that include either the distal half site (-328 to -348), the proximal half site (-316 to -336), or the tandem repeat element (-311 to -351). These oligonucleotides were radiolabeled and incubated with affinity purified GST-C/EBPδ fusion protein in gel-shift assays. As shown in Figure 6, C/EBPδ bound preferentially to the tandem repeat element with high affinity (lanes 9 and 12), whereas either of the single C/EBP binding sites showed relatively low binding affinity for C/EBPδ (lane 3, distal half site; lane 6, proxi-

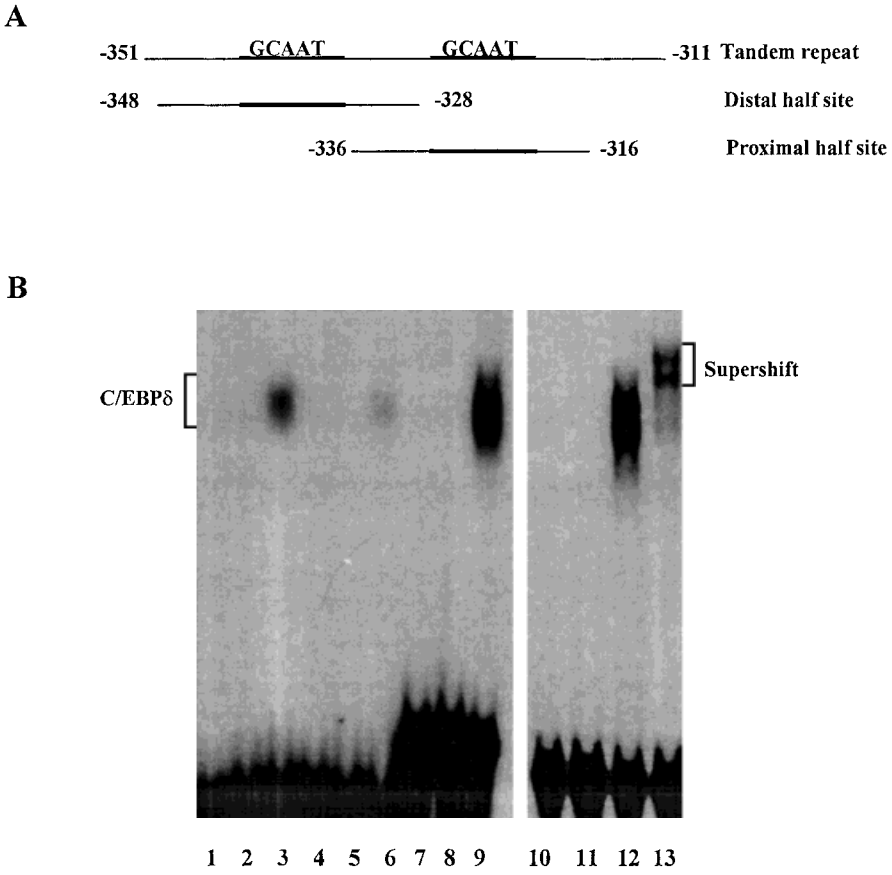

Fig. 6. C/EBPδ preferentially binds to a 40-bp DNA element containing a tandem repeat of C/EBP binding sites within the PPARγ2 promoter. **A**: Diagram showing the regions of PPARγ2 promoter used as probes in gel shift assay. **B**: Electrophoretic mobility shift assay. Double stranded oligonucleotide probes of distal (**lanes 1–3**), proximal (**lanes 4–6**), and tandem repeat (**lanes 7–13**) of the C/EBP binding sites were end-labeled and incubated with purified GST-C/EBPδ fusion protein. The DNA-protein complexes were subjected to non-denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Lanes 1, 4, 7, and 10 contained no protein; Lanes 2, 5, 8, and 11 contained GST protein; Lanes 3, 6, 9, and 12 contained GST-C/EBPδ fusion protein. The binding specificity was confirmed by supershift of the DNA-protein complex using anti-C/EBPδ antibody (lane 13). Lanes 10–12 are repeats of lanes 7–9 on a separate gel.

mal half site), indicating that the tandem repeat element is a novel C/EBP binding element. Furthermore, development of the retarded band was supershifted by the anti-C/EBPδ monoclonal antibody. Taken together, these results demonstrated that C/EBPδ is a mediator that relays the dexamethasone effect to PPARγ2, although it can not be ruled out that other dexamethasone-responsive proteins may also influence PPARγ2 expression, since some activity remained after deletion of the tandem repeats (Fig. 3).

DISCUSSION

Glucocorticoids are widely used to maintain organ-transplants, and are useful in the management of rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, and leukemia [Baxter et al., 1979]. However, long term administration of glucocorticoids causes serious skeletal problem. Fat accumulates in the bone marrow, and bone density decreases [Meunier et al., 1971]. This eventually results in fractures in 30–50% of the patients who receive chronic glucocorticoid therapy [Elmstedt, 1981]. Bone is dynamically formed by osteoblasts and resorbed by osteoclasts throughout life. The bone mass is balanced by accurate coordination between osteoblastic and osteoclastic activity. Compelling evidence indicates that glucocorticoids induce differentiation of bone marrow stromal cells, stem cells of osteoblasts, into adipocytes [Cui et al., 1997]. If this is the situation, glucocorticoid-induced adipogenesis can effectively displace stromal cells from the osteoblast differentiation pathway and impair the bal-

anced bone metabolism. The intracellular mechanism responsible for this action, however, is not understood. Because PPARγ2 is a key adipocyte regulator, and because C/EBPδ is induced rapidly in response to glucocorticoid stimulation (Fig. 5), we hypothesized that C/EBPδ might be the mediator that relays the glucocorticoid signal to PPARγ2. To test this hypothesis we studied the effects of glucocorticoid on PPARγ2 and C/EBPδ expression, and further, characterized the binding of C/EBPδ to PPARγ2 promoter and its biological function in regulating PPARγ2 gene expression. Although C/EBPα is also an important factor, it coordinates PPARγ2 synergizing adipogenic effects [Hu et al., 1995], it is unlikely an initial inducer, because it is expressed just prior to the initiation of transcription of most adipocyte-specific genes [Mandrup et al., 1997; Lane et al., 1996]. C/EBPβ and C/EBPδ function early in the program leading to adipocyte differentiation [Cao et al., 1991], and both of them are involved in induction of PPARγ2 [Wu et al., 1996; Cao et al., 1991]. Of these two, C/EBPβ is induced by cAMP [Cao et al., 1991; Wu et al., 1996] and C/EBPδ is induced by glucocorticoids (Fig. 5), so it is logical that C/EBPδ is the mediator that relays the effects of glucocorticoids to PPARγ2. Importantly, C/EBPδ binds the PPARγ2 promoter (Fig. 6) and transactivates PPARγ2 gene transcription (Fig. 3). It is also likely that C/EBPβ binds to the tandem repeat sequence and heterodimerizes with C/EBPδ [Wu et al., 1996].

Once PPARγ2 gene is activated during adipogenesis, its expression level is maintained to the terminal differentiation. However, C/EBPδ expression is transiently regulated by glucocorticoids (Fig. 5), which is not sufficient to maintain the level of PPARγ2 gene expression. To reach the relative constant level of PPARγ2 gene expression (Fig. 2B), it is likely that C/EBP isoforms are also involved. For example, GST-C/EBPβ binds to the tandem repeat element equally well as C/EBPδ (data not shown). It is also important to know that glucocorticoid-induced adipocyte differentiation requires presence of induction medium including insulin and IBMX (Figs. 1 and 2). Neither induction medium nor glucocorticoids alone have such effect. IBMX has been shown to increase expression of C/EBPβ, and this increase is required for subsequent PPARγ gene expression. Insulin, on the other hand, regulates C/EBPα gene expression. Furthermore, glucocorticoid dexamethasone can stimulate C/EBPα [Crosson et al., 1997; Ramos et al., 1996] and C/EBPβ [Boudreau et al., 1996] expression in certain cell types. Therefore, the tandem repeat of C/EBP sites may serve as the key C/EBP regulatory element in maintaining PPARγ2 gene expression during the course of adipocyte differentiation.

Our understanding of transcriptional control of adipogenesis is based on information from in vitro studies. Preadipocyte, fibroblast, and multipotential mouse C3H10T1/2 cell lines have been studied extensively. Yeh et al. [1995] showed a modest stimulation of adipocyte conversion when 3T3-L1 preadipocytes were infected with a retrovirus-directed C/EBPδ expression vector (MFG-Cδ), which is consistent with our observations (Fig. 3). Recent in vivo studies in mice deficient in C/EBPβ, C/EBPδ [Lekstrom-Himes et al., 1998], or both C/EBPβ and C/EBPδ [Tanaka et al., 1997], and transgenic mice expressing A-ZIP/F [Moitra et al., 1998], are defective in lipid storage and carbohydrate metabolism but express C/EBPα and PPARγ2 normally. A-ZIP/F is a dominant-negative protein that prevents the DNA binding of bZIP transcription factors of both the C/EBP and Jun families. These in vivo results highlight the importance of the micro-environment, as well as the importance of the communications between extracellular stimuli and adipogenesis-signaling network that determines the fate of the stromal cells as to proliferate or differentiate. Based on this information, we speculate that the initiation of adipocyte differentiation requires an elevated expression of PPARγ2 and/or C/EBPα to surpass a threshold. The signals that lead to break this threshold could be a high nutritional dietary, or physipathological conditions that increase the production of hormones. Laboratory mice deficient in C/EBPβ and/or C/EBPδ express normal, but not elevated PPARγ2 and C/EBPα and do not accumulate fat. In the in vitro situation, the cells are forced to overexpress C/EBPβ and C/EBPδ by a cocktail of stimulants that are provided at much higher concentrations than are present under physiological conditions. The elevated C/EBPβ and C/EBPδ synergistically induce the expression of PPARγ2 and C/EBPα results in adipocyte differentiation.

Our studies demonstrate that glucocorticoids effectively induce human bone marrow stromal cells to differentiate into adipocytes. This pro-

cess is mediated, at least in part, by a cascade reaction in which the glucocorticoid activates C/EBPδ transcription. C/EBPδ then binds to the PPARγ2 promoter and transactivates PPARγ2 expression. Finally, this activated key regulator activates the entire adipocyte differentiation program. While there clearly must also be balancing processes, since osteoblasts do not disappear after glucocorticoid treatment, this would promote unbalanced stem cell differentiation in favor of fat cells. This would have the effect of depleting stem cells needed to differentiate into other cell types, particularly osteoblasts.

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