Blockage of oncostatin M-induced LDL receptor gene transcription by a dominant-negative mutant of C/EBP β

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OM (oncostatin M) activates the human LDLR [LDL (low-density lipoprotein) receptor] gene transcription in HepG2 cells through the SIRE (sterol-independent regulatory element) of LDLR promoter. The SIRE sequence consists of a C/EBP (CCAAT/enhancer-binding protein)-binding site and a CRE (cAMP-response element). Our previous studies [Zhang, Ahlborn, Li, Kraemer and Liu (2002) *J. Lipid Res.* **43**, 1477–1485; Zhang, Lin, Abidi, Thiel and Liu (2003) *J. Biol. Chem.* **278**, 44246–44254] have demonstrated that OM transiently induces EGR-1 (early growth response gene product 1) expression and EGR-1 activates LDLR transcription primarily through a protein–protein interaction with C/EBP β , which serves as a co-activator of EGR-1. In the present study, we examined the direct role of C/EBP β as a transactivator in OM-regulated LDLR gene transcription independent of EGR-1. We show that OM induces C/EBP β expression with kinetics slower than EGR-1 induction. A significant increase in C/EBP β protein level is detected by 2 h of OM treatment and remains elevated for 24 h. Chromatin immunoprecipitation assays demonstrate that the amount of C/EBP β

bound to the LDLR SIRE sequence is increased 2.8-fold of control by 2 h of OM treatment, reached the highest level of 8-fold by 4 h, and slowly declined thereafter. To further examine the requirement of C/EBP β in OM-stimulated LDLR expression, we developed a His-tagged dominant-negative mutant of C/EBP β (His–C/EBP β -P4; where P4 is plasmid 4 in our mutation series), consisting of the DNA-binding and leucine zipper domains of C/EBP β (amino acids 246–345). Expression of His–C/EBP β -P4 in HepG2 cells significantly diminishes the OM-induced increase in LDLR promoter activity and the elevation of endogenous LDLR mRNA expression. Taken together, these new findings identify C/EBP β as an OM-induced transactivator in LDLR gene transcription and provide a better understanding of the molecular mechanism underlying the sterol-independent regulation of LDLR expression.

Key words: CCAAT/enhancer-binding protein (C/EBP β), chromatin immunoprecipitation, early growth response gene product 1 (EGR-1), low-density lipoprotein (LDL) receptor, oncostatin M, sterol-independent regulatory element.

INTRODUCTION

C/EBP (CCAAT/enhancer-binding protein) transcription factors comprise a family of related basic leucine zipper DNA-binding proteins that bind to its recognition motif on target genes as homodimers or heterodimers with its own family members or with CREB [CRE (cAMP-response element)-binding protein] family members that all possess the bZIP DNA-binding structure [1–3]. The C/EBP family includes C/EBP α , C/EBP β , C/EBP γ , C/EBP ϵ and C/EBP δ [2,3]. NF-IL6 [nuclear factor that binds to an IL-1 (interleukin-1)-responsive element in the IL-6 gene] is the human homologue of rat C/EBP β [4]. Despite the highly similar DNA-binding domains and dimerization domains, C/EBP family members have been shown to differentially regulate gene transcription.

The involvement of C/EBP β in regulating the transcription of human LDLR [LDL (low-density lipoprotein) receptor] gene was previously identified by our laboratory in studies to define a sterol-independent regulatory pathway utilized by cytokine OM (oncostatin M) to activate LDLR gene expression in HepG2 cells [5,6]. OM increases LDLR mRNA expression independent of intracellular levels of sterols. Through deletional and mutational analyses, we localized the OM-responsive sequence to the LDLR

promoter region –17 to –1 and designated this regulatory *cis*-acting element the SIRE (sterol-independent regulatory element), which lies downstream of the classical SRE-1 (sterol response element 1) of the LDLR promoter [7]. The SIRE motif consists of a C/EBP-binding site and a CRE. Mutations within the SIRE sequence either at the C/EBP-binding site or at CRE completely abolish the OM-induced LDLR promoter activity without affecting the basal transcriptional rate. By performing a series of *in vitro* and *in vivo* experiments, we identified the transcription factor EGR-1 (early growth response gene product 1) as the OM-induced transactivator that binds to the SIRE motif of the LDLR promoter [8]. We further demonstrated that EGR-1 regulates LDLR transcription through a novel activation mechanism that is different from its traditional act of DNA binding [9]. Whereas EGR-1 has very weak binding affinity to the SIRE sequence by itself, its interaction with LDLR promoter is greatly enhanced in the presence of nuclear proteins of HepG2. Mammalian two-hybrid system revealed that EGR-1 specifically interacts with C/EBP β , but not with C/EBP α nor with CREB or with other SIRE-binding proteins, and this interaction is mediated through the transactivation domain of EGR-1. However, it was not understood how the transient expression of EGR-1 could result in an activation of LDLR transcription for a much extended

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CHOP, C/EBP homologous protein; CRE, cAMP-response element; CREB, CRE-binding protein; DAPI, 4',6-diamidino-2-phenylindole; EGR-1, early growth response gene product 1; EMEM, Eagle's minimum essential medium; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDL, low-density lipoprotein; LDLR, LDL receptor; IL, interleukin; NF-IL6, nuclear factor that binds to an IL-1-responsive element in the IL-6 gene; OM, oncostatin M; SIRE, sterol-independent regulatory element; SRE-1, sterol response element 1; SREBP, sterol-regulatory-element-binding protein; SV40, simian virus 40.

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time in OM-treated cells. This raised one possibility that other transactivators might be utilized by OM to increase LDLR transcription at the later time point of OM treatment when EGR-1 expression level has declined.

In the present study, we demonstrate that C/EBP β expression and interaction with the SIRE motif are induced by OM in HepG2 cells with distinct kinetics than OM induction of EGR-1. By developing and utilizing a dominant-negative mutant of C/EBP β , we provide strong evidence to demonstrate that C/EBP β directly participates in LDLR transcription as an OM-induced transactivator in addition to its role as the cofactor for EGR-1. The persistent association of C/EBP β with LDLR promoter *in vivo* upon OM stimulation is likely to be responsible for maintaining high level of LDLR transcription in HepG2 cells.

MATERIALS AND METHODS

Cells and reagents

The human hepatoma cell line HepG2 was obtained from A.T.C.C. (Manassas, VA, U.S.A.) and was cultured in EMEM (Eagle's minimum essential medium) supplemented with 10% (v/v) FBS (fetal bovine serum; Summit Biotechnology, Fort Collins, CO, U.S.A.). Antibodies to C/EBP β (sc-150), His₆ tag (sc-803) and EGR-1 (sc-110) were obtained from Santa Cruz Biotechnology. Anti-actin was obtained from Chemicon.

Plasmid constructions

The construction of a His-tagged full-length C/EBP β named His-C/EBP β -P1 (where P1 is plasmid 1 in our mutation series) has been previously described [9]. To construct His₆-tagged truncated C/EBP β fusion proteins, different coding regions of C/EBP β were amplified by PCR using pEF-NFIL6 as the template and the PCR products were cloned directly into the pcDNA4/HisMax-TOPO vector. All vectors were sequenced to confirm the correct sequence and direction. Expressions of His-C/EBP β -P1 (amino acids 24–345), His-C/EBP β -P2 (amino acids 24–145), His-C/EBP β -P3 (amino acids 24–245) and His-C/EBP β -P4 (amino acids 246–345) in HepG2 cells were confirmed by immunoblotting with anti-His₆ monoclonal antibody.

Quantification of LDLR mRNA expression by Northern-blot analysis and real-time PCR

Analysis of LDLR and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNAs by Northern blotting and quantitative real-time PCR was conducted as we previously described [10].

Western-blot analysis

HepG2 cells seeded in 60 mm culture dishes were incubated in 0.5% FBS overnight. OM was added to the cells for indicated time points and cells were lysed in 100 μ l of cell lysis buffer [20 mM Hepes, pH 7.9, 0.2% Nonidet P40, 10% (v/v) glycerol, 400 mM NaCl and 0.1 mM EDTA] with protease inhibitor cocktail (no. 1836153; Roche). 50 μ g of each cell lysate was used for Western blotting.

Preparation of nuclear extracts and electrophoretic mobility-shift assays

HepG2 cells were transfected with His-C/EBP β -P4 plasmid or mock transfected using FuGENE^{TM} 6 transfection reagent (no. 1814443; Roche). Two days after transfection, cells were collected and nuclear extracts were prepared by the modified method of Dignam et al. [11] as we have previously described

[7]. SIRE oligonucleotide probe [7] was end-labelled with T4 polynucleotide kinase in the presence of [γ -³²P]ATP. Each binding reaction was composed of 10 mM Hepes (pH 7.8), 2 mM MgCl₂, 2 mM dithiothreitol, 80 mM NaCl, 10% glycerol, 1 μ g of poly(dI-dC) · (dI-dC), 1 μ g of BSA and 10 μ g of nuclear extract in a final volume of 20 μ l. Nuclear extracts were incubated without or with anti-His₆ antibody for 30 min before addition of 0.5 ng of ³²P-labelled double-stranded SIRE probe [(40–80) $\times$ 10³ c.p.m.] for another incubation of 10 min at room temperature (20°C). Reaction mixtures were loaded on to a 6% (w/v) polyacrylamide gel and run in TGE buffer (50 mM Tris base, 400 mM glycine and 1.5 mM EDTA, pH 8.5) at 30 mA for 2.5–3 h at 4°C. Gels were dried and visualized by a phosphorimager.

ChIP (chromatin immunoprecipitation) assays

HepG2 cells were untreated or treated with OM (50 ng/ml) for the indicated time points and thereafter were cross-linked with 0.37% formaldehyde at 37°C for 10 min. Total cell lysate was isolated, and the genomic DNA was sheared to sizes between 200 and 600 bp by sonication. ChIP assays with rabbit antibodies to C/EBP β or with normal rabbit IgG as a negative control were performed according to the method of Upstate Biotechnology using aliquots of lysate obtained from 5 $\times$ 10⁶ cells. The immune complex was heated at 65°C for 4 h to revert the cross-linking between DNA and proteins. DNA was purified by repeated phenol/chloroform extraction and ethanol precipitation. The purified DNA (designated as bound) was dissolved in 20 μ l of Tris buffer (10 mM Tris, pH 8.5). The DNA isolated using the same procedure without the immunoprecipitation step was designated as the input DNA and was diluted 100 times prior to PCR. The bound and the input DNA were analysed by PCR (31 cycles) with primers that amplify a 180 bp fragment of the human LDLR proximal promoter region from –124 to +54, relative to the major transcription start site. The PCR product was visualized on a 2% (w/v) agarose gel stained with ethidium bromide. The intensity of the PCR products was scanned with a Bio-Rad Fluoro-S MultiImager system and quantified by the Quantity One program. Different amounts of template DNA were tested in the PCR to ensure a linear range of DNA amplification.

Immunostaining

HepG2 cells untransfected or transfected with His-C/EBP β -P4 were cultured on coverslips. The cells were washed with ice-cold PBS and fixed in cold methanol (–20°C) for 10 min. After two washes with PBS, cells were incubated for 1 h in blocking buffer (0.4% Triton X-100, 0.1% gelatin and 1% BSA in PBS). The cells were incubated with 2 μ g/ml rabbit anti-His₆ IgG for 1 h at room temperature. After washing with PBS, cells were incubated with Texas Red-conjugated secondary antibody (1:200 dilution) for 1 h at room temperature. After two washes with PBS, cells were stained with DAPI (4',6-diamidino-2-phenylindole) at a concentration of 1 μ g/ml. After rinsing with PBS and drying the coverslips, the coverslips were mounted in an antifading reagent (Molecular Probes, Eugene, OR, U.S.A.). Images were recorded by a Penguin 600CL digital camera connected to an inverted fluorescent microscope.

Transient transfection and dual luciferase reporter assays

HepG2 cells were seeded in 48-well plates at 9 $\times$ 10⁴ per well in 10% FBS. On the next day, cells were transfected with plasmid DNA (100 ng/well) by using FuGENE^{TM} 6 transfection reagent. The DNA proportion of the analysing firefly luciferase reporter to expression vector and to *Renilla* luciferase reporter, pRL-SV40 (where SV40 is simian virus 40), was 65:25:10. For analysis of

Figure 1 Kinetics of LDLR mRNA expression in OM-treated HepG2 cells

HepG2 cells cultured in EMEM containing 0.5% FBS supplemented with sterols were incubated with 50 ng/ml OM for different times as indicated. Total RNA was isolated and 15 μ g per sample was analysed for LDLR mRNA by Northern blotting. The membrane was stripped and hybridized to a human GAPDH. The difference in LDLR mRNA levels was also independently examined by quantitative real-time PCR assays. LDLR mRNA levels were corrected by measuring GAPDH mRNA levels. The abundance of LDLR mRNA in untreated cells was defined as 1, and the amounts of LDLR mRNA from treated cells were plotted relative to that value. The results shown are representative of three separate experiments.

the LDLR promoter, transfected cells were incubated in a medium containing 0.5% FBS and sterols (10 μ g/ml cholesterol + 1 μ g/ml 25-hydroxycholesterol). OM at the concentration of 50 ng/ml was added 20 h after transfection, and cells were lysed 4 h later for analysing luciferase activities using the Dual Luciferase Assay system obtained from Promega. For analysis of pS100A9Luc promoter activity, cells were lysed 48 h after transfection. Triplicate wells were assayed for each transfection condition, and three to five independent transfection assays were performed for each reporter construct.

RESULTS

Induction of a transient expression of EGR-1 and a sustained increase in C/EBP β expression by OM

HepG2 cells were stimulated with 50 ng/ml OM for different periods from 30 min to 24 h. Total RNA was isolated by the end of treatment and levels of LDLR mRNA in cells were determined by Northern-blot analysis and quantitative real-time PCR (Figure 1). The two independent methods produced similar results showing that OM induced a rapid elevation of LDLR mRNA levels with an over 4-fold increase by 1 h and the level of LDLR mRNA remained elevated throughout the 24 h treatment. To correlate the changes in LDLR transcription with the expression level of EGR-1 protein, cells were treated with OM for the indicated time points and total cell lysates were harvested for Western-blot analysis with anti-EGR-1 antibody. Figure 2(A) shows that EGR-1 protein

Figure 2 Western-blot analyses of EGR-1 and C/EBP β

Cells cultured in a medium containing 0.5% FBS were stimulated with OM for the indicated times. At the end of treatment, cells were scraped into lysis buffer and cell extracts were prepared. Soluble proteins (50 μ g/lane) were subjected to SDS/PAGE. Detections of EGR-1 (A) and C/EBP β (B) were performed by immunoblotting. Membranes were reprobed with anti-actin antibody to correct for protein loading. Signal intensities of EGR-1 and C/EBP β were quantified using Kodak Imaging Station 400 and were normalized to actin signal. NS, non-specific.

expression was increased by 6.5-fold by 15 min, by 8.3-fold by 30 min and by 7.6-fold by 1 h of OM treatment as compared with that in control cells. This strong increase in EGR-1 expression was transient and the abundance of EGR-1 protein returned to the baseline level by 2 h of OM treatment, whereas the level of LDLR mRNA was still highly elevated.

To determine whether C/EBP β protein expression followed a similar kinetics as EGR-1 in OM-treated cells, Western blotting of the cell lysate with anti-C/EBP β antibody was performed and it revealed a different expression kinetics. The protein level of C/EBP β was unchanged within 1 h of OM treatment, started to increase by 2 h, and continuously inclined during the OM treatment (Figure 2B). These results together indicate that OM treatment leads to a transient stimulation of EGR-1 expression that is followed by a sustained induction of C/EBP β expression.

Stimulation of the binding of C/EBP β to LDLR promoter *in vivo* by OM

Our previous study detected the *in vivo* association of EGR-1 with LDLR promoter containing the SIRE site within 1 h of OM stimulation [8]; after that the binding of EGR-1 to LDLR promoter was no longer detectable. To determine whether the increased C/EBP β protein expression in OM-treated cells results in enhanced bindings of C/EBP β to the LDLR promoter time-dependently, we performed ChIP assay to examine the effect of OM on the association of C/EBP β with LDLR promoter in intact cells. Figure 3 shows that the binding of C/EBP β to LDLR promoter was strongly induced by OM in a time-dependent manner. The amount of C/EBP β bound to the LDLR promoter was slightly increased at 1 h of OM treatment. A significant increase (2.8-fold) was detected at 2 h of OM treatment, and the binding of C/EBP β to SIRE reached the highest level of 8-fold at 4 h and slowly declined to a level of 3.5-fold over control by 24 h. These results clearly demonstrate that the interaction of C/EBP β

Figure 3 ChIP analysis for C/EBP β association with the LDLR promoter in HepG2 cells

Rabbit anti-C/EBP β antibody or normal rabbit IgG were used in a ChIP analysis followed by PCR to amplify a 180 bp region surrounding the SIRE site from genomic DNA isolated from control and OM-stimulated HepG2 cells at the indicated time points. The PCR product was separated on a 2% agarose gel, stained with ethidium bromide and quantified by a Bio-Rad Fluoro-S Multimager system. Bound represents the DNA co-immunoprecipitated with antibody, whereas input represents the starting material before immunoprecipitation.

with LDLR promoter is continuous and is concomitant with the increased expression of LDLR mRNA expression.

Attenuation of OM-mediated LDLR transcription by a dominant-negative mutant of C/EBP β

In order to firmly demonstrate a critical role of C/EBP β in OM-induced LDLR transcription, we sought to develop dominant-negative mutants of C/EBP β . To this end, we constructed four vectors of His-tagged full-length and different domains of C/EBP β and employed these constructs in transient transfection studies. Figure 4(A) describes domain structures of C/EBP β and the truncated proteins. Expressions of the His-tagged proteins with correct molecular masses were examined by transient transfection and Western-blot analysis. Immunoblotting with anti-His₆ antibody detected high expression levels of His-C/EBP β -P1, -P3 and -P4 in transfected cells, whereas the level of His-C/EBP β -P2 was relatively lower (Figure 4B).

To determine the functions of the fusion proteins in regulating the basal transcriptional activity of LDLR, these vectors were individually co-transfected with an LDLR promoter luciferase reporter pLDLR234Luc and the normalizing vector pRL-SV40 into HepG2 cells. Two days after transfection, the LDLR promoter-driven firefly luciferase activity was determined and was normalized with the *Renilla* luciferase activity to correct for transfection efficiency. Expression of the full-length His-C/EBP β markedly increased LDLR promoter activity, consistent with our previous findings [9] using the wild-type C/EBP β expression vector. The basal LDLR promoter activities were not altered in His-C/EBP β -P2 and -P3 transfected cells, but were slightly increased in pHis-C/EBP β -P4-transfected cells (Figure 5A).

To examine effects of truncated C/EBP β proteins on OM-induced LDLR transcription, LDLR promoter activities were examined after transfection and stimulation of transfected cells with OM for 4 h. The results of Figure 5(B) were derived from four independent transfections and showed that the inducing effect of OM on LDLR promoter activity was not altered by pHis-C/EBP β -P2 and -P3 constructs but was significantly reduced by the expression of C/EBP β -P4 ($P < 0.05$). We further demonstrate that His-C/EBP β -P4 inhibits OM activity on LDLR promoter in a dose-dependent manner (Figure 5C).

To determine whether the stimulating activity of OM on endogenous LDLR mRNA expression is also inhibited by His-C/EBP β -P4 expression, HepG2 cells seeded in dishes were transfected with the control vector pHis-LacZ or pHis-C/EBP β -

Figure 4 Construction and expression of His-tagged truncated C/EBP β

(A) Schematic representations of C/EBP β functional domains and His-tagged proteins. P, proline-rich; S, serine-rich; L, leucine. (B) Western blotting with anti-His₆ antibody. HepG2 cells were transfected with individual vectors or mock-transfected. Total cell lysates were harvested after 48 h of transfection and soluble proteins (50 μ g/lane) were subjected to SDS/PAGE. Detections of different forms of His-tagged C/EBP β were performed by immunoblotting using anti-His₆ antibody. The asterisks indicate the tagged proteins.

P4 respectively. One day after transfection, cells were trypsinized and reseeded equally into two dishes for each vector transfection. On the next day, cells were untreated or treated with OM for 5 h and harvested for total RNA isolation. Quantitative real-time PCR was conducted to determine the LDLR and GAPDH mRNA levels. The results of Figure 5(D) were derived from three independent transfections and showed that OM treatment led to a 3.5-fold increase in levels of LDLR mRNA in pHis-LacZ-transfected cells and only a 1.8-fold increase in His-C/EBP β -P4-transfected cells. Considering the low transfection efficiency of HepG2 cells, these results corroborated the finding of LDLR promoter activity and suggest that His-C/EBP β -P4 acts as a dominant-negative form of C/EBP β . These results together provide strong evidence for a critical role of C/EBP β in OM-regulated LDLR transcription.

Nuclear localization and SIRE-binding activity of His-C/EBP β -P4

His-C/EBP β -P4 contains the DNA-binding and dimerization domains of human C/EBP β . To determine its subcellular localization, immunostainings of cells mock-transfected and transfected with pHis-C/EBP β -P4 were conducted using a rabbit anti-His₆ IgG as the primary antibody and a mouse anti-rabbit secondary antibody conjugated with Texas Red. Figure 6 shows that a strong red-fluorescent signal was detected in the nucleus of cells transfected with His-C/EBP β -P4 and a very faint red signal was shown in the nucleus of untransfected cells due to the background staining.

To examine the direct binding of His-C/EBP β -P4 to the SIRE sequence of LDLR promoter, HepG2 nuclear extracts were

Figure 5 Inhibition of OM-mediated activation of LDLR transcription by His-C/EBP β -P4

(A) Effects of the full-length and truncated C/EBP β on the basal activity of LDLR promoter. LDLR promoter activity was examined by co-transfection of pLDLR234Luc with pHis-LacZ as the control vector or with different pHis-C/EBP β vectors. The normalized luciferase activity in pHis-LacZ-transfected cells was defined as 1, and the normalized luciferase activities from pHis-C/EBP β -transfected cells were plotted relative to that value. The results shown are representative of four to six separate experiments. (B) Effects of OM on LDLR promoter activities in cells expressing the truncated C/EBP β s. The results (means $\pm$ S.E.M.) shown were derived from four independent transfection assays. (C) Dose-dependent inhibitory effect of pHis-C/EBP β -P4 on OM activity. The pLDLR234Luc was co-transfected with different amounts of pHis-C/EBP β -P4 or pHis-LacZ as the control vector to reach an equal amount of total DNA under each transfection condition. (D) Inhibition of OM activity on LDLR mRNA induction by His-C/EBP β -P4. HepG2 cells were transfected with pHis-LacZ or pHis-C/EBP β -P4. On the next day, transfected cells were reseeded equally into two dishes for each transfection. OM or its dilution buffer (1 mg/ml BSA in PBS) as control was added to cells for 5 h and total RNA was isolated from all dishes. Real-time quantitative PCR was conducted to measure LDLR and

isolated from control and pHis-C/EBP β -P4-transfected cells. A ^{32} P-labelled SIRE probe was incubated with 10 μ g of each nuclear extract in the absence or presence of anti-His $_6$ antibody. After a 10 min incubation at room temperature, reaction mixtures were separated by PAGE and results were visualized by a Bio-Rad phosphorimager. Figure 7 shows that two major DNA protein complexes were detected in both control and transfected extracts, and these complexes were not supershifted by anti-His $_6$ antibody. However, nuclear extract of transfected cells formed one additional complex of faster mobility with the labelled SIRE probe and this complex was totally supershifted by anti-His $_6$ antibody, thereby identifying this band as the His-C/EBP β -P4-SIRE complex. This result clearly demonstrates the strong binding of this truncated C/EBP β to the LDLR promoter SIRE motif and suggests that it may inhibit the OM activity on LDLR transcription by competing or blocking the binding of endogenous C/EBP β to the SIRE site.

Inhibition of C/EBP-mediated transcription of S100A9 by His-C/EBP β -P4

The facts that His-C/EBP β -P4 is localized in the nucleus and is able to bind the LDLR SIRE sequence containing a classical C/EBP motif suggest that this truncated protein may have general inhibitory effects on gene transcriptions mediated by the C/EBP family. To test this, we utilized the S100A9 promoter reporter pGL3-S100A9Luc, which was previously constructed and studied in our laboratory [12]. S100A9 belongs to the family of small calcium-binding proteins [13]. The luciferase reporter of S100A9 promoter contains a 1431 bp fragment of the S100A9 5'-upstream sequence [12]. A canonical C/EBP-binding site has been identified in the proximal section of the promoter and shown to interact with C/EBP β and C/EBP α [14]. Effects of C/EBP α and C/EBP β on S100A9 promoter activities were examined in the absence and presence of His-C/EBP β -P4. Figure 8 shows that S100A9 promoter activity was increased by 39.5-fold over control by C/EBP β and co-expression of His-C/EBP β -P4 with C/EBP β reduced more than 70% of the stimulating activity of C/EBP β . Likewise, the transactivating activity of C/EBP α was greatly attenuated by His-C/EBP β -P4. Similar to the case of LDLR promoter, a small increase in the basal activity of S100A9 promoter was observed in His-C/EBP β -P4-transfected cells. Nevertheless, these results clearly demonstrate that His-C/EBP β -P4 acts as a dominant-negative mutant to members of C/EBP family.

DISCUSSION

Our previous studies have demonstrated that OM stimulates the transcription of human LDLR gene in HepG2 cells through a sterol-independent regulatory mechanism involving activation of the zinc finger transcription factor EGR-1 [8]. EGR-1 indirectly binds to LDLR promoter at the OM-responsive SIRE motif through its specific association with C/EBP β but not with any other SIRE-binding proteins [9]. In the present study, from three different lines of investigation, we identify a new function of C/EBP β in LDLR transcription as an OM-induced transactivator by itself in addition to its role as an EGR-1 co-activator.

GAPDH mRNA levels. The normalized LDLR mRNA levels in control cells were defined as 1 and the amounts of LDLR mRNA from OM-treated cells were plotted relative to that value. The results (means $\pm$ S.E.M.) shown were derived from three independent transfection assays.

Figure 6 Immunostaining of His-C/EBP β -P4

HepG2 cells were mock-transfected (A–C) or transfected with pHis-C/EBP β -P4 (D–F). Two days after transfection, cells were fixed with cold methanol and stained with anti-His₆ antibody and Texas Red-conjugated rabbit anti-mouse IgG (red) (B, E) as well as DAPI to visualize the DNA (blue) (A, D). Cell images under fluorescent microscope were recorded by a CCD (charge-coupled-device) digital camera PENGUIM 600CL and processed by Adobe Photoshop.

Figure 7 Binding of His-C/EBP β -P4 to SIRE DNA

10 μ g of control HepG2 nuclear extract (Control NE) or 10 μ g of nuclear extract from pHis-C/EBP β -P4-transfected HepG2 cells was incubated with ³²P-labelled SIRE oligonucleotide probe in the absence or presence of 2 μ l of anti-His₆ antibody.

First, we show that OM treatment elicits a co-ordinated induction of EGR-1 and C/EBP β expression. EGR-1 is rapidly induced within 15 min of treatment and returned to the baseline level by 2 h. In contrast with the rapid and transient increase in EGR-1 expression, the cellular abundance of C/EBP β was not changed when EGR-1 was highly induced but significantly elevated at 2 h of OM treatment and the expression level of C/EBP β remains elevated more than 3-fold during the 24 h period of the OM treatment. IL-6, another member of the gp130 cytokine family has been shown to induce C/EBP β mRNA expression in mouse hepatocytes [4,15,16]. To our best knowledge, the present study is the first to demonstrate that OM up-regulates C/EBP β expression in hepatoma-derived HepG2 cells.

Secondly, we demonstrate a strong and sustained binding of C/EBP β to LDLR promoter *in vivo*. The kinetics of this binding is concomitant with the elevated level of LDLR mRNA. Inter-

Figure 8 Blocking C/EBP-activated transcription of S100A9

HepG2 cells were co-transfected with pGL3-S100A9Luc with pNF-NF16, or with pCMV-C/EBP α in the presence of pHis-C/EBP β -P4 or pHis-LacZ as the control vector.

estingly, the ChIP assay detected increased binding of C/EBP β to LDLR promoter by 1 h of OM treatment when the cellular level of C/EBP β has not changed. Our previous studies have shown an increased level of ERK (extracellular-signal-regulated kinase)-induced phosphorylation of C/EBP β after 1 h exposure to OM in HepG2 cells [9]. Therefore we speculate that the early increase in the DNA-binding activity of C/EBP β is probably caused by increased phosphorylation of this protein by OM. It is noteworthy that the fold increase of LDLR-bound C/EBP β (8-fold) is significantly higher than the fold increase in the total cellular abundance of C/EBP β detected by Western blotting (3.3-fold at maximum). This difference suggests that the phosphorylated form of C/EBP β caused by ERK has stronger DNA-binding and transactivating activities than unphosphorylated C/EBP β in control cells. This is consistent with other reports that demonstrate a higher transactivating activity of C/EBP β when it is phosphorylated by ERK or by protein kinase C [17–19]. Our previous studies have shown that activation of ERK by OM in HepG2 cells is rapid and sustained [20]. The activation

of ERK has been shown to be a crucial event for the OM-mediated stimulation of LDLR transcription [8]. Thus it is possible that the increased protein abundance and the higher phosphorylation level of C/EBP β both contribute to the increased LDLR mRNA expression in OM-treated cells.

Finally, to obtain a definitive approval for the requirement of C/EBP β in OM-induced LDLR transcription, we took the approach of dominant-negative mutants as inhibitors to C/EBP β . Initially, we developed three His-tagged truncated forms of C/EBP β that contain the transactivation domain only (His-C/EBP β -P2, amino acids 24–145), the transactivation domain and the phosphorylation domain (His-C/EBP β -P3, amino acids 24–245), and the DNA-binding and dimerization domains (His-C/EBP β -P4, amino acids 246–345). Expressions of His-C/EBP β -P2 and -P3 affected neither the basal activity nor the OM-induced activity of LDLR transcription. However, expression of His-C/EBP β -P4 did not lower the basal transcription but greatly reduced the OM-induced LDLR promoter activity and mRNA expression, thereby providing direct evidence for the active role of C/EBP β in OM-mediated stimulation of LDLR transcription.

The fact that expression of His-C/EBP β -P4 also strongly inhibited C/EBP β as well as C/EBP α -induced transcriptional activations of S100A9 indicates that His-C/EBP β -P4 is a dominant-negative mutant of the C/EBP family. Therefore this truncated form of C/EBP β could be a useful molecular tool to study gene regulation mediated by members of the C/EBP family. The C/EBP family contains two natural truncated forms of C/EBP, namely C/EBP γ (Ig/EBP) and CHOP (C/EBP homologous protein) (gadd153) [2]. These factors both lack the transactivation domain and have the DNA-binding domain and the dimerization domain. However, they are structurally different from His-C/EBP β -P4. The amino acid sequence of the DNA-binding domain of C/EBP β is slightly different from CHOP and the sequence of C/EBP β dimerization domain is subtly different from C/EBP γ . C/EBP γ alone has no transcriptional activity and it was shown to modulate C/EBP activity in a cell- and isoform-specific manner. In HepG2 cells, C/EBP γ was an ineffective repressor despite forming C/EBP heterodimers [21]. CHOP has been shown to modulate C/EBP activity either negatively or co-operatively depending on the specific binding site sequence of target genes [22,23]. In addition to C/EBP γ and CHOP, HepG2 cells naturally express p20-C/EBP β [LIP (liver-enriched inhibitory protein)] through a leaky ribosome scanning mechanism [24]. The p20-C/EBP β contains the C-terminal 145 amino acids initiated at the third in-frame AUG of C/EBP β . It has been shown to bind C/EBP site as homodimer or heterodimer with C/EBP β and inhibits C/EBP-mediated transcription [24,25]. Our gel shift assays showed that expression of pHis-C/EBP β -P4 only produced one extra band and the band was entirely supershifted by anti-His₆ antibody, suggesting that His-C/EBP β -P4 may preferentially form a homodimer by itself. Additional experiments are required to determine the ability of His-C/EBP β -P4 to heterodimerize with C/EBP family members.

Based on the results of the present study and our previous studies of EGR-1 induction [8], we propose an action model of OM in the regulation of LDLR transcription as illustrated in Figure 9. In untreated cells, the SIRE motif is occupied by unphosphorylated C/EBP β and CREB. During the early phase of OM induction, EGR-1 is expressed and it is tethered to the LDLR promoter primarily through its association with C/EBP β . The phosphorylation of C/EBP β further strengthens the association of EGR-1 and C/EBP β . Along with CREB, these transactivators form an activating complex at the SIRE site, resulting in a higher level of LDLR transcription. In the later phase of OM induction, EGR-1 expression is extinguished but C/EBP β expression and

Basal Transcription of the LDLR:

OM-Stimulated Transcription: Early Phase

OM-Stimulated Transcription: Late Phase

Figure 9 An action model for OM-stimulated LDLR transcription

In the model, the binding of Sp1 to repeats 1 and 3 and SREBP binding to the SRE-1 site in repeat 2 are not changed during OM stimulation. At the basal state of transcription, unphosphorylated C/EBP β and CREB occupy the SIRE site. During the early phase of OM stimulation, EGR-1 is induced and binds to phosphorylated C/EBP β . A triplex containing EGR-1, phosphorylated C/EBP β and CREB is formed at the SIRE site, leading to an increased transcriptional rate of LDLR. At the later phase of OM stimulation, EGR-1 is absent and more phosphorylated C/EBP β associates with the LDLR promoter, thereby keeping the transcription rate from decreasing. R1, repeat 1; P, phosphorylation.

phosphorylation have been stimulated that lead to an increased binding of C/EBP β to the SIRE site, thereby retaining the higher level of LDLR transcription.

In summary, we have demonstrated that C/EBP β plays a critical role in OM-regulated LDLR transcription separating from its role as EGR-1 cofactor. This work provides a better understanding at the molecular level of the SRE-1/SREBP (sterol-regulatory-element-binding protein)-independent regulation of LDLR transcription in liver-derived cells. Recently, we demonstrated the LDL-cholesterol-lowering effect of OM in hyperlipidaemic hamsters [26]. A complete understanding of the working mechanisms of OM in regulation of LDLR expression in cell culture model and in animal models is of importance for the potential application of this cytokine as an effective cholesterol-lowering bioagent in the treatment of hypercholesterolaemia.

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