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CAAT/Enhancer Binding Proteins Directly Modulate Transcription from the Peroxisome Proliferator-Activated Receptor γ2 Promoter

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The CCAAT/enhancer binding proteins (C/EBPs) and the peroxisome proliferator-activated receptors (PPARs) together regulate adipogenesis. The current work uses co-transfection studies to examine the C/EBP dependence of PPARγ2 transcription. Both C/EBPα and C/EBPδ expression vectors activated transcription from a PPARγ2 promoter/luciferase expression vector by 5-6 fold in UMR106 cells. The simultaneous transfection of the C/EBP homologous protein (CHOP) (also known as growth arrest DNA damage protein 153 or *gadd153*) inhibited this C/EBP-dependent activation in a concentration dependent manner. The CHOP protein is known to heterodimerize with other C/EBP proteins to form transcriptionally inactive complexes. Mutation of the two C/EBP DNA recognition elements at −340 bp and −327 bp within the PPARγ2 promoter reduced the inductive effects of both C/EBPα and C/EBPδ. These findings demonstrate that proteins within the C/EBP family directly modulate transcription from the PPARγ2 promoter.

The peroxisome proliferator-activated receptors (PPARs) are a novel class of steroid receptors which are encoded by three mammalian genes: PPARα, PPARγ, and PPARδ (1,2). One of these genes, PPARγ, utilizes alternative promoter sites (3) to express two isoforms, PPARγ1 (3,4), which is found in the liver and other organs, and PPARγ2 (5), which is selectively expressed in adipose tissues. In general, the PPARs regulate transcription of genes related to lipid metabolism (1,2); in particular, the PPARγ2 protein regulates adipogenesis. The PPARs are activated by specific ligands which include natural compounds, such as long chain fatty acids, and synthetic compounds, such as the oral anti-diabetic thiazolidinedione drugs (1,2). Ligands for PPARγ2 induce adipocyte differentiation in cells naturally expressing the PPARγ2 protein or where it has been introduced by transfection (1,2,5,6,7).

Likewise, the CCAAT/enhancer binding proteins (C/EBPα, C/EBPβ, and C/EBPδ) are transcriptional regulators of adipogenesis (8,9). Studies have shown that transfection of C/EBP cDNA expression vectors would convert fibroblasts into pre-adipocytes (10,11) and that anti-sense C/EBP vectors inhibited adipogenesis in pre-adipocyte models (12,13). Additional experiments demonstrated that a synergy exists between C/EBP and PPAR proteins; co-transfection of C/EBP and PPARγ2 expression vectors was sufficient to induce adipogenesis in the absence of exogenous ligands (14).

However, the C/EBP family is not limited to positive acting transcription factors. The murine C/EBP homologous protein (CHOP), known as growth arrest DNA damage protein 153 (*gadd153*) in the hamster, is a negative regulatory transcription factor. CHOP was originally cloned from fully mature adipocytes (15) while *gadd153* was identified by differential hybridization of a cDNA library prepared from growth arrested Chinese hamster ovary cells (16, 17). The CHOP/gadd153 protein contains the amino acid sequences necessary to form heterodimers with other C/EBP proteins (15,18) but lacks a DNA binding domain. Consequently, the expression of CHOP/*gadd153* has been found to inhibit adipocyte differentiation (19).

Recent studies have investigated the mechanisms regulating PPARγ2 transcription. Sequence analysis of the murine PPARγ2 promoter has identified two C/EBP recognition elements (GCAAT) at positions −340bp and −327 bp relative to the transcriptional

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start site (20,21). Stable transfection of the full length C/EBP expression vectors into 3T3 fibroblasts has been found to increase PPARγ2 expression during adipocyte differentiation (22-24). These experiments demonstrate that C/EBP proteins regulate PPARγ2 transcription. The current work extends these findings by directly examining the expression from a PPARγ2 promoter/reporter construct transfected in the absence and presence of C/EBP expression vectors.

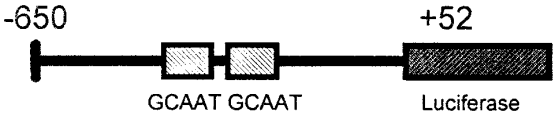

FIG. 1. The murine PPARγ2 promoter expression construct. The fragment containing −609 bp to +52 bp of the murine PPARγ2 promoter was subcloned between the SalI and KpnI sites of the p19Luc vector. The location of C/EBP recognition sites (GCAAT) at −340 bp and −327 bp are indicated.

MATERIALS AND METHODS

Materials. All reagents were purchased from Fisher Scientific (Dallas TX) or Sigma Chemical Co. (St. Louis MO) unless otherwise noted. The rat osteosarcoma-derived UMR106 cells (ATCC CRL 1661) (25) were obtained from the American Type Culture Collection (Rockville MD). Oligonucleotides were synthesized by Dr. Ken Jackson, Oklahoma Center for Molecular Medicine and the William K. Warren Medical Research Institute, OUHSC. The SV40 promoter/β-galactosidase vector was obtained from Promega (Madison, WI).

Vector construction. The murine PPARγ2 promoter was cloned by polymerase chain reaction from genomic DNA using oligonucleotide primers based on the published sequence (19) with an additional 5′ SalI site and 3′ KpnI site for subcloning purposes:

bp −609 TTTGTCGAC GAATTTGGATAGCAGTAACATTTTGGACC bp −581
bp +52 TTTGGTAC CAGAGATTTGCTGTAATTCACACTGGTG bp +25

The PCR fragment was blunt ended by incubation with the Klenow fragment of DNA polymerase, cloned into the SmaI site of the pBluescript SKII vector (Stratagene, San Diego CA), and the sequence confirmed using the Sequenase Version 2 enzyme (Amersham, Arlington Hts. IL) (26). Mutagenesis of the promoter in the pBluescript vector was performed using the QuikChange Mutagenesis Kit (Stratagene, San Diego, CA) according to the manufacturers instructions; PCR reactions were performed for 18 cycles with settings of 95°C, 30 sec; 55° C, 1 min; 68° C, 7 min. The following oligonucleotide primers were used (mutated sites are underlined):

bp −357 GGTGTGTATTTTACTAGTATTTTAAAAAAAAATCAATATTGAAC −313 bp
bp −313 GTTCAATATTGATTTTTTTTTAAAATACTAGTAAAATACACACC −357 bp

The promoter fragments were excised by SalI/KpnI restriction digestion and subcloned between the SalI and KpnI sites of the p19Luc luciferase reporter vector polylinker (27). The full length murine coding sequences indicated were excised with restriction the enzymes listed from the following vectors: C/EBPα (EcoRI/HindIII fragment from MSV/CEBP), C/EBPδ (EcoRI/BamHI fragment from MSV/CEBPδ) (MSV/CEBP) vectors provided courtesy of Dr. S. McKnight, UT Southwestern) (8,28) and CHOP (NcoI/BglII fragment from CHOP10 7ZAd) (provided courtesy of Dr. D. Ron, NYU) (15). Each fragment was blunted with the Klenow fragment of DNA polymerase I, ligated with BstXI linkers and subcloned into the BstXI site of the eukaryotic expression vector, pEF-BOS (provided courtesy of Dr. K. Oritani, OMRF) (29).

Cell culture and co-transfection protocol. The UMR106 cell line was maintained in Dulbecco's Modified Eagle's Medium (high glucose DMEM) supplemented with 10% fetal bovine serum (FBS)(Hyclone, Logan UT), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. Each experiment contained duplicate or triplicate plates and was repeated a minimum of three times. Cells were plated in 35 mm dishes at a density of 3.1×10^4 cells/cm^2, incubated for 18 hours, rinsed with DMEM medium without fetal bovine serum and maintained in 1 ml of serum-free DMEM medium. The cationic detergent, polyethylenimine (Fluka Chemical Co., Switzerland) was prepared in advance as a stock solution of 0.09% (wt/vol) pH 7.0 and sterile filtered prior to use (30). The DNA (6 μg/plate) was pre-incubated with the polyethylenimine (12 μl/plate) in polystyrene tubes in a volume of 100 μl of 0.15 M NaCl for 45 min. A total of 1 μg of PPARγ2 promoter/luciferase reporter construct, 1 μg SV40/β galactosidase reporter construct and 2 μg of each C/EBP expression construct in the pEF-BOS vector were used; the empty pEF-BOS vector was used to maintain a constant amount of DNA in each transfection assay. The DNA was added directly to the cells and these were incubated in the absence of serum for 5 hrs. At that time, the cultures were supplemented with 1 ml DMEM supplemented with 20% FBS (final concentration, 10% FBS), incubated overnight, and the medium replaced with 10% FBS/DMEM the following morning. After an additional 24 hour incubation, the cells were harvested in 100 μl of 25 mM glycylglycine, 15 mM $MgSO_4$, 1 mM dithiothreitol and 1% Triton X-100 and centrifuged at 8000 × g for 6 min at 4° C. Aliquots of the supernatant were assayed for luciferase activity in duplicate using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego CA) according to previously published methods (31). Aliquots of the supernatant were assayed for β-galactosidase activity by incubation in a volume of 300 μl containing a final concentration of 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 0.66 mg/ml of o-nitrophenyl-β-D-galactopyranoside (ONPG) for 30 min to 2 hr at 37° C. The reaction was terminated by the addition of 500 μl of 1 M sodium carbonate and the OD_{420} measured; values were converted to β-galactosidase units by comparison to a standard curve created by assays using 0 to 6 milliunits of the pure β-galactosidase enzyme (Promega, Madison WI). Luciferase activity per unit volume of cell lysate was normalized relative to the β-galactosidase activity per unit volume to control for transfection efficiency differences between plates.

Statistical analysis. Results were compared by repeated measures one way analysis of variance (ANOVA) and the Student-Newman-Keuls test.

RESULTS AND DISCUSSION

Induction of the murine PPARγ2 promoter by C/EBPα and C/EBPδ. The initial experiments examined the role of C/EBP proteins in the regulation of PPARγ2 transcription. All cultures were transfected with a PPARγ2 promoter (−602 to +52)/luciferase reporter construct (Figure 1) and an SV40/β-galactosidase vector to control for transfection efficiency. Cultures were co-transfected with EF promoter-driven expression vectors containing the full-length coding cDNAs for murine C/EBPα, C/EBPδ and/or CHOP (Fig-

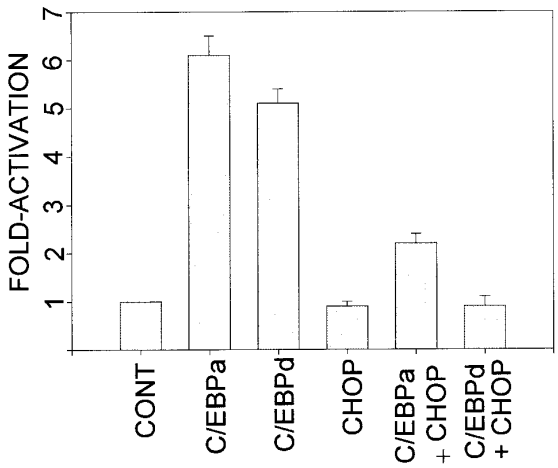

FIG. 2. C/EBPα and C/EBPδ activate the PPARγ2 promoter in co-transfection experiments. UMR106 rat osteoblast-like cells were co-transfected with an SV40 promoter/β-galactosidase vector, the PPARγ2 reporter construct and eukaryotic expression vectors for each of the C/EBP family members. Luciferase activity was normalized relative to β-galactosidase activity to control for transfection efficiency. The level of luciferase activity in the absence of cotransfected C/EBP cDNAs was defined as "1"; -fold activation was determined relative to this level of activity. Values indicated in the figure are the mean ± S.E.M. from n = 4 individual experiments. All cultures were transfected with equal quantities of plasmid DNA. The empty pEF-BOS expression vector was used to balance between individual experimental conditions to rule out any effect of the vector itself on these results.

ure 2). To rule out any artifactual effect of the EF promoter, transfections included the empty pEF-BOS vector as necessary to achieve equal plasmid concentrations between experiments. Transcription from the PPARγ2 promoter increased 5-6-fold above baseline in the presence of either C/EBPα or C/EBPδ. The presence of the full-length CHOP expression vector had no significant effect by itself. However, when CHOP was co-transfected at equal plasmid concentrations with either C/EBPα or C/EBPδ, transcription activation was significantly ($p<0.05$) reduced.

Dose-dependent inhibition of C/EBPα and C/EBPδ by CHOP. To confirm the negative regulatory effect of CHOP, co-transfections were performed with a constant amount of the C/EBPα (Figure 3A) or C/EBPδ (Figure 3B) expression vectors and increasing amounts of the CHOP vector. Again, the final concentration of plasmid was held constant in all co-transfections by the addition of the empty expression vector, pEF-BOS, to appropriate plates. The CHOP vector significantly ($p<0.05$) inhibited transcription from the PPARγ2 promoter relative to the maximum levels of C/EBPα and C/EBPδ induction (defined as 100%) in a dose-dependent manner. These findings demonstrate that the CHOP protein can negatively regulate C/EBP-activated transcription from the PPARγ2 promoter.

Mutagenesis of the C/EBP consensus recognition elements in the PPARγ2 promoter. The PPARγ2 promoter contains two C/EBP consensus recognition elements at −340 bp and −327 bp relative to the transcriptional start site. Site-directed mutagenesis was performed to determine if these consensus elements mediated transcriptional activation by C/EBPα and C/EBPδ. Co-transfections were performed with both the wild type and mutated luciferase reporter constructs (Figure 4). Mutation of the two C/EBP consensus recognition elements in the PPARγ2 promoter reduced both C/EBPα and C/EBPδ activation by >50% as compared to the wild type promoter construct. These results are

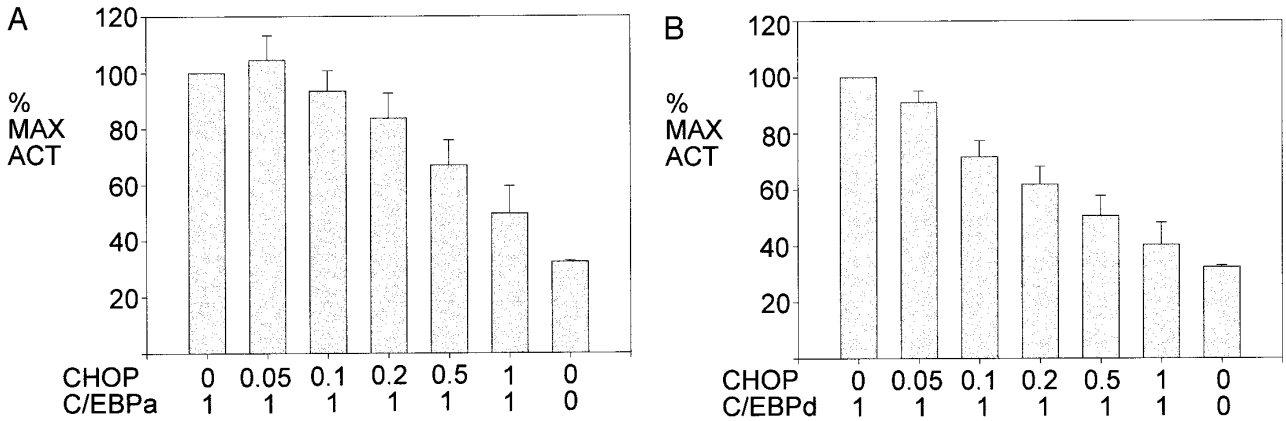

FIG. 3. CHOP inhibits activation of the PPARγ2 promoter by C/EBPα and C/EBPδ. The UMR106 cells were co-transfected with the SV40 promoter/β-galactosidase vector, the PPARγ2 promoter/luciferase reporter construct, and either the C/EBPα (A) or C/EBPδ (B) expression constructs in the presence of increasing concentrations of the CHOP expression construct. Values are reported relative to the maximal level of activation in the presence of C/EBP expression constructs alone (defined as 100%). The total amount of DNA in each transfection was kept constant by the addition of the appropriate amount of the empty pEF-BOS vector. Luciferase values were normalized relative to the β-galactosidase activity. Values indicate the mean ± S.E.M. from n = 3 individual experiments.

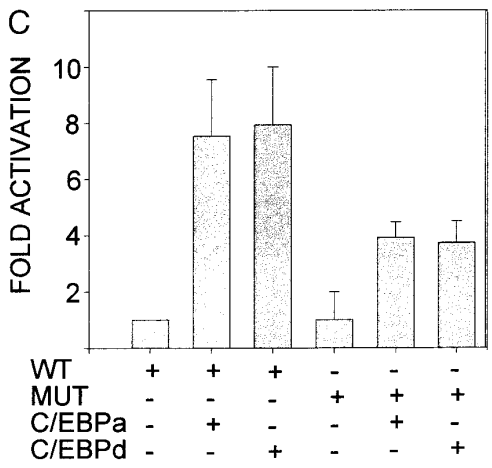

FIG. 4. Mutation of the C/EBP recognition elements reduces activation of the PPARγ2 promoter by C/EBPα and C/EBPδ. The sequence of the mutated (Mut) murine PPARγ2 promoter between −345 bp and −320 bp is shown in Materials and Methods. The wild-type and mutated luciferase expression constructs were transfected with the SV40 promoter/β-galactosidase vector alone or in the presence of either the C/EBPα or C/EBPδ expression constructs. Fold-activation of the normalized luciferase values were determined relative to the luciferase expression in the absence of co-transfected C/EBP expression constructs. Values reported are the mean ± S.E.M. from n = 4 individual experiments.

consistent with a direction action by C/EBP proteins on PPARγ2 transcription.

Conclusions. In the current work, we have examined the mechanism regulating transcription from the PPARγ2 promoter. We find that both C/EBPα and C/EBPδ induce expression from a PPARγ2 promoter reporter construct in co-transfection experiments. These results support and extend previous reports that C/EBP transfection increased levels of the endogenous PPARγ2 mRNA in 3T3-L1 cells (22-24). In our co-transfection studies, the positive effects of C/EBPα and C/EBPδ were reduced when the negative heterodimeric partner, CHOP/gadd153, was simultaneously expressed or when the C/EBP DNA recognition elements in the PPARγ2 promoter were mutated. These findings are consistent with previous observations demonstrating that CHOP/gadd153 protein inhibited adipogenesis (19). Multiple agents causing stress to cellular metabolism are known to increase *CHOP/gadd153* expression through a mechanism involving the endoplasmic reticulum and protein processing (16,17,32-36). The possibility exists that CHOP/gadd153 and C/EBP proteins act together in a feedback loop to modulate PPARγ2 transcription in adipocytes.

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