Identification of Egr1 as the oncostatin M-induced transcription activator that binds to sterol-independent regulatory element of human LDL receptor promoter

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Abstract **Previously, we identified the low density lipoprotein receptor (LDLR) promoter region −17 to −1 as a novel sterol-independent regulatory element (SIRE) that mediates the stimulating effect of oncostatin M (OM). The goal of this study was to identify the OM-induced transcription activator that binds to the SIRE sequence. By conducting a electrophoretic mobility shift assay (EMSA) followed by UV crosslinking and SDS-PAGE, we show that a protein with a molecular mass of 85 kDa was present in the OM-induced SIRE DNA-protein complex. Western blotting and supershift assays reveal that the 85 kDa factor is early growth response gene 1 (Egr1). The interaction of Egr1 with the SIRE sequence was further confirmed in vivo by chromatin immunoprecipitation assays. The functional role of Egr1 in LDLR transcription was assessed by cotransfection of an Egr1 expression vector with an LDLR promoter reporter construct. We show that overexpression of Egr1 significantly increases LDLR promoter activity when cotransfected with CCAAT/enhancer binding protein β (c/EBPβ) or with cAMP-responsive element binding protein (CREB) expression vectors. Our studies clearly demonstrate that Egr1 is the OM-induced transcription factor that binds to the SIRE sequence of the LDLR promoter and also suggest that Egr1 may have a functional role in OM-induced upregulation of LDLR transcription through interaction with other SIRE binding proteins such as c/EBPβ or CREB.**—Zhang, F., T. E. Ahlborn, C. Li, F. B. Kraemer, and J. Liu. **Identification of Egr1 as the oncostatin M-induced transcription activator that binds to sterol-independent regulatory element of human LDL receptor promoter.** ***J. Lipid Res.*** **2002.** 43: **1477–1485.**

Supplementary key words cytokines • early growth response gene 1 • gene transcription • transcriptional activation • signal transduction

Transcription of the low density lipoprotein receptor (LDLR) is largely controlled by a cholesterol-mediated feedback mechanism through interaction of a sterol regulatory element-1 (SRE-1) and SRE binding proteins (SREBPs) (1–9). In addition to this sterol-dependent pathway that is regulated by intracellular cholesterol levels, cumulative evidence from both in vivo studies and cell culture models suggests the existence of a sterol-independent regulatory pathway for LDLR transcription that is modulated by cytokines, growth factors, hormones, and secondary messengers (10–19). Some of these modulators appear to increase LDLR transcription under cholesterol-repressed conditions, and their activities do not require SRE-1. However, in contrast to the well-characterized mechanism of cholesterol regulation, the molecular and cellular mechanisms underlying sterol-independent regulation have not been clearly defined. This is at least in part due to the lack of understanding at the promoter level of the transcription factors and their interacting *cis*-acting elements that play critical roles in sterol-independent regulation.

Recently, we identified a *cis* regulatory element in the human LDLR promoter that is responsible for cytokine oncostatin M (OM), CCAAT/enhancer binding protein (c/EBP), and cAMP-stimulated transcription of LDLR (20). This regulatory sequence designated as the sterol-independent regulatory element (SIRE) lies down stream of the SRE-1 and Sp1 sites. It is located in the LDLR promoter region −17 to −1 that overlaps the previously described TATA-like sequences (−23 to −8). The SIRE sequence consists of a putative binding site for c/EBP (−17 to −9) and a cAMP-responsive element (CRE; −8 to −1). Mutations within the SIRE sequence have no effect on 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 sin-

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Abbreviations: Ap1, activator protein 1; ATF, activating transcription factor; C/EBP, CCAAT/enhancer binding protein; CRE, cAMP-responsive element; CREB, cAMP-responsive element binding protein; Egr1, early growth response gene 1; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal regulated kinase; LDLR, low density lipoprotein receptor; OM, oncostatin M; SIRE, sterol-independent regulatory element; SRE-1, sterol regulatory element-1; SREBP, SRE-1 binding protein.

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gle base) within the SIRE motif completely abolish OM- and c-AMP-induced promoter activity and the activation synergy between OM and cAMP. Electrophoretic mobility shift assay (EMSA) using a double-stranded oligonucleotide containing the SIRE sequence detected three specific DNA-protein complexes (C1, C3, C4) from nuclear extract prepared from untreated HepG2 cells. OM stimulation induced formation of one additional complex, C2 (20). Although supershift assays demonstrated the presence of several family members of cAMP-responsive element binding protein (CREB), c/EBP, and c-jun in the complexes of C1 and C3, none of the tested antibodies, individually or in combination, could completely supershift or block the binding of the OM-induced complex C2.

In this study, using both in vitro and in vivo DNA binding assays, we demonstrate that the unknown DNA binding protein present in the OM-induced C2 complex is early growth response gene 1 (Egr1). Egr1 is a zinc finger transcription factor that regulates transcription of a variety of genes through specific binding to its recognition sequence (GCGGGGGCG) residing in the promoter region of target genes (21). Intriguingly, the LDLR promoter does not contain a consensus or a homologous Egr1 binding site. The interaction of Egr1 with the SIRE motif appears to require both the c/EBP binding site and the CRE site. Moreover, LDLR promoter activity is markedly stimulated by cotransfection of Egr1 with c/EBPβ or with CREB expression vectors. Our studies suggest that Egr1 might be a new candidate in the regulation of LDLR transcription. Egr1 may mediate the OM-induced upregulation of LDLR transcription through interaction with other SIRE binding proteins such as c/EBPβ or CREB.

MATERIALS AND METHODS

Cells and reagents

The human hepatoma cell line HepG2 and the human breast carcinoma-derived cell line T47D were obtained from American Type Culture Collection (Manassas, VA). HepG2 cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). T47D-TAM67 cells were generated in our laboratory by stable transfection of plasmid TAM67 into T47D cells (22). These cells were routinely cultured in RPMI medium supplemented with 10% FBS and 200 μg/ml of G418. Antibodies specific to the following proteins were obtained from Santa Cruz Biotechnology for use in EMSA: c/EBPβ, CREB-1, activating transcription factor (ATF)-1, ATF-2, ATF-3, c-jun, c-fos, Egr1, Egr2, and Egr3. Egr1-blocking peptide (Santa Cruz, sc-110P) contains an epitope of 18 amino acids mapping to the carboxyl terminus of human Egr1. The polyclonal anti-Egr1 antibody (sc-110) was generated by immunizing rabbits with this peptide. The plasmids pCMV-Egr1 and pCMV5, generously provided by Dr. Gerald Thiel at University of the Saarland Medical Center (Homburg, Germany) have been previously described (23). The plasmids pCIATF2 and pCIATF3 were gifts from Dr. Shigetaka Kitajima at Tokyo Medical & Dental University (Tokyo, Japan). The plasmid pEF-NFIL6, encoding the human homolog of rat c/EBPβ, was a gift from Dr. Shizuo Akira (Hyogo College of Medicine, Hyogo, Japan). The plasmid RSV-CREB was kindly provided by Dr. Linda M. Boxer at the VA Palo Alto Health Care System.

Preparation of nuclear extracts

HepG2 cells were cultured in EMEM supplemented with 10% FBS at a density of 4.5×10^6 cells per 100 mm dish for 3 days. Human recombinant OM at a concentration of 50 ng/ml or OM dilution buffer (1 mg/ml BSA in PBS) were then added to the cells for the indicated lengths of time prior to harvesting nuclear extract by the modified method of Dignam et al. as previously described (20, 24).

EMSA

Oligonucleotide probes were annealed and end-labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. Each binding reaction was composed of 10 mM HEPES (pH 7.8), 2 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 80 mM NaCl, 10% glycerol, 1 μg of poly (dI-dC), 1 μg BSA, and 6 μg nuclear extract in a final volume of 20 μl. Nuclear extracts were incubated with 0.4–0.5 ng of ^{32}P-labeled double-stranded synthetic oligonucleotide probe ($40–80 \times 10^3$ 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. In competition analysis, nuclear extracts were incubated with 2- to 50-fold molar excess of unlabeled competitor DNA for 5 min prior to the addition of the labeled probe. For supershift assays, antibody was incubated with nuclear extract for 30–60 min at room temperature prior to the addition of the probe. **Table 1** describes the sense sequences of EMSA probes and sequences of other oligonucleotides used in this study.

UV crosslinking and SDS-PAGE

An EMSA was performed with labeled MU13 probe as described above. The wet gel was exposed to a short-wave UV box from a distance of 2–3 cm at 4°C for 1 h. The gel was then briefly exposed to a PhosphorImager screen to locate the complexes. The region of the gel containing the C2 complex was cut out,

TABLE 1. Sequences of oligonucleotides used in EMSA and ChIP assay

	Nucleotide Sequence (5′ to 3′)
EMSA oligonucleotides	
SIRE-wt	CATTGAAA*TGCTGTAAA*TGACGTGGGCCCC
SIRE-mu1	CATTGAA**cg***GCTGTAAA*TGACGTGGGCCCC
SIRE-mu5	CATTGAAA*TGCTGTA**ct***TGACGTGGGCCCC
SIRE-mu7	CATTGAAA*TGCTGTAAA*TG**cg**GTGGGCCCC
SIRE-mu12	CATTGAAA*TGCTGTAAA***g**GACGTGGGCCCC
SIRE-mu13	CATTGAAA*TGCTGTAAA*T**c**ACGTGGGCCCC
SIRE-mu23	CATTGAAA*TGCTGTAAA*T**a**ACGTGGGCCCC
Ap1 consensus	CGCTTGATGACTCAGCCGGAA
C/EBP consensus	TGCAGATTGCGCAATCTGCA
CRE consensus	AGAGATTGCCTGACGTCAGAGAGCTAG
Egr1 consensus	GGATCCAGCGGGGGCGAGCGGGGGCGA
Sp1	TTCGAAACTCCTCCCCCTGCTAG
LDLR ChIP primers	
Sense strand	CGATGTCACATCGGCCGTTCG
Antisense strand	CACGACCTGCTGTGTCCTAGCTGGAA

Ap1, activator protein 1; C/EBP, CCAAT/enhancer binding protein; CRE, cAMP-responsive element; Egr1, early growth response gene 1; EMSA, electrophoretic mobility shift assay; LDLR, low density lipoprotein receptor; SIRE, sterol-independent regulatory element.

The c/EBP site is italic, the CRE site is underlined, the mutated nucleotides are small bold letters, and the consensus binding sequences are double underlined.

and the complex was eluted at room temperature overnight in elution buffer containing 50 mM Tris-HCl (pH 7.9), 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl, and 50 μg/ml γ-globulin. The eluted proteins were precipitated with four volumes of dry ice-cold acetone, washed with ethanol, and air-dried. After resuspension in Laemmili loading buffer and heating, SDS-PAGE was performed, and the labeled proteins were visualized by a PhosphorImager.

Western blot analysis

An EMSA was performed with a SIRE probe as described above. The wet gel without UV crosslinking was briefly exposed to a screen of PhosphorImager to locate the complexes. The region of the gel containing the C2 complex was cut out, and the complex was eluted and loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane and blotted with rabbit anti-Egr1 polyclonal antibody (sc-110, 1:1000 dilution) using an enhanced chemiluminescence (ECL) detection system (Amersham).

Chromatin immunoprecipitation assays with antibodies to human Egr1 and CREB

HepG2 cells were untreated or treated with OM (50 ng/ml) for 1 h and thereafter were crosslinked 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 bp to 600 bp by sonication. Chromatin immunoprecipitation (ChIP) assays with rabbit antibodies to Egr1 (sc-110) and CREB (sc-186) 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 reverse the crosslinking 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 EB buffer [10 mM Tris (pH 8.5)]. The DNA isolated using the same procedure with omission of the immunoprecipitation 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, circumventing the SIRE sequence. The PCR conditions were 94°C for 5 min, 94°C for 30 s, 68°C for 30 s, 72°C for 30 s, and 72°C for 5 min. The 180 bp PCR product was visualized on 2% agarose gels stained with ethidium bromide. The intensity of the PCR products was scanned with a BioRad Fluro-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.

RNA isolation and northern blot analysis

Cells were lysed in Ultraspec RNA lysis solution (Biotecxs Laboratory, Houston, TX), and total cellular RNA was isolated according to the vendor's protocol. Approximately 15 μg of each total RNA sample was used to analyze LDLR mRNA or Egr1 mRNA. The RNA blots were first hybridized to a 0.84 kb ^{32}P-labeled human LDLR probe, and then stripped and reprobed with a 3 kb human Egr1 cDNA probe. Finally, the membrane was probed with a human GAPDH probe to ensure that equivalent amounts of RNA were being analyzed. Hybridization signals were visualized by a BioRad PhosphorImager and were quantified by the Quantity One program.

Site-directed mutagenesis

The mutant LDLR promoter reporter (pLDLR-SIREMU6) was generated by site-directed mutagenesis on template DNA (pLDLR234Luc) with the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA). The correct mutation at the SIRE CRE site (TGACGT→gGACGT) was verified by dideoxy sequencing.

Transient transfection assays

HepG2 cells seeded in 24-well plates were transiently transfected with plasmid DNA by the method of calcium phosphate coprecipitation (20). In assays of cotransfection, the plasmid DNA ratio of pLDLR234Luc to expression vectors was 2:1. After transfection, cells were cultured in 10% FBS EMEM for 24 h, then switched to cholesterol-containing medium (0.5% lipoprotein-depleted serum plus 10 μg/ml cholesterol and 1 μg/ml 25-hydroxylcholesterol) overnight in order to demonstrate a sterol-independent regulation. Before harvesting, cells were stimulated with OM for 4 h. Total cell lysates were collected, and luciferase and β-galactosidase activities were assayed. Absolute luciferase activity was normalized against β-galactosidase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition, and at least three independent transfection assays were performed for each reporter construct.

T47D-TAM67 cells were transiently transfected with Effectene reagent (Qiagen, Valencia, CA). For cotransfection experiments, the plasmid DNA ratio of pLDLR234Luc to expression vectors was 2:1, as it was in HepG2 cells. A renilla luciferase reporter, pRL-SV40, was used as an internal control for transfection efficiency in T47D-TAM67 cells. After transfection, luciferase activities were measured using the Promega Dual Luciferase Assay System. Absolute promoter firefly luciferase activity was normalized against renilla luciferase activity to correct for transfection efficiency. Triplicate wells were assayed for each transfection condition and at least three independent transfection assays were performed for each reporter construct.

RESULTS

Identification of Egr1 as the OM-induced SIRE binding protein

Using supershift assays, we have previously demonstrated that CREB/ATFs, c/EBPβ, and c-jun bind to the SIRE motif of the LDLR promoter in a constitutive manner, whereas an unknown transcription factor present in the C2 complex only interacts with this promoter region upon OM stimulation (20). In order to completely rule out the possibility that the OM-induced factor is related to the c/EBP or CRE/activator protein 1 (Ap1) binding proteins, we conducted EMSA with OM-treated extract and labeled SIRE probe in the presence of competing oligonucleotides containing consensus sequences for Ap1, CRE, and c/EBP. As shown in **Fig. 1**, addition of the Ap1 oligonucleotide completely eliminated the binding of C1 to the labeled probe, but it did not compete for the binding of C2. The ability of CRE oligonucleotide to compete for the binding of C1 and C3 was even stronger than the wild-type unlabeled SIRE probe, but it only slightly competed for the binding of C2. The c/EBP oligonucleotide competed for the binding of C1 and C3 in a manner similar to the wild-type unlabeled probe, but it failed to eliminate the binding of C2. In contrast to these consensus oligonucleotides, an oligonucleotide containing the Sp1 binding site did not compete for the binding of any of the complexes. Based on these results and the previous supershift assays, we conclude that the OM-induced C2 com-

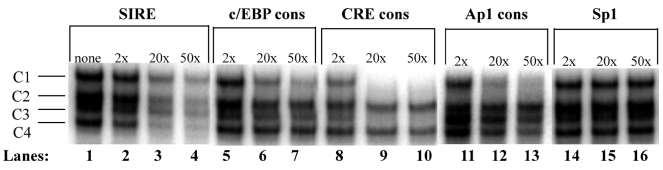

Fig. 1. Electrophoretic mobility shift assay (EMSA) competition analysis of sterol-independent regulatory element (SIRE) with oligonucleotides containing consensus binding sites for CCAAT/enhancer binding protein (c/EBP), cAMP-responsive element (CRE), or Ap1. Oligonucleotides containing consensus recognition sequence for Ap1, c/EBP, or CRE were synthesized and used in the competition binding assays at the same molar ratio as the wild-type competitor DNA. An unrelated oligonucleotide containing an Sp1 binding site of the repeat 3 sequence of the low density lipoprotein receptor (LDLR) promoter was included as a nonspecific competitor.

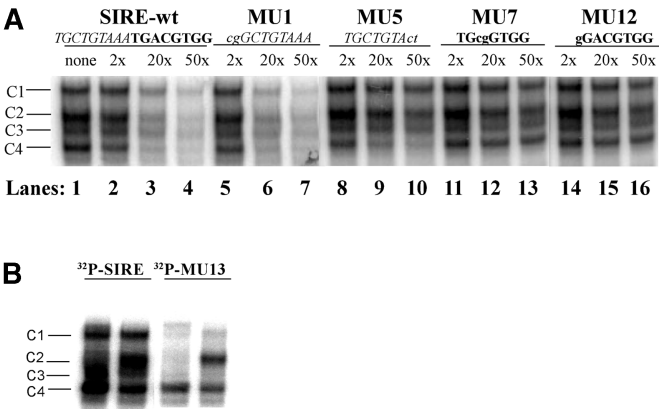

Fig. 2. Examination of the mutational effects of the c/EBP binding site or the CRE site on the binding of the oncostatin M (OM)-induced transactivator to the SIRE DNA. A: Gel shift assays with OM-treated nuclear extract and the ^{32}P-labeled SIRE probe were conducted in the presence of increasing concentrations of oligonucleotides that contain base substitutions at either the c/EBP binding site or the CRE site. The c/EBP site is italic, the CRE site is bold, and the mutated nucleotides are small letters. B: The DNA/protein complexes formed with the labeled MU13 probe were compared with that formed with the labeled wild-type SIRE probe.

plex contains a unique transactivator that does not belong to the c/EBP, CREB, or Ap1 family. These studies also suggest that C4 contains a factor unrelated to these known DNA binding proteins. Because C4 is not induced by OM, its identity was not further investigated in this study.

To determine whether the binding of C2 to the SIRE probe was mediated through the c/EBP site or through the CRE site, radiolabeled SIRE probe was incubated with OM-treated nuclear extract in the presence of different amounts of unlabeled oligonucleotides containing base substitutions at either the c/EBP binding site (MU1, MU5) or the CRE site (MU7, MU12). A change of two nucleotides at the region 5′ adjacent to the c/EBP site (MU1) did not affect the ability of the oligonucleotide to compete for binding, whereas a change of two bases within the 3′ c/EBP site (MU5) significantly diminished the ability of the mutated oligonucleotide to compete with the SIRE probe to C1, C2, and C3. Similarly, alteration of two bases (MU7) or even a single base (MU12) within the CRE site completely abrogated the binding of all four complexes to the mutated SIRE sequence (**Fig. 2A**). These data suggest that the OM-induced factor interacts with both the c/EBP site and the CRE site of the SIRE motif.

The effects of mutations were further evaluated by competition binding assays with additional single base substitutions at the CRE site. We found that oligonucleotide MU23 with a mutation at the second nucleotide in the CRE site (TGACGT→TaACGT) did not compete for the binding of any of the complexes. By contrast, the oligonucleotide MU13, with an alteration of the same nucleotide from G to C (TGACGT→TcACGT), was able to compete for the binding of C2 in a manner similar to the wild-type SIRE. However, the ability of MU13 to compete for the binding of other complexes was diminished, suggesting that MU13 may have a higher binding affinity for C2 than to other complexes. Therefore, gel shift assays using ^{32}P-labeled MU13 and wild-type SIRE were performed. As shown in Fig. 2B, the overall binding of nuclear extract to the MU13 probe was less efficient as compared with the wild-type SIRE sequence; however, the OM-induced C2 complex was predominant and was clearly separated from other complexes. In an attempt to characterize protein components in the C2 complex, we performed UV crosslinking after EMSA. The C2 band was then excised off the gel, and the protein components were analyzed by denaturing SDS-PAGE. **Figure 3A** shows that one major band with slower mobility and one minor band of faster mobility were crosslinked to the labeled DNA. After correction for the bound probe, the molecular masses of these two proteins appear to be 85 kDa and 30 kDa approximately. The 30 kDa protein is possibly ATF-3, as we have detected a faint supershift band by anti-ATF-3 antibody from the C2-MU13 complex (data not shown).

Previously, we have shown that the transcription factor Egr1 was rapidly induced by OM in HepG2 cells (25). Because Egr1 has a molecular mass of 82 kDa, similar to the molecular weight of the major protein that was crosslinked to the MU13 probe, we performed a Western blot analysis to determine the reactivity of the 85 kDa protein to the Egr1 antibody. In this experiment, we used a ^{32}P-labeled oligonucleotide containing an Egr1 binding site as a positive control. OM-treated nuclear extract was incubated with the labeled MU13 probe and Egr1 probe, respectively. The C2-MU13 complex and the Egr1 DNA protein complex were isolated and loaded onto SDS-PAGE along with the total cell lysates isolated from untreated and OM-treated HepG2 cells. The proteins were then transferred to a nitrocellulose membrane and probed with anti-Egr1 antibody. As shown in Fig. 3B, Egr1 protein was detected from the C2 complex as well as from the DNA-protein complex formed with the Egr1 probe. A small amount of Egr1 protein was detected from untreated cell lysate, whereas an abundant level of Egr1 protein was detected from OM-treated cell lysate. To prove that Egr1 is indeed the OM-induced transactivator that interacts with the SIRE sequence, a supershift assay was conducted with

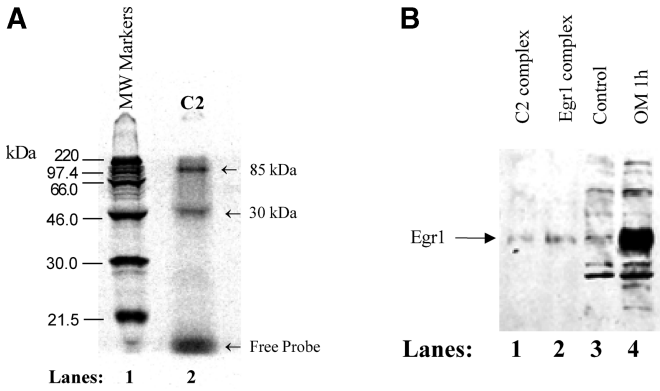

Fig. 3. Analysis of protein components of the C2 complex. A: UV crosslinking and SDS-PAGE. EMSA with OM-treated nuclear extract and the labeled MU13 probe was conducted. After electrophoresis, the wet gel was irradiated by a short-wave UV lamp, and the region of the gel containing the C2 complex was cut out. The complex was eluted at room temperature overnight and loaded onto a 7.5% denaturing SDS-polyacrylamide gel. The positions of ^{14}C-labeled molecular mass markers are shown in lane 1, and the proteins detected from the C2 complex are shown in lane 2. B: Western blot analysis of the C2 complex with anti-early growth response gene (anti-1Egr1) antibody. EMSA using ^{32}P-MU13 and ^{32}P-Egr1 probes was conducted with OM-treated nuclear extract. After electrophoresis, the C2 complex (lane 1) and the Egr1 binding complex (lane 2) were directly isolated from the wet gel without UV irradiation. The proteins eluted from the complexes were loaded onto a 7.5% denaturing SDS-polyacrylamide gel along with 50 μg of total cell lysates of untreated (lane 3) and OM 1 h-treated (lane 4) HepG2 cells. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and probed with a rabbit anti-Egr1 antibody.

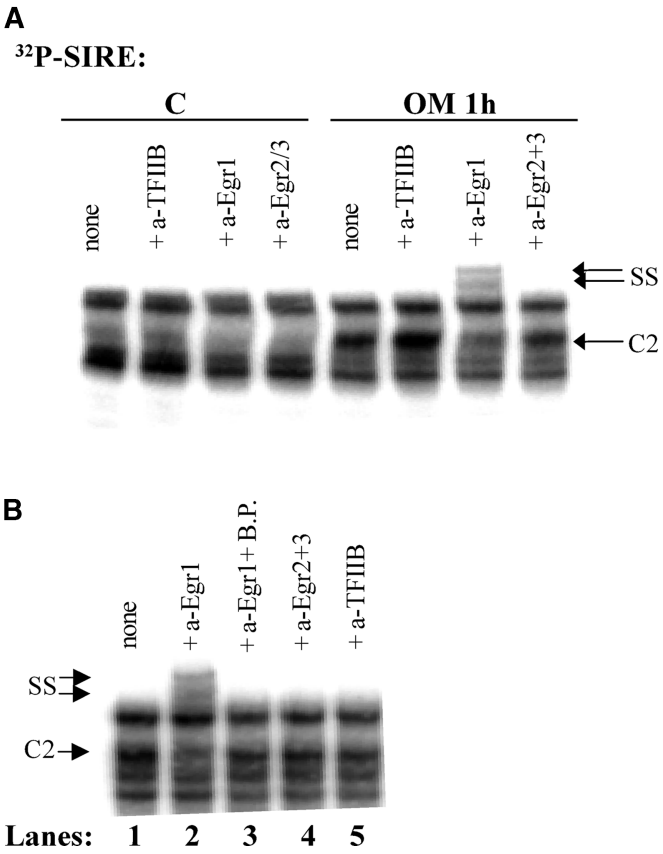

Fig. 4. Supershift assay with anti-Egr1 antibody. A supershift assay using labeled SIRE probe was conducted with antibodies directed to members of Egr1 family (Egr1, 2, and 3) and an unrelated DNA binding protein TFIIB in the absence (A) and the presence (B) of an Egr-1 blocking peptide.

labeled wild-type SIRE probe and the nuclear extracts of control and OM-treated cells. Anti-Egr1 antibody did not produce a supershift band with untreated extract, but it supershifted the C2 complex of OM-treated extract almost completely. In contrast, antibodies to other members of the Egr1 family and to an irrelevant DNA binding protein (TFIIB) had no effect (**Fig. 4A**). Incubation of the reaction mixture of the supershift with an Egr1-blocking peptide totally inhibited the interaction of the Egr1 antibody with the C2 complex (Fig. 4B, lane 3), further illustrating the specific interaction between the C2 complex and the anti-Egr1 antibody.

ChIP assays demonstrate the binding of Egr1 to the LDLR promoter in OM-stimulated cells in vivo

The above findings were obtained from DNA binding assays conducted under in vitro conditions with isolated nuclear extracts. To confirm the interaction of Egr1 with the LDLR promoter, we performed ChIP assays to directly detect the binding of Egr1 to the LDLR promoter SIRE sequence in intact cells. Control and OM-stimulated HepG2 cells were briefly treated with formaldehyde to crosslink DNA binding proteins to chromatin. The isolated chromatin was subjected to sonication followed by immunoprecipitation with rabbit anti-Egr1 and anti-CREB antibodies. Because CREB binding to the SIRE element is constitutive, as indicated by the supershift assays (20), we included anti-CREB IgG in the ChIP assay to serve as a positive control. In addition, normal rabbit IgG was used in the experiments as an irrelevant negative control. DNA from the immunoprecipitate was isolated. From this DNA, a 180 bp fragment of the LDLR proximal promoter region surrounding the SIRE sequence was amplified. **Figure 5** shows that the levels of CREB crosslinked to the SIRE sequence were slightly higher than the IgG control and were not changed by OM stimulation. In contrast, the level of Egr1 was significantly increased in OM-stimulated cells as compared with the control. Analysis of the material before the immunoprecipitation, designated as input DNA, showed equal levels of LDLR promoter DNA from untreated and OM-treated cells. Thus, the results of ChIP assays strongly support our in vitro finding that the OM-induced transcription factor that binds to the SIRE sequence in the LDLR promoter is Egr1.

Induction of Egr1 mRNA precedes the upregulation of LDLR transcription by OM in HepG2 cells

The next sets of experiments were designed to examine the kinetics of OM-induced Egr1 expression and LDLR upregulation. First, time-dependent induction of Egr1 binding to the SIRE sequence and Egr1 binding to a ca-

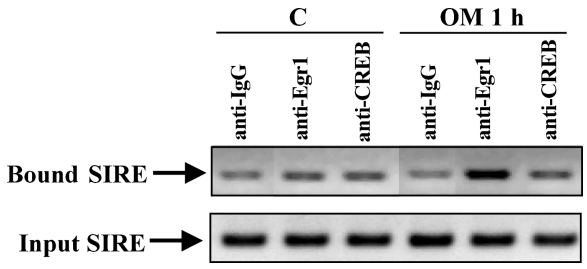

Fig. 5. Chromatin immunoprecipitation (ChIP) analysis for Egr1 and cAMP-responsive element binding protein (CREB) association with the LDLR promoter in control and OM-treated HepG2 cells. Antibodies to Egr1 and CREB were used in a ChIP analysis followed by PCR to amplify a 180 bp region surrounding the SIRE site from genomic DNA isolated from unstimulated and OM 1 h-stimulated HepG2 cells. Normal rabbit IgG was included in the assay as a negative control for nonspecific binding. The PCR product was separately on a 2% agarose gel, stained with ethidium bromide and quantified by a BioRad Fluro-S MultiImager system. "Bound" represents the DNA coimmunoprecipitated with antibody, while "input" represents the starting material before immunoprecipitation. The data shown are representative of three separate assays.

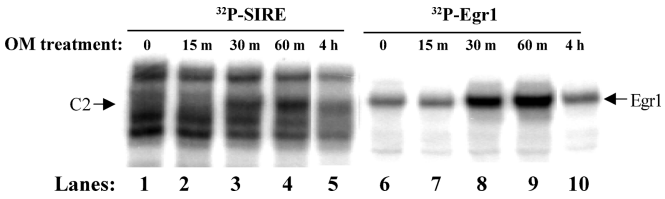

Fig. 6. Kinetics of the OM-induced Egr1 binding activities to the SIRE probe and to the Egr1 probe. HepG2 cells were treated with OM for the indicated lengths of time and harvested for isolation of nuclear extracts. EMSAs were conducted with labeled SIRE probe (lanes 1–5) and labeled Egr1 probe (lanes 6–10). A total of 6 μg of nuclear extract per sample was used in each binding reaction.

nonical Egr1 binding site were compared by using nuclear extracts of HepG2 cells that were treated with OM for different lengths of time. The results showed that the kinetics of C2 formation (**Fig. 6**, lanes 1–5) were identical to the kinetics of induced Egr1 DNA binding activity (Fig. 6, lanes 6–10). Second, to further examine the correlation between Egr1 expression and the activation of LDLR transcription, Northern blot analysis was performed to detect Egr1 mRNA and LDLR mRNA in HepG2 cells after OM treatment. **Figure 7A** shows that Egr1 mRNA became detectable after 15 min of OM treatment. The level of Egr1 mRNA was markedly increased by 30 min and reached its highest level at 1 h, then rapidly declined. However, a small amount of Egr1 mRNA was still detectable after 8 h. In comparison, the level of LDLR mRNA was increased 2.6-fold by 1 h; it reached its maximal level of 7.9-fold by 2 h and remained elevated for 8 h. These data show that transcription of Egr1 and LDLR is coordinately regulated by OM. More importantly, these results demonstrate that the induction of Egr1 mRNA precedes the upregulation of LDLR transcription by OM in HepG2 cells. The observation that the LDLR mRNA stayed at the induced level while Egr1 mRNA already declined suggests that, in addition to Egr1, other factors may be induced by OM at longer time periods that are responsible for the maintenance of the high LDLR mRNA expression. Alternatively, the half-life of Egr1 protein might be prolonged by OM, which is sufficient to maintain transcription of the LDLR gene.

Blockade of the ERK MAP kinase pathway abolishes the stimulating effects of OM on Egr1 mRNA and LDLR mRNA expression

Our previous studies have shown that the effect of OM on LDLR transcription can be abrogated by blocking the extracellular signal regulated kinase (ERK) signaling pathway with an inhibitor of MAP kinase kinase (MEK) (26). To determine whether a MEK inhibitor has a similar effect on the activity of OM on Egr1 mRNA expression, HepG2 cells were stimulated with OM for 1 h in the presence of different concentrations of U0126, a specific and potent MEK inhibitor. Figure 7B shows that U0126 had nearly identical dose-dependent inhibitory effects on OM-

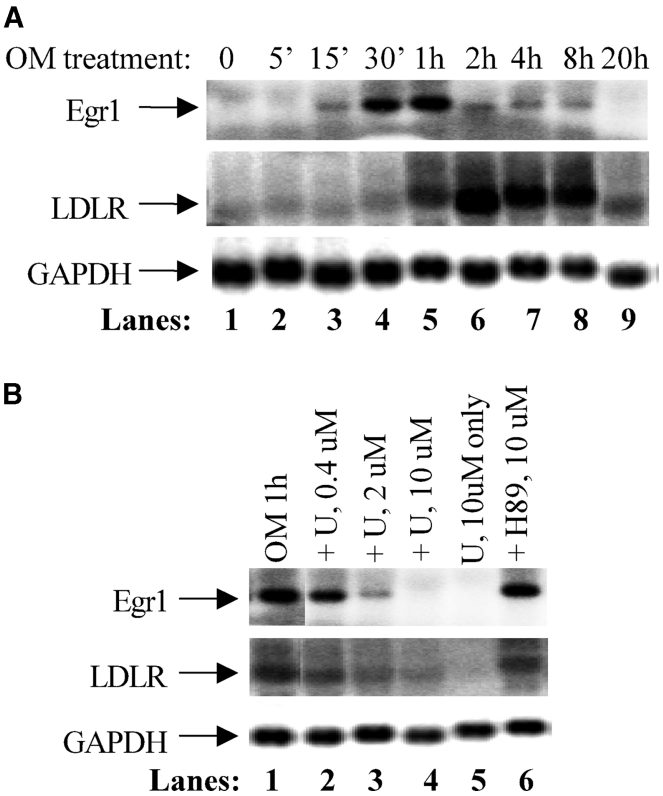

Fig. 7. Effects of OM on Egr1 mRNA and on LDLR mRNA expression. A: HepG2 cells cultured in EMEM containing 10% FBS were incubated with 50 ng/ml OM for different times as indicated. Total RNA was isolated, and 15 μg per sample was analyzed for LDLR mRNA by Northern blot. The membrane was stripped and rehybridized to a human Egr1 cDNA probe. Finally, the membrane was probed with a human GAPDH probe. B: HepG2 cells were treated with OM for 1 h in the absence or the presence of different concentrations of U0126 (lanes 1–4), or the presence of 10 μM H89 (lane 6). Total RNA was then isolated and analyzed for Egr1 and LDLR mRNA expression.

induced LDLR and Egr1 mRNA expression. In contrast, H89, a selective inhibitor of protein kinase A, had no effect on OM-mediated Egr1 mRNA or LDLR mRNA induction. These data provide additional evidence to support a functional role of Egr1 in OM-induced LDLR transcription.

Effect of overexpression of Egr1 on LDLR promoter activity

Finally, to directly examine the function of Egr1 as a transactivator in LDLR transcription following its binding to the SIRE site, LDLR promoter reporter pLDLR234Luc was transiently transfected with an Egr1 expression vector pCMV-Egr1 or with a control empty vector pCMV5 into HepG2 cells. In addition, we transfected cells with plasmid RSV-βGal as an internal control for variability in tranfection efficiency. The basal LDLR promoter activity was increased approximately 2-fold ($P = 0.012$) by pCMV-Egr1 transfection and was further increased to a small extent by OM induction (20% increase, $P = 0.2$), as compared with Egr1 control vector pCMV5 (**Fig. 8**, Mock). Because the EMSA data showed that the binding of Egr1 to the SIRE sequence requires bindings of c/EBPβ and CREB/ATFs, we cotransfected pCMV-Egr1 with equal amounts of expression vectors encoding for ATF-2, ATF-3, or c/EBPβ, respectively. Cotransfection of pLDLR234Luc with ATF-2 increased the basal LDLR promoter activity 2-fold in the presence or absence of pCMV-Egr1. Cotransfection of ATF-3 and pLDLR234Luc with or without pCMV-Egr1 did not affect the basal or OM-induced LDLR promoter activities. In contrast to ATF-2 and ATF-3, cotransfection of a c/EBPβ expression vector (pEF-NFIL6) increased pLDLR234Luc activity 4.3-fold. Interestingly, coexpression of Egr1 with c/EBPβ seemed to increase basal LDLR promoter activity (30% increase, $P = 0.06$) as well as OM-induced LDLR promoter activity (36% increase, $P = 0.03$), albeit to a moderate level.

To further evaluate the effect of Egr1 expression on LDLR transcription, we employed a second independent cell line, T47D-TAM67, a stable cell line derived from T47D breast cancer cells. **Figure 9** shows that OM increased the LDLR promoter activity to a similar extent in HepG2 and T47D-TAM67 cells. Importantly, in both cell lines, the effects of OM were completely abolished by a single base mutation (TGCTGTAAATGACGTGG→TGCTGTAAAgGACGTGG) at the SIRE motif. The same mutation also eliminated the binding of the OM-induced C2 complex in the gel shift assay (Fig. 2A, lanes 14–16).

Therefore, we examined the effects of Egr1 and other SIRE binding proteins on LDLR promoter activity in T47D-TAM67 cells by transient transfection of pLDLR234Luc plasmid with pCMV5 or with pCMV-Egr1 in the absence or presence of other transactivators. **Figure 10** shows that expression of Egr1 alone did not increase basal or OM-induced LDLR promoter activity. Expression of ATF-2 or ATF-3 slightly increased LDLR promoter basal activity (2-fold), which was not affected by Egr1 overexpression. Interestingly, LDLR promoter basal activity was increased 11-fold ($P < 0.001$) by cotransfection with c/EBPβ alone and was increased 26-fold ($P < 0.001$) by coexpression of Egr1 with c/EBPβ. OM stimulation further increased the luciferase activity. Similar to c/EBPβ, cotranfection of pLDLR234Luc with a CREB expression vector increased

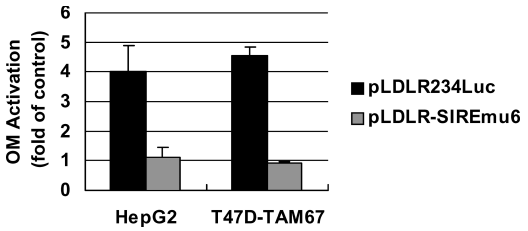

Fig. 9. A SIRE mutation abolishes OM induction of LDLR promoter activity in HepG2 and T47D-TAM67 cells. LDLR promoter wild-type vector pLDLR234Luc and the SIRE mutant vector pLDLR-SIREmu6 were separately transfected into HepG2 and T47D-TAM67 cells. Twenty hours after transfection, cells were treated with OM (50 ng/ml) for 4 h prior to cell lysis. The normalized luciferase activity is expressed as the fold of luciferase activity in untreated control cells.

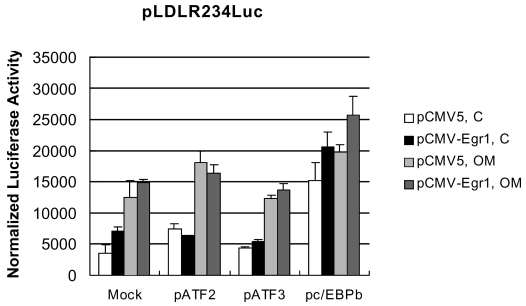

Fig. 8. Analysis of LDLR reporter luciferase activities in HepG2 cells. The LDLR promoter reporter pLDLR234Luc was cotransfected with pCMV-Egr1 or with pCMV5 in the absence (mock) or the presence of an equal amount (50 ng) of ATF-2, ATF-3, or c/EBPβ expression vectors. Forty hours after transfection, cells were treated either with OM (50 ng/ml) or with OM dilution buffer (1 mg/ml BSA in PBS) for 4 h prior to harvesting cell lysates. Luciferase activity was normalized against β-galactosidase activity.

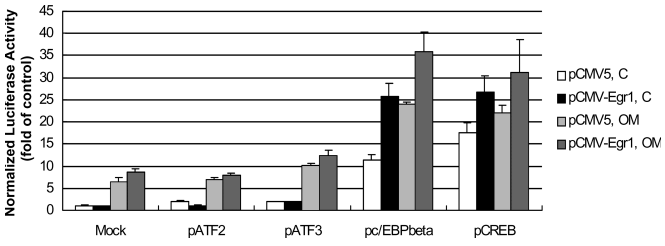

Fig. 10. Activation of LDLR promoter activity by coexpression of Egr1 with c/EBPβ or with CREB in T47D-TAM67 cells. The LDLR promoter reporter pLDLR234Luc was cotransfected with pCMV-Egr1 or with pCMV5 in the absence (mock) or the presence of equal amounts of ATF-2, ATF-3, c/EBPβ, or CREB expression vectors into T47D-TAM67 cells. Forty hours after transfection, cells were treated either with OM (50 ng/ml) or with OM dilution buffer for 4 h prior to harvesting cell lysates. The normalized luciferase activity is expressed as the fold of luciferase activity in untreated control cells (mock).

luciferase activity 17-fold ($P < 0.001$) in the absence of pCMV-Egr1. When Egr1 and CREB were cotransfected simultaneously, LDLR basal promoter activity was increased 27-fold ($P < 0.001$).

DISCUSSION

The zinc finger transcription factor Egr1 (27), also known as zif268 (28), knox24 (29), NGF1-A (30), and TIS8 (31), belongs to a group of immediate-early genes whose transcription is rapidly activated by extracellular stimuli in the absence of new protein synthesis. Egr1 couples extracellular signals to long-term cellular responses by alteration of transcription of late response genes through specific binding to its recognition sequence (5′-GCGGGGGCG-3′) residing in the promoter region of target genes. In this study, however, we report for the first time that Egr1 interacts with the LDLR promoter through the SIRE sequence that does not contain a canonical or a homologous Egr1 binding site.

We have demonstrated the presence of Egr1 in the OM-induced C2 complex that specifically binds to the SIRE probe by supershift assays using Egr1 antibody and isolated nuclear extracts. Importantly, applying a ChIP assay that detects the binding of a transcription factor to its canonical sequence on chromatin in intact cells, we demonstrated the OM-stimulated binding of Egr1 to the LDLR promoter in vivo, which fully supports our in vitro DNA binding studies.

The function of Egr1 as a transactivator in LDLR promoter activity was first evaluated in HepG2 cells by overexpression of Egr1. Without cotransfection of other SIRE binding proteins, we detected a small stimulatory effect of pCMV-Egr1 on pLDLRLuc activity. Similarly, we observed a moderate increase in the LDLR promoter activity in cells cotransfected with pCMV-Egr1 and a c/EBPβ expression vector in both untreated and OM-treated cells. By contrast, cotransfection of pCMV-Egr1 with ATF-2 or ATF-3 expression vectors did not increase LDLR promoter activity at all. These observations were recapitulated in a second independent cell line T47D-TAM67 in which we detected a large synergistic effect on LDLR promoter activity by cotransfection of Egr1 with c/EBPβ or with CREB.

There are several reasons that could explain why in both cell systems we did not detect a strong induction of LDLR promoter activity by overexpression of Egr1 alone. First, it is possible that c/EBPβ and/or CREB are cofactors for Egr1, and the activity of Egr1 on the LDLR promoter may require cooperation with c/EBPβ or CREB. A similar scenario has been reported for the interaction of c/EBPβ and c-Myb. For example, for regulation of myeloid-specific genes, overexpression of c-Myb alone did not affect promoter reporter activity, and overexpression of c/EBPβ resulted in a moderate increase. However, promoter activity was markedly activated by cotransfection of expression vectors for c-Myb and c/EBPβ (32). Second, a posttranslational modification of Egr1, such as phosphorylation induced by OM, may be critical for Egr1 to regulate LDLR transcription. The exogenously expressed Egr1 is not phosphorylated and it may not be able to interact with cofactors efficiently, thereby failing to elicit a strong transcriptional response. Third, Egr1 might not be the only factor induced by OM that interacts with the SIRE sequence. Stimulation of LDLR transcription through the SIRE sequence could involve the induced binding of some unidentified factors besides Egr1. Additional experiments will be needed to answer these questions.

Egr1 expression and transactivation are subject to regulation by the MAP kinase ERK signaling pathway. Activation of ERK kinase activity leads to the transcription of the Egr1 gene (33). The transcriptional activity of Egr1 protein is further enhanced by ERK-mediated phosphorylation (34). In HepG2 cells, OM rapidly activates the ERK signaling pathway. Within 5 min of OM treatment, ERK1 and ERK2 become phosphorylated and stay in the phosphorylated form for at least 1 h. Our previous studies have shown that the activation of LDLR transcription by OM can be totally inhibited by blocking the ERK signaling pathway with the MEK inhibitor U0126 (26). In addition, treatment of HepG2 cells with U0126 eliminated the formation of the C2 complex (20). These data suggest that the transcription factor in the C2 complex is a substrate of ERK. In this study, we have demonstrated that U0126 has the same dose-dependent inhibitory effect on OM-induced Egr1 and LDLR transcription. Moreover, induction of Egr1 mRNA expression precedes the transcriptional activation of the LDLR gene. These sequential events strongly suggest that activation of the MEK/ERK pathway leads to an increased LDLR transcription through the specific interaction of Egr1 with the SIRE site of the promoter.

In considering a physiological rationale why activation of the Raf-1/MEK/ERK cascade would activate LDLR transcription, it should be noted that in general, cytokines stimulate hepatic lipogenesis and VLDL secretion. One could speculate that in parallel to this increase in lipoprotein secretion, cytokines might stimulate LDLR transcription as a compensatory mechanism whereby the liver increases reuptake of newly secreted lipoproteins, thus maintaining intracellular cholesterol homeostasis.

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