Specific Interaction of Egr1 and c/EBPβ Leads to the Transcriptional Activation of the Human Low Density Lipoprotein Receptor Gene*

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The sterol-independent regulatory element (SIRE) of the LDL receptor (LDLR) promoter mediates oncostatin M (OM)-induced transcription of the LDLR gene through a cholesterol-independent pathway. Our prior studies have detected specific associations of the zinc finger transcription factor Egr1 with the SIRE sequence in OM-stimulated HepG2 cells. Because the SIRE motif is composed of a c/EBP binding site and a cAMP response element, both of which are quite divergent from the classical GC-rich Egr1 recognition sequences, we hypothesized that Egr1 may regulate LDLR transcription through interacting with members of the c/EBP and CREB families. Here, we show that treating HepG2 cells with OM specifically leads to prominent increases of the CREB families. Here, we show that treating HepG2 cells through interacting with members of the c/EBP and CREB families. Here, we show that treating HepG2 cells with OM specifically leads to prominent increases of the levels of c/EBPβ and Egr1 bound to the LDLR promoter in vivo. In vitro, the binding of Egr1 to the SIRE sequence is weak, but is strikingly enhanced in the presence of HepG2 nuclear extract. Mammalian two-hybrid assays demonstrate that the N-terminal transactivation domain of Egr1 specifically interacts with c/EBPβ but not with c/EBPα or CREB. The OM treatment further enhances this interaction, resulting in a large increase in the Egr1 transactivating activity. The direct protein to protein contact between Egr1 and c/EBPβ is also demonstrated by co-immunoprecipitation experiments. Furthermore, we show that a mutation of the phosphorylation motif of c/EBPβ diminished the OM-stimulated interaction of Egr1 and c/EBPβ. Taken together, we provide strong evidence that Egr1 regulates LDLR transcription via a novel mechanism of protein-protein interaction with c/EBPβ.

Regulation of the human LDL receptor (LDLR) gene transcription in liver cells involves cholesterol-dependent and -independent mechanisms. The sterol-regulatory element (SRE-1) plays a key role in the cholesterol-mediated negative feedback regulation of the LDLR transcription by interacting with the SRE-1-binding proteins (SREBPs), whose nuclear translocation is controlled by intracellular cholesterol levels (1). The sterol-independent regulatory element (SIRE) has been recently identified and shown to mediate the stimulatory effects of cytokine oncostatin M (OM), cAMP, and c/EBPβ on LDLR transcription in HepG2 cells in a cholesterol-independent fashion (2). The SIRE motif (TGCTGTAAATGACGTGG) is located at the promoter region of −17 to −1, downstream of the SRE-1 and Sp1 binding sites. It is composed of a c/EBP binding site (−17 to −9) and a CRE site (−8 to −1). Mutations within the SIRE sequence do not affect the cholesterol-mediated suppression and only slightly lower basal promoter activity to levels 60–80% of the wild-type sequence. However, alterations of nucleotides (even a single base) within the SIRE motif can totally prevent the OM-induced increase in the LDLR promoter activity.

In vitro DNA binding assays (EMSA) have detected the binding of several basic leucine zipper DNA-binding proteins including c/EBPβ, CREB, ATF1, ATF2, ATF3, and E-Jun to the SIRE sequence (2). These DNA-protein complexes can be detected in unstimulated as well as OM-stimulated nuclear extracts of HepG2 cells. However, stimulation of HepG2 cells with OM induces the formation of a new DNA protein complex, previously referred as the C2 complex. By conducting EMSA, followed by UV-cross-linking and Western blot analysis, we have demonstrated that the unknown DNA-binding protein present in the OM-induced C2 complex is Egr1 (3). By using the chromatin immunoprecipitation (ChIP) assay we further showed the specific association of Egr1 with the LDLR promoter in intact HepG2 cells after OM stimulation. Northern blot analysis showed that the expression of Egr1 is rapidly induced by OM and its expression precedes the up-regulation of LDLR transcription by OM in HepG2 cells. Additional evidence to link Egr1 with the OM-induced LDLR transcription is the dependence of Egr1 induction and LDLR induction on the activation of the MEK/ERK signaling cascade by OM (3, 4). Treatment of HepG2 cells with MEK1 inhibitor U0126 not only eliminated Egr1 expression but also abolished the OM activity on LDLR transcription. The functional role of Egr1 in LDLR transcription was assessed by cotransfection of an Egr1 expression vector with an LDLR promoter reporter construct. We showed that overexpression of Egr1 alone does not lead to a significant increase of the LDLR transcription. However, the LDLR promoter activity is markedly up-regulated by coexpression of Egr1 with c/EBPβ in HepG2 cells. These results suggest a cofactor dependence of Egr1 in the regulation of LDLR transcription.
Egr1 and c/EBPβ Interaction in Regulation of LDLR Transcription

**Materials and Methods**

**Cells and Reagents**—The human hepatoma cell line HepG2 was obtained from American Type Culture Collection (Manassas, VA) and was cultured in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO). The human embryonal kidney (HEK)-derived cell line HEK293 was obtained from ATCC and was cultured according to the manufacturer’s instructions. Antibodies specific to the following proteins were obtained from Santa Cruz Biotechnology: c/EBPβ (sc-15384), c/EBPδ (sc-15380), and c/EBPγ (sc-15364). The monoclonal antibody (catalog no. 2921) that recognizes the phospho-Thr-Pro motif of ERK substrates was obtained from Cell Signaling Technology (Beverly, MA).

**Plasmid Constructions**—For construction of the vp16AD and the c/EBPβ fusion proteins, different coding regions of c/EBPβ were amplified by PCR using pEF-NFIL6 as the template and appropriate 5’- and 3’-primers flanked with BamH1 sites. The primer sequences for cloning and other assays are listed in Table I. The GC-rich PCR system (Roche Applied Science) was used to amplify c/EBPδ. The PCR reactions of 35 cycles were carried out at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s with a final extension at 72 °C for 7 min. The PCR fragments were directly cloned into pCR2.1-Topo vector. The insert was isolated with BamH1 digestion and cloned into pVP16 vector (Clontech) at the BamH1 site. The clones encoding the vp16AD-c/EBPβ fusion proteins with the right insert orientation and in the correct reading frame were verified by sequencing. For the construction of a His-tagged c/EBPβ, the coding region (amino acids 24–345) was PCR-amplified and cloned directly into the pcDNA4/HisMax-TOPO vector. The resulting plasmid pcDNA4/His-c/EBPβ-P1 expresses c/EBPβ with its N terminus tagged with six histidine residues. The expression of His-c/EBPβ in HepG2 cells is confirmed by immunoblotting with anti-His monoclonal antibody. The plasmid His-c/EBPβ-T235A was obtained by performing site-directed mutagenesis with pcDNA4/His-c/EBPβ-P1 as the template DNA. The expression vector pCMV-FLAG-Egr1-DN, encoding the dominant-negative mutant of Egr-1, was constructed by inserting a Bsal/BglII fragment of the murine Egr-1 cDNA (nucleotides 2640–3382, GenBank™ accession no. M22328) into plasmid pXFLAG-CMV-NLS. The protein expressed from this vector contains a triple FLAG sequence MDYKDHDGDYKDHDLDYKDDDDK on the N terminus. Egr1 and c/EBPβ are fused in HepG2 cells is confirmed by immunoblotting with anti-His monoclonal antibody. The plasmid His-c/EBPβ-T235A was obtained by performing site-directed mutagenesis with pcDNA4/His-c/EBPβ-P1 as the template DNA. The expression vector pCMV-FLAG-Egr1-DN, encoding the dominant-negative mutant of Egr-1, was constructed by inserting a Bsal/BglII fragment of the murine Egr-1 cDNA (nucleotides 2640–3382, GenBank™ accession no. M22328) into plasmid pXFLAG-CMV-NLS. The protein expressed from this vector contains a triple FLAG sequence MDYKDHDGDYKDHDLDYKDDDDK on the N terminus. Egr1 and c/EBPβ are fused in HepG2 cells is confirmed by immunoblotting with anti-His monoclonal antibody. The plasmid His-c/EBPβ-T235A was obtained by performing site-directed mutagenesis with pcDNA4/His-c/EBPβ-P1 as the template DNA. The expression vector pCMV-FLAG-Egr1-DN, encoding the dominant-negative mutant of Egr-1, was constructed by inserting a Bsal/BglII fragment of the murine Egr-1 cDNA (nucleotides 2640–3382, GenBank™ accession no. M22328) into plasmid pXFLAG-CMV-NLS. The protein expressed from this vector contains a triple FLAG sequence MDYKDHDGDYKDHDLDYKDDDDK on the N terminus. Egr1 and c/EBPβ are fused in HepG2 cells is confirmed by immunoblotting with anti-His monoclonal antibody. The plasmid His-c/EBPβ-T235A was obtained by performing site-directed mutagenesis with pcDNA4/His-c/EBPβ-P1 as the template DNA. The expression vector pCMV-FLAG-Egr1-DN, encoding the dominant-negative mutant of Egr-1, was constructed by inserting a Bsal/BglII fragment of the murine Egr-1 cDNA (nucleotides 2640–3382, GenBank™ accession no. M22328) into plasmid pXFLAG-CMV-NLS. The protein expressed from this vector contains a triple FLAG sequence MDYKDHDGDYKDHDLDYKDDDDK on the N terminus.
highly purified and already activated ERK1/ERK2 was purchased from Stratagene. An aliquot of Egr1 in vitro translation product was mixed on ice with 4 μl of 10 mM kinase reaction buffer, 1 μl of the activated ERK, and 2 μl of [γ-32P]ATP in a reaction volume of 40 μl. The kinase reaction was executed by incubation of the mixture at 37 °C for 90 min. The standard ERK substrate PHAS-I was used in a separate reaction as a positive control. The phosphorylated products were examined by SDS-PAGE and phosphorimaging. After detection of phosphorylated Egr1, the phoshorylation reaction was repeated using unlabelled ATP.

Electrophoretic Mobility Shift Assays (EMSA)—Oligonucleotide probes were annealed and end-labeled 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 (v/v), 0.1 μg oligonucleotide (d-dc), 1 μg of bacterial serum albumin, and 0.5 ng of 32P-labeled double-stranded synthetic oligonucleotide probe (40–80 × 10^6 cpm) for 10 min at room temperature. The reaction mixtures were loaded onto a 6% polyacrylamide gel and run in TGE buffer (50 mM Tris base, 400 mM glycine, 1.5 mM EDTA, pH 8.5) at 30 mA for 2.5–3 h at 4 °C. Gels were dried and visualized on a phosphorimager. For supershift assays, antibody was incubated with samples for 30 min at room temperature prior to the addition of the probe.

Chromatin Immunoprecipitation (ChIP) Assays—HepG2 cells were untreated or treated with OM (50 ng/ml) for 1 h 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 of between 200 and 600 bp by sonication. ChIP assays with rabbit antibodies to c/EBPβ, Egr1, CREB, ATF3, and with normal rabbit IgG as a negative control were performed according to the protocol of Upstate Biotechnology using aliquots of lysate obtained from 5 × 10^6 cells. The immunocomplex 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 μl of Tris buffer (10 mM Tris, pH 8.5). The DNA isolated using the same procedure with omission of the cross-linking step was designated as the input DNA and was diluted 100× prior to PCR. The bound and the input DNA were analyzed 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 conditions were 94 °C for 5 min, 94 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The 180-bp PCR product was visualized on a 2% agarose gel stained with ethidium bromide. The intensity of the PCR products was scanned with a BioRad Fluoro-S MultiImager System and quantified by the Quantity One Program. Different amounts of template DNA were tested in the PCR reaction to ensure a linear range of DNA amplification.

Transient Transcription and Dual Luciferase Reporter Assays—HepG2 cells were seeded in 48-well plates at 9 × 10^4 per well in 10% fetal bovine serum the day before transfection. A total of 100 ng of DNA per well was transfected into cells using the FuGENE 6 transfection reagent (Roche Applied Science, 1814443). The DNA ratios of the analyzing firefly luciferase reporter to expression vector and to the phosphorylation reaction containing the translation product of Egr1 in vitro translation product in the presence of [γ-32P]ATP, and the kinase reaction was executed by incubation of the reaction mixture at 37 °C for 90 min. The standard ERK substrate PHAS-I was used in a separate reaction as a positive control. The phosphorylated products were then examined by SDS-PAGE and phosphorimaging. Fig. 1B shows that a 32P-labeled protein migrated as 82 kDa was only detected in the reaction containing the translation product of Egr1 (lane 3). After validation of these methods, we reproduced Egr1 as well as ERK-phosphorylated Egr1 protein using unlabeled methionine and ATP for the subsequent DNA binding assays.

To be able to compare the binding affinity of Egr1 to the SIRE sequence with its binding to the classic EBS, a 32P-labeled SIRE probe and a 32P-labeled Egr1 probe were incubated with equal amounts of the Egr1 translation product in the absence or presence of untreated HepG2 nuclear extract, which does not contain a detectable level of the Egr1 protein. After a 15-min incubation at room temperature, reaction mixtures were separated by polyacrylamide gel electrophoresis, and results were visualized by a BioRad PhosphoImager. Fig. 1C shows that the binding of Egr1 to the labeled Egr1 probe was very strong and was not affected by the presence of HepG2 nuclear extract (compare lanes 1 and 2). In contrast, the binding of Egr1 to the labeled SIRE probe was weak by itself, however, a strong binding with an increase of 8-fold was detected in the presence of HepG2 nuclear extract (compare lanes 5 and 6). The Egr1-SIRE complex could be totally supershifted by anti-Egr1 antibodies (Fig. 1D, compare lanes 2 and 4).

RESULTS

A Strong Interaction of Egr1 with SIRE DNA Requires Cooperation from other SIRE-binding Proteins—In order to determine whether Egr1 can bind to the SIRE motif by itself or it requires cooperation from other SIRE-binding proteins, we first produced Egr1 protein by using the TntC-toupled reticulocyte lysate system. The plasmid pCITEzif encoding Egr1 (8) or a control plasmid expressing LacZ was added to the rabbit reticulocyte lysate containing T7 polymerase and [35S]methionine. After reactions, translation products were examined by SDS-PAGE and autoradiography. As shown in Fig. 1A, a major band with the molecular mass of 82 kDa, corresponding to the reported molecular mass of Egr1 protein on SDS-PAGE (80–82 kDa) was detected in the reaction mixture containing the Egr1 plasmid (lane 1) and was not present in the sample without Egr1 DNA (lane 3). Some proteins with lower molecular mass in the reaction mixture of Egr1 were also shown, which might represent degraded translation products.

It has been previously reported that Egr1 contains a single ERK phosphorylation motif (PHSP, amino acids 24–27) residing in the N-terminal transactivation domain (AD) (9). We were interested in determining whether ERK-induced phosphorylation of Egr1 affects the binding of Egr1 to the SIRE motif. To produce the phosphorylated Egr1, the highly purified and already activated ERLK1/ERK2 was incubated with an aliquot of Egr1 in vitro translation product in the presence of [γ-32P]ATP, and the kinase reaction was executed by incubation of the reaction mixture at 37 °C for 90 min. The standard ERK substrate PHAS-II was used in a separate reaction as a positive control. The phosphorylated products were then examined by SDS-PAGE and phosphorimaging. For detection of phosphorylated His-c/EBPβ or His-c/EBPβ-T325A, HEK293 cells were untransfected or transfected with pcDNA4/His-c/EBPβ-P1 and pcDNA4/His-c/EBPβ-T235A plasmids. Two days after transfection, cells were trypsinized, resuspended into two dishes, and cultured overnight in medium containing 0.5% fetal bovine serum. Transfected cells were stimulated with OM for 1 h and harvested for immunoprecipitation with anti-His antibody. The immunoprecipitation complexes were analyzed by Western blotting with the monoclonal antibody that recognizes the phospho-Thr-Pro motif.

For other Western blot analyses, cells were seeded in 60-mm dishes and were lysed in 100 μl of whole cell lysis buffer (20 mM HEPES pH 7.9, 0.2% Nonidet P-40, 10% glycerol, 400 mM NaCl, and 0.1 mM EDTA) with protease inhibitor mixture (Roche Applied Science, 1836153). 50 μg of protein from whole cell lysate per sample were used for Western blotting.
ERK-directed phosphorylation of PHAS-I (lane 1) and PHAS-I (lanes 3 and 4) showed the kinase activity of ERK as a positive control for the kinase activity of ERK. C, autoradiograph showing the SDS-PAGE separation of in vitro translation products labeled with [35S]methionine. B, autoradiograph showing the SDS-PAGE separation of the in vitro phosphorylation mixtures containing in vitro translated Egr1 with activated ERK (lane 3) and in vitro translated Egr1 without activated ERK (lane 4). The ERK-directed phosphorylation of PHAS-I (lane 1) was included in the experiment as a positive control for the kinase activity of ERK. C, EMSA with in vitro translated Egr1. 32P-labeled Egr1 or SIRE oligonucleotide probe was incubated with 2 µl of in vitro translated Egr1 mixture in the absence or the presence of 6 µg of control HepG2 nuclear extract (con NE) in a final volume of 20 µl (lanes 1 and 2; 3 and 5). In lanes 3, 4, 7, and 8, the translated Egr1 was first phosphorylated by ERK and subsequently mixed with the probes. Equal amounts of Egr1 protein were used under all conditions. The data shown are representative of three separate assays. D, supershift assay with anti-Egr1 antibody. HepG2 nuclear extract was incubated with the 32P-labeled SIRE probe without (lanes 1 and 3) or with 2 µl of Egr1 (lanes 2 and 4) in the absence (lanes 1 and 2) or the presence of 2 µl of anti-Egr1 antibody (lanes 3 and 4).

whereas no supershifted band was detected in the reaction only containing the nuclear extract of untreated HepG2 (Fig. 1D, lane 3). The EMSA also revealed that phosphorylation of Egr1 by ERK has no effects on the interaction of Egr1 with Egr1 probe (Fig. 1C, lanes 3 and 4) or the SIRE probe (Fig. 1C, lanes 7 and 8). To further examine the effects of phosphorylation on the DNA binding activities of Egr1, HEK293 cells were transfected with pCMV-Egr1 or pCMV-Egr1-T26A. Two days after transfection, nuclear extracts were prepared from the transfected cells and the EMSA was performed with the nuclear extracts that express the wild-type Egr1 or the phosphorylation-deficient Egr1. The results showed that mutation of T26A on Egr1 did not diminish its DNA binding activity to EBS or SIRE (data not shown). Altogether, these experiments convincingly demonstrate that the binding of Egr1 with SIRE DNA is not affected by ERK-directed phosphorylation but it is strongly enhanced by other SIRE-binding proteins, possibly through protein to protein interaction.

**Egr1 Interacts Specifically with c/EBPβ**—To obtain insights into which SIRE-binding protein may interact with Egr1 at the SIRE motif, initially we examined the in vivo binding of c/EBPβ, CREB, ATF3, as well as Egr1 to the LDLR promoter in control and OM 1-h-stimulated HepG2 cells by ChIP assays. Fig. 2 shows that levels of CREB (lane 2) and ATF3 (lane 4) bound to the SIRE sequence were slightly higher than the IgG control (lane 5) and were not changed by OM stimulation. However, in addition to the increased binding of Egr1 (lane 3), the amount of c/EBPβ (lane 1) bound to the LDLR promoter containing the SIRE site was also markedly increased in OM-stimulated cells. These results indicate that while CREB and ATF3 have the ability to bind SIRE DNA in vitro, Egr1 and c/EBPβ are associated with the LDLR promoter in living cells after OM stimulation, and are thus likely to be the true activators that mediate OM-induced LDLR transcription through the SIRE site.

To determine whether c/EBPβ functions as a Egr1 coactivator, we performed mammalian two-hybrid assays by using a series of constructs of GAL4-Egr1 fusion proteins (Fig. 3A) and the luciferase reporter pFR-Luc that contains 5 GAL4 binding sites. HepG2 cells were transiently transfected with pFR-Luc along with GAL4-Egr1 fusion constructs in the absence or presence of expression vectors for c/EBPβ (pEF-NFIL-6) or CREB (RSV-CREB). The plasmid pRL-SV40 expressing the renilla luciferase was also included as a control vector to normalize the variation of transfection efficiency. 20-h post-transfection, OM was added to cells for 4 h prior to cell lysis. Fig. 3B shows that the transactivating activities of the GAL4 fusion proteins containing the partial AD of Egr1 (pM-Egr1–1) and the whole AD of Egr1 (pA-Egr1–2) were greatly increased by coexpression of c/EBPβ but not by coexpression of CREB, and OM stimulation further increased the stimulatory effect of c/EBPβ. The activity of the fusion protein pM-Egr1–3 that contains the AD and the repression domain (RD) of Egr1 (7, 10) was not increased by c/EBPβ, suggesting that the presence of the repression domain may interfere with the accessibility of c/EBPβ to the Egr1 AD.

To further evaluate the specificity of the Egr1–c/EBPβ interaction, we included the constructs of pBP-p53 and pAD-SV40T in the mammalian two-hybrid assays. As shown in Fig. 3C, the activity of GAL4-Egr1–2 is increased by pEF-NFIL-6 but was not increased by pAD-SV40T that expresses the v16 AD and SV40 T antigen fusion protein. On the other hand, the activity of GAL4 and p53 fusion protein pBD-p53 was only increased by pAD-SV40T and was not increased by pEF-NFIL6. Importantly, OM has no stimulatory effect for the interaction of p53 and SV40 T antigen. These results confirm the specific inter-
action between Egr1 and c/EBPβ and further indicate a stimulatory role of OM in this interaction.

So far our data have shown that a strong binding of Egr1 to the SIRE DNA requires the presence of other SIRE-binding proteins and that c/EBPβ increases the transactivating activity of Egr1 containing only the N-terminal transcriptional activation domain. These results together suggest that Egr1 may be involved in the regulation of LDLR transcription through a new mechanism that does not require its DNA binding activity. To test this, we constructed an expression vector pCMV-Egr1-DN that encodes a fusion protein containing a FLAG tag, followed by a nuclear localization sequence (NLS) derived from the large T antigen, and the C terminus of Egr-1, including the entire DNA binding domain (DBD) of Egr1. We reasoned that this vector may compete the binding of Egr1 to EBS and thus act as a dominant-negative mutant to inhibit the Egr1 transactivating activity through the classic mode of Egr1, but it may not affect Egr1 activity on LDLR transcription if its DNA binding activity is not involved. Indeed, Fig. 4 shows that the OM-induced increase in the promoter activity of an Egr1 reporter pEBS4Luc was significantly decreased by cotransfection of pEBS4Luc with pCMV-Egr1-DN, whereas the stimulation of LDLR promoter activity by OM was not diminished at all by the expression of the Egr1 DBD, indicating that the Egr1 inhibitor blocked OM-induced binding of Egr1 to the EBS in pEBS4Luc promoter but it did not prevent OM-induced association of Egr1 with the LDLR promoter.

Next, we examined the effects of other members of the c/EBP and CREB family on LDLR transcription and the interaction with Egr1. The upper panel of Fig. 5A shows that among the transcription factors examined, LDLR promoter activities were increased by exogenous expression of c/EBPβ and c/EBPα, whereas c/EBPδ and CREB/ATF had no effect. Interestingly, unlike c/EBPβ and c/EBPα, c/EBPβ did not increase the transactivating activity of Egr1 in the mammalian two-hybrid assay (Fig. 5B, lower panel). We also analyzed the endogenous expressions of c/EBPβ, c/EBPα, and c/EBPδ in HepG2 cells by Western blot and found that c/EBPβ protein was readily detected whereas expression levels of c/EBPα and c/EBPδ were below the detection limit. However, expressions of these two proteins were seen after transfection of their expression vectors respectively in HepG2 cells (Fig. 5B). Together, these results demonstrating the absence of c/EBPα and c/EBPδ protein expression in HepG2 cells and the lack of their interaction with Egr1 further indicate the specific roles of c/EBPβ and Egr1 in the OM-mediated up-regulation of the LDLR transcription through the SIRE element.
An Intact c/EBPβ Is Required for Its Interaction with Egr1—While sequences of the DBD of the c/EBP family are highly homologous, the N-terminal transactivation domains among different members of this family are quite divergent (11–13). The fact that c/EBPα or c/EBPδ could not interact with Egr1 suggests that the NH2-terminal AD of c/EBPβ may play a critical role in the interaction with Egr1. In an attempt to define the domain requirement, we made four fusion constructs that express the AD of vp16 and the full-length or the truncated c/EBPβ (Fig. 6A) and tested their abilities to activate the transactivating activity of Egr1. Fig. 6B shows that the Egr1 transactivating activity is increased 7.1-fold by the fusion protein containing a full-length c/EBPβ. Deletion of the C-terminal domains reduced the activity of c/EBPβ to 2.3-fold whereas deletion of the N-terminal AD nearly abolished the activation. These data indicate that the N-terminal activation domain alone is not sufficient to enhance the Egr1-transactivating ability; an intact c/EBPβ protein is needed. It is possible that maintaining the correct conformation of c/EBPβ is important for its interaction with Egr1. This may also explain the relatively lower activity of the vp16 AD-c/EBPβ fusion protein as compared with the wild-type c/EBPβ (pEF-NFIL6), because of the attachment of the vp16 AD to the N terminus of the protein.

To determine whether Egr1 and c/EBPβ can directly interact in the absence of other SIRE-binding proteins we performed immunoprecipitation assay using [35S]methionine labeled in vitro translated products of Egr1 and a His-tagged c/EBPβ. In vitro translated His-LacZ was included as a negative control. Using rabbit anti-His antibody to immunoprecipitate, Egr1 translated His-LacZ was included as a negative control. vitr

ERK-induced Phosphorylation of c/EBPβ Enhances the Interaction of c/EBPβ with Egr1—Previously we have shown that ERK is rapidly activated by OM (4) and blocking this activation totally abolishes the OM-induced Egr1 mRNA expression (3), the formation of the C2 complex with SIRE DNA (2), and the up-regulation of the LDLR expression (3, 4). These prior studies combined with the results described above imply a functional role of ERK activation in the interaction of Egr1 with c/EBPβ. Both Egr1 and c/EBPβ contain a single ERK phosphorylation site (9, 13). The phosphorylation motif of Egr1 (PHSP, amino acids 24–27) is located in the N-terminal AD that mediates the interaction with c/EBPβ. To determine whether the phosphorylation status is important for Egr1 to interact with c/EBPβ, we conducted the mammalian two-hybrid assays using the GALA fusion proteins containing the wild-type Egr1 (p-Egr1–2) and the mutated Egr1 (p-Egr1–2-S26A). In this assay, ERK activation is either induced by OM or by cotransfection with the plasmid MAPKKKAN3-S218E-S232D that encodes a constitutively active form of MEK1 (14). Fig. 7 shows that the c/EBPβ-induced activation of Egr1 transactivation activity was not diminished by the Egr1 mutant. The c/EBPβ-induced increase in the luciferase activities were further increased by OM treatment in cells expressing either the Egr1 wild type or the phosphorylation-inactive mutant. Expression of the wild-type MEK1 did not produce an effect, however, expression of the constitutively active MEK1 caused a similar enhancement on the transactivating activities of both the wild-type and the mutant Egr1 in the presence of c/EBPβ without OM treatment. These data demonstrate the nonessential role of Egr1 phosphorylation and point to a functional role of ERK-induced phosphorylation of c/EBPβ.

It has been documented in other studies that c/EBPβ transactivating activity is stimulated by ERK-directed phosphorylation of Thr-235 (15, 16). By conducting the mammalian two-hybrid assays expressing the wild-type c/EBPβ (pcDNA4/His-c/EBPβ) and the T235A mutants of c/EBPβ (pcDNA4/His-c/EBPβ-T235A, pCMV-NFIL6-T235A), we show that the T235A mutation did not affect the interaction of c/EBPβ with Egr1 but lost response to OM stimulation (Fig. 8A).

We further tested the c/EBPβ mutant on the OM induction of LDLR promoter activity. The OM activity on the LDLR promoter was relatively reduced but not eliminated by cotransfection with the mutated c/EBPβ as compared with the wild-type vector (Fig. 8B). The residual activity is likely caused by OM-induced Egr1 expression that can interact with unphosphorylated c/EBPβ, leading to increased LDLR promoter activity.
expression levels of c/EBPβ/H9252

C

c/EBPα phosphorylation site, 293 cells were transfected with pcDNA4/His-

bit anti-c/EBPβ substrates. The membrane was subsequently probed with rab-

that only recognizes the phospho-Thr-Pro motif of the ERK-directed

phosphorylation of c/EBPβ/H9252 shows that the phospho-c/EBPβ was only detected in cells

transfected with the wild-type His-c/EBPβ (lane 4), and OM treatment significantly increased its abundance (lane 5). In contrast, this band was not present in untransfected cells (lane 1) and was not detected in cells transfected with the His-c/EBPβ-T235 mutant (lanes 2 and 3). These results demonstrate that the OM-induced phosphorylation occurs at the ERK recognition site. All together, the results of Fig. 8 indicate that the ERK-induced phosphorylation of c/EBPβ has a functional role in OM-mediated activation of the LDLR transcription.

**DISCUSSION**

Egr1, also known as ZFP268, NGFI-A, Knox-24, and TIS8, is an 80–82-kDa nuclear phosphoprotein (5). Egr1 belongs to a group of genes termed the immediate early genes. A variety of environmental signals, including growth factors, hormones, neurotransmitters, and cytokines, induce Egr1 (9, 17–21). The prompt induction of Egr1 expression is poised to initiate genetic programs that would couple a stimulus with an appropriate cellular response. It is well known that Egr1 binds to a GC-rich motif (5′-GGG(T/C)GGGCG-3′) through its three zinc
finger DNA binding domains and modulates transcriptions of a number of genes that participate in different cellular functions (8, 22–25). However, in this study we provide compelling evidence from five different lines of investigation to demonstrate a different activation mechanism of Egr1 in the regulation of LDLR transcription via a protein-protein interaction with c/EBPβ, a transcription factor belonging to the basic leucine zipper transcription factor family.

First, EMSA showed that compared with the strong binding of Egr1 to the canonical EBS, the interaction of Egr1 alone with the SIRE sequence is trivial. However, in the presence of HepG2 nuclear extract that contains c/EBPβ, the amount of Egr1 recruited to the SIRE DNA increased 8-fold, a level of binding compatible to the classical EBS. This indicates that Egr1 is tethered to the SIRE sequence by other proteins present in the HepG2 nuclear extract. Second, ChIP assay demonstrates the co-occupation of SIRE motif by Egr1 and c/EBPβ after OM stimulation in intact HepG2 cells, thereby suggesting that...

Fig. 7. Blocking ERK-directed phosphorylation of Egr1 does not affect Egr1 association with c/EBPβ. The mammalian two-hybrid reporter pFR-Luc and pEF-NFIL6 were cotransfected with the wild type or S26A mutated GAL4-Egr1–2 plus expression vectors for the wild-type MEK1 or the constitutively activated MEK1. After transfection, cells were untreated or treated with OM for 4 h prior to cell lysis.

Fig. 8. The phosphorylation-defective c/EBPβ mutant lacks the response to OM stimulation. A, mammalian two-hybrid assays were conducted by using pm-Egr1–2 with different amounts of the wild type or the Thr235A mutants of c/EBPβ. The asterisk sign indicates the statistically significant difference (p < 0.05) of the Egr1 transactivating activities between OM and control. B, HepG2 cells were transfected with pLDLR234Luc with different amounts of plasmid DNA encoding the wild-type c/EBPβ (pEF-NFIL6) or the phosphorylation-defective c/EBPβ mutant (pCMV-NFIL6-T235A). The OM-induced increases of luciferase activity are 4.9- and 3.7-fold in cells co-transfected with the wild-type c/EBPβ and are 2.5- and 2.0-fold in cells co-transfected with the mutant c/EBPβ. C, detection of OM-induced phosphorylation of endogenous c/EBPβ. HepG2 cells were untreated or treated with OM for 1 h. Total cell lysates (500 μg per sample) were incubated with 2 μg of rabbit anti-c/EBPβ antibodies and preceded with immunoprecipitation. The immunoprecipitation complexes were analyzed with immunoblotting with antibody recognizing only phosphorylated c/EBPβ. Aliquots of total cell lysates were probed with rabbit anti-c/EBPβ antibodies to detect the expression levels of total c/EBPβ protein. D, detection of OM-induced phosphorylation of transfected His-c/EBPβ. HEK293 cells were mock-transfected, transfected with the wild-type His-c/EBPβ, and transfected with the His-c/EBPβ-T235A mutant. Two days later, cells were trypsinized and reseeded into two dishes for each transfection. After an overnight incubation in medium with 0.5% fetal bovine serum, cells were treated with OM for 1 h. Immunoprecipitation with anti-His antibody was performed for each total cell lysate containing 1 mg of protein, and the immunocomplexes were analyzed by Western blotting to detect phosphorylated His-c/EBPβ. The anti-c/EBPβ and anti-actin antibodies were used to analyze total lysates. The asterisk sign indicates the nonspecific band that is equally present in every immunoprecipitation sample.
c/EBPβ is the transcription factor that recruits Egr1 to the SIRE motif. Third, the mammalian two-hybrid assay using a series of constructs of GAL4-Egr1 fusion proteins demonstrates that among members of the c/EBP and CREB families that all have the ability to bind the SIRE DNA under in vitro conditions, only c/EBPβ increases the transactivating activity of the GAL4-Egr1 fusion proteins that do not contain Egr1 DBD. Fourth, by using the approach of in vitro translation coupled with immunoprecipitation, we demonstrate a direct contact between Egr1 and c/EBPβ in the absence of other SIRE-binding proteins. The last evidence to rule out the involvement of the EBS4Luc luciferase reporter driven by 4 EBS repeats, is obtained by using a dominant-negative inhibitor of Egr1 (pCMV-Egr1-DN). The construct expressing the zinc finger domains of Egr1 inhibited the OM-induced activation of the pEBS4Luc luciferase reporter driven by 4 EBS repeats, whereas the inhibitor had no diminishing effect on OM-induced reporter driven by the LDLR promoter.

Although we have focused the study of Egr1-c/EBPβ interaction on the regulation of LDLR transcription that does not have an EBS, it is possible that this interaction may also play a role in transcriptions of other Egr1-regulated genes. We have found that the pEBS4Luc reporter activity can be moderately increased by coexpression with c/EBPβ but not with c/EBPα (data not shown). The question of whether endogenous cellular genes containing EBS can be modulated by c/EBPβ through its interaction with Egr1 needs further investigation.

ERK activation is a critical event in the OM-stimulated LDLR expression and Egr1 expression. Egr1 and c/EBPβ both contain a single ERK recognition motif. Previously we have postulated that the OM-induced ERK activation probably has dual effects on Egr1 in relation to LDLR transcription. ERK activates Egr1 promoter activity (13, 21, 27), resulting in prompt accumulation of Egr1 mRNA and protein in nucleus. Subsequently, the Egr1 protein could be further phosphorylated by ERK. These two combined events may account in part for the increased binding of Egr1 to the LDLR promoter. In this study, we show that in vitro phosphorylation of Egr1 with activated ERK did not increase Egr1 binding affinity to SIRE DNA or to EBS. Additionally, we found that the interaction between Egr1 and c/EBPβ is stimulated by OM and by the constitutively activated MEK1 to similar degrees by using the wild type or mutated Egr1S262A mutant. These results together indicate that the phosphorylation site on Egr1 is nonessential for Egr1 transcriptional activity. In contrast to Egr1, the ERK-directed phosphorylation site on c/EBPβ is likely functionally involved in the interaction between c/EBPβ and Egr1, as we have shown that mutation of this site abolished the OM stimulatory effect on the Egr1 transactivating activity. Furthermore, transfection of pCMV-c/EBPβ-T235A lowered the effect of OM on induction of LDLR promoter activity. By utilizing a monoclonal antibody that only recognizes the Thr-235-phosphorylated c/EBPβ, we were able to demonstrate that OM treatment leads to an elevated phosphorylation level of c/EBPβ. Altogether, these data indicate the double roles of ERK in the OM-mediated up-regulation of LDLR expression. ERK activation leads to Egr1 expression and phosphorylation of c/EBPβ, both of which are important steps in the signaling cascade that ultimately changes the transcriptional rate of the LDLR in OM-treated HepG2 cells.

In conclusion, we have demonstrated a novel activation mechanism for Egr1 to regulate LDLR gene transcription that is different from its traditional act of DNA binding. Instead, Egr1 is recruited to the SIRE motif through its interaction with c/EBPβ. The interaction of Egr1 and c/EBPβ is further strengthened by the ERK-induced phosphorylation of c/EBPβ, thereby leading to the formation of a transactivating complex at the SIRE site and resulting in a rapid increase of LDLR gene transcription.

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