

Sterol regulatory element binding proteins (SREBP)-1a and SREBP-2 are linked to the MAP-kinase cascade

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Abstract The classic sterol regulatory *cis* element (*sre-1*) in the LDL receptor promoter mediates sterol regulatory element binding protein (SREBP)-binding and the effects of insulin and platelet derived growth factor (PDGF). To elucidate whether SREBP-1a and SREBP-2 play a direct role in insulin and PDGF action, stable cell lines of HepG2 deficient in either SREBP-1 or SREBP-2 were used. Transfection of these cells with the wild-type promoter fragment of the low density lipoprotein (LDL) receptor gene showed that the effects of insulin and PDGF were significantly reduced in both, SREBP-1- as well as SREBP-2-deficient cells. Insulin and PDGF action could be reconstituted again in these deficient cell lines by reintroducing SREBP-1a or SREBP-2. Preincubation of cells with either the phosphatidylinositol (PI)-3 kinase inhibitor wortmannin or the mitogen-activated protein (MAP) kinase cascade inhibitor PD 98059 showed that the latter abolished the stimulatory effects of insulin and PDGF on LDL receptor promoter activity completely, whereas wortmannin had no effect. Overexpression of upstream activators of the MAP kinases, like MEKK1 or MEK1, stimulated LDL receptor promoter activity several fold in an *sre-1* related manner. These effects could be enhanced by coexpression of the transcriptional active N-terminal domains of SREBP-1a and SREBP-2. Using the heterologous Gal-4 system, we could show that intracellular activation of the MAP kinase cascade by ectopic expression of MEKK1 or MEK1 has a direct stimulatory effect on the transcriptional activity of SREBP-1a and SREBP-2. Experimental evidence for a direct link between MAP kinases and SREBPs was obtained due to the MAP kinases ERK1 and ERK2 phosphorylating recombinant GST-fusion proteins of SREBP-1a and SREBP-2, *in vitro*. We conclude that SREBP-1a and SREBP-2 mediate different regulatory effects converging at *sre-1* and that they appear to be linked to the MAP kinase cascade, possibly being direct substrates of ERK1 and ERK2.—Kotzka, J., D. Müller-Wieland, G. Roth, L. Kremer, M. Munck, S. Schürmann, B. Knebel, and W. Krone. Sterol regulatory element binding proteins (SREBP)-1a and SREBP-2 are linked to the MAP-kinase cascade. *J. Lipid Res.* 2000. 41: 99–108.

Supplementary key words SREBPs • MAP kinase cascade • transcription factors • insulin • growth factors

Alterations of cellular cholesterol homeostasis appear to play a major role in the pathogenesis of atherosclerosis. A breakthrough in the understanding of cellular cholesterol metabolism was the characterization of the low density lipoprotein (LDL) receptor and its mutations in patients with familial hypercholesterolemia and premature atherosclerosis (1, 2). In accordance with that, several large prospective clinical trials have shown recently that treatment of patients with (3–5) and without (6, 7) coronary heart disease (CHD) with statins to lower plasma cholesterol levels leads to a significant reduction of cardiovascular morbidity and mortality. One key question in the understanding of cellular cholesterol homeostasis is, what happens to cell metabolism when intracellular cholesterol concentration is reduced. In this respect a new and broader perspective in the understanding has been developed by the group of Brown and Goldstein (for review, see ref. 8). They have identified and characterized a family of cholesterol-sensitive transcription factors, called sterol regulatory element binding proteins (SREBPs). These intracellular proteins appear to transmit the signal of membrane-embedded cholesterol level to the nucleus regulating the expression rate of multiple genes.

The promoter of the LDL receptor gene contains a sterol regulatory element (*sre-1*/ATCACCCAC), which is regulated by the intracellular content of sterols. This DNA sequence in the promoter of the LDL receptor gene can bind three transcription factors: SREBP-1a, SREBP-1c, and SREBP-2. These transcription factors are activated by a novel cholesterol-regulated proteolytic mechanism (9) that controls the cytosolic release of these proteins and thereby, for example, the transcription rate of the LDL receptor gene. These transcription factors are localized in

Abbreviations: SREBP, sterol regulatory element binding protein; LDL, low density lipoprotein; LDLR, LDL receptor; PDGF, platelet-derived growth factor; CHD, coronary heart disease; EGF, epidermal growth factor; MAP, mitogen-activated protein; PCR, polymerase chain reaction; FCS, fetal calf serum; IGF, insulin-like growth factor; GST, glutathione-S-transferase.

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the endoplasmic reticulum. Decrease of intracellular sterol levels can activate protease activities, which cleave these transcription factors in the endoplasmic reticulum. The N-terminal domains with a molecular mass of approximately 68 kDa translocate by unknown mechanisms into the nucleus. In the nucleus the N-terminal domain of the activated transcription factors binds to sterol responsive elements not only in the LDL receptor gene but also in many others coding for enzymes involved not only in cholesterol metabolism, but also in fatty acid and triglyceride metabolism, and possibly others. It has been shown that beside the LDL receptor gene promoter, SREBPs can regulate transcription of genes, e. g., coding for the HMG-CoA reductase (10), HMG-CoA synthase (11), farnesyl diphosphate synthase (12, 13), acetyl CoA carboxylase (14), fatty acid synthase (15), glycerol-3-phosphate acyltransferase (16), stearoyl-CoA desaturase 2 (17), and interestingly, the gene of SREBP-2 (18).

Using different deletion mutations of the LDL receptor promoter, we (19) and others (20) could show that the sterol regulatory *cis* element of the LDL receptor gene promoter *sre-1* is also the *cis* element for insulin as well as insulin-like growth factor (IGF-1) and is part of the regulatory element mediating the effects of platelet derived growth factor (PDGF) as well as epidermal growth factor (EGF). By use of SREBP-1- and SREBP-2-deficient human hepatoma cell lines as well as recombinant glutathione-S-transferase (GST)-fusion proteins we show that SREBPs mediate different gene regulatory effects converging at *sre-1* and that they are linked to the MAP kinase cascade, possibly being direct substrates of ERK1 and ERK2.

MATERIALS AND METHODS

Plasmids and molecular cloning

Construction of the LDLR promoter reporter gene plasmids containing a functional intact sterol regulatory element (*sre*)-1 flanked by two SP1 elements (phLDL4) or fragments containing an inactivated *sre-1* either by mutation (phLDL7) or deletion (phLDL10) were described previously (19). Expression vectors pFC-MEKK for dominant-active MEKK1 (aa 380-672) and pFC-MEK1 for activated MEK1 (Δ 32-51/S218E/S222E) under control of CMV promoter were purchased from Stratagene. Reporter plasmid pFR-Luc, which contains five Gal4 DNA binding sites cloned upstream of a minimal promoter element and the firefly luciferase gene, was obtained from Stratagene.

Total RNA was isolated from HepG2 cells by guanidine isothiocyanate extraction protocol described previously (19). Using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech) the RNA was reverse transcribed according to the manufacturers protocol. A cDNA fragment corresponding to the amino acids 1-460 of SREBP-1a (21) and to the amino acids 1-468 of SREBP-2 (22), corresponding to transcriptional active aminoterminal domain (NT), was amplified by PCR from this single stranded cDNA using the sequence-specific 5'-primer (GGGGATCCCCATGGACGAGCCACCCCTTCA) and 3'-primer (GGAATTCCTCAGTCAGGCTCCGAGTCACTGCCA) for SREBP-1a and using the sequence-specific 5'-primer (GGGGATCCCGATGGACGACAGCGGCGGCT) and 3'-primer (GGAATTCCTCAGTCTGGCTCATCTTTGACCTT) for SREBP-2, which contain additional bases (underlined

sequence) to generate a BamHI and a EcoRI restriction site, respectively. These fragments were inserted in frame to glutathione-S-transferase into pGEX 3X (Pharmacia Biotech). The SREBP-1a-NT and SREBP-2-NT expression vectors were constructed by ligating the N-terminal domain of SREBP-1a (1380 bp) or SREBP-2 (1404 bp) as a BamHI/EcoRI fragment into the BamHI and EcoRI site of pcDNA3 (Invitrogen). To construct Gal4-SREBP-1a-NT and Gal4-SREBP-2-NT the corresponding fragment was ligated as a BamHI/EcoRI fragment into the BamHI and EcoRI site of expression vector pFA-CMV (Stratagene) containing the DNA binding domain of yeast transcription factor Gal4 (aa 1-147). Then, for in frame insertion, the construct was digested with BamHI, refilled with Klenow and religated. PCR-amplified GAPDH fragment (nt 271-824) was subcloned into pcDNA3 in antisense orientation to the T7 RNA promoter (pcGAPDH(-)). The sequences of the constructs were confirmed by using a model 373A DNA sequencer (Applied Biosystem Inc.).

Construction of a SREBP-2 antisense expression vector

A SREBP-2 cDNA fragment from amino acids 47 to 187 (22) was amplified from HepG2 cDNA using the sequence-specific 5'-primer (GTAGGATCCAGAACAGCTGTGTAGCTCCTTTCTGTC) and 3'-primer (ATTGAAGCTTTGGACTTGAGGCTGAAGGAC) which contain additional bases (underlined sequence) to generate a BamHI or a HindIII restriction site, respectively. This fragment was cloned into the pcDNA3 vector (Invitrogen) using the polylinker HindIII and BamHI restriction sites to generate pcSREBP-2(-).

Stable transfection of HepG2 cells with pcSREBP-2(-)

HepG2 cells were transfected with pcSREBP-2(-) DNA by electroporation as described below. After this, cells were selected by growth in medium containing 800 μ g/ml G418 (Sigma-Aldrich). After 20 days, individual G418-resistant colonies were selected and expanded in medium containing 500 μ g/ml G418.

RNase protection assay

After linearization of pcGAPDH(-) with NciI and pcSREBP-2(-) with EcoRI, antisense RNA was transcribed with [α P³²]UTP (>800 Ci/mmol) and bacteriophage T7 RNA polymerase (Promega). Specific activities (SREBP-2(-): 5×10^8 cpm/ μ g, GAPDH(-) 8×10^7 cpm/ μ g) were mixed with 15 μ g total RNA derived from either HepG2/pCDNA3, SREBP-1(-) or SREBP-2(-) cells and subjected to RNase protection assay (RiboQuant™ assay system (Pharmingen)). Each assay tube contained a cRNA probe for SREBP-2 and a cRNA probe complementary to the mRNA of GAPDH with the specific activity adjusted to give comparable signals to the tested SREBP-2 mRNA. After denaturation at 85°C for 5 min, hybridization was performed at 42°C overnight. Samples were RNase digested (12 ng RNaseA, 37.5 U RNaseT1/ reaction; 45 min 37°C) and thereafter reactions were terminated with proteinase K digestion followed by precipitation. The protected fragments were resuspended in loading buffer, denatured at 90°C, and separated on 8 m urea/6.4% PAA gels. Gels were dried and subjected to autoradiography at -80°C.

RT-PCR of the LDL receptor

One hundred ng total RNA of extracted from HepG2/pCDNA3, SREBP-1(-) or SREBP-2(-) cells was reverse transcribed with 0.1 pg d(N)₆ oligo in a total volume of 15 μ l using the Pharmacia first strand kit according to manufacturer's instructions. Ten μ l of each reaction was used for combined PCR amplification of a LDL receptor fragment from nt 1055-1424 using 30 pmol of the sequence-specific 5'-primer (GCCAGCGAAGATGCGAAGATAT) and 3'-primer (TGCTGATGACGGTGTCA TAGGAA) and of a GAPDH fragment from nt 462-815 using 30

pmol of the sequence-specific 5'-primer (CATGTTTCGTCATGGGTGTGAACC) and 3'-primer (CAGGTGAGGTCCACCACTGACAC). After 30 amplification steps, 10 μ l of the PCR products was separated on 3% agarose gels in 1 \times TAE as running buffer.

Cell culture and transient transfection assay

HepG2 cells were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS) (GibcoBRL) and antibiotics (GibcoBRL). HepG2/pCDNA3 cells and HepG2 cells with drastically reduced intracellular mRNA level of SREBP-1 (21) (SREBP-1(-)) or SREBP-2 (SREBP-2(-)) were cultured in RPMI 1640 medium supplemented with 10% (v/v) FCS, antibiotics and 500 μ g/ml G418. Before electroporation, cells were released by trypsinization, washed, and suspended

in Opti-MEM (GibcoBRL) supplemented with 10% (v/v) FCS. Cell suspensions (2×10^5 cells/well) were mixed with vectors as indicated in figure legends. Samples were transferred to an electroporation cuvette (interelectrode distance 0.4 cm) and pulsed for 18 msec in GenePulser II (Bio-Rad). Before seeding on 6-well plates (Costar), cell suspension was diluted with RPMI 1640 with 10% (v/v) FCS and antibiotics. For endogenous induction using expression vectors coding for dominant active MEKK1 or activated MEK1, cells were harvested 16 h after electroporation. For the treatment with insulin and PDGF, cells were cultured in serum-free medium on day 2 after electroporation for 16 h. Before harvesting, cells were incubated without or with insulin (10^{-7} m) or PDGF-AB (3.3×10^{-9} m) (Sigma-Aldrich) for 4 h. Luciferase activity was measured according to the supplier's instruc-

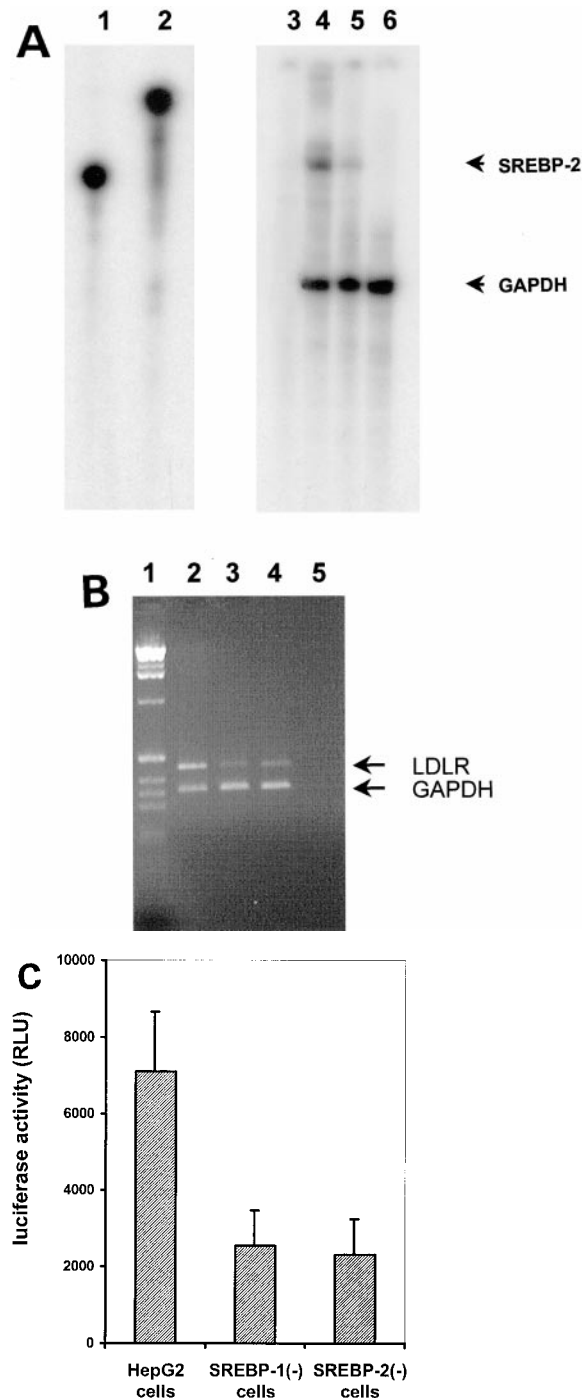


Fig. 1. A) Depletion of SREBP-2 mRNA in SREBP-2(-) cells. Fifteen μ g total RNA from either HepG2/pCDNA3, SREBP-1(-) or SREBP-2(-) cells was subjected to RNase protection assay. Each assay tube contained α - 32 P UTP [>800 Ci/mmol]-labeled cRNA probes for SREBP2 and GAPDH with the specific activity adjusted to give comparable signals to the tested SREBP mRNA. After RNase digestion, protected fragments were separated on 8 m urea/6.4% PAA gels. Gels were dried and subjected to autoradiography at -80°C . A typical result of three independent experiments is shown. Protected fragments are indicated by an arrowhead; (lane 1: 32 P-labeled cRNA probe for GAPDH, lane 2: 32 P-labeled cRNA probe for SREBP-2, lane 3: tRNA, lane 4: HepG2/pCDNA3, lane 5: SREBP-1(-), lane 6: SREBP-2(-)). B) Different expression of LDLR mRNA in HepG2 cells deficient for SREBP-1 or SREBP-2 in comparison to HepG2 cells. RT-PCR analyses of 100 ng total RNA of HepG2/pCDNA3, SREBP-1(-) or SREBP-2(-) cells were reverse transcribed and subsequently amplified with combined LDLR- and GAPDH-specific primers. PCR products were separated on 3% agarose gels in 1 \times TAE as running buffer. The specific products of LDLR and GAPDH are indicated (lane 1: 1Kb DNA ladder (GibcoBRL); lane 2: HepG2/pCDNA3; lane 3: SREBP-1(-); lane 4: SREBP-2(-); lane 5: negative control (PCR without cDNA)). A representative result of eight independent experiments is shown. C) Basal LDLR promoter activity in HepG2 cells deficient for SREBP-1 or SREBP-2 in comparison to HepG2 cells. HepG2/pCDNA3, SREBP-1(-) or SREBP-2(-) cells were transiently transfected with LDLR promoter construct phLDL4 (1 μ g/well). After electroporation cells were placed in 6-well plates and incubated in 10% FCS for 24 h. Then, cells were further incubated for 16 h in lipid- and serum-free medium to induce full endogenous SREBP activity. Luciferase activity (indicated as RLU) was measured as described under Materials and Methods. Results are given as means (\pm SD) of five independent experiments, each performed in triplicate.

tions (Promega). Transfection efficiency was monitored by cotransfection of β -galactosidase expression vector pSV- β Gal (Promega). β -Galactosidase activity was determined by galactose-light assay (Tropix). The data represent relative luciferase activity as x-fold induction of either endogenous or exogenous stimulation relative to unstimulated cells as indicated in the legends.

Protein kinase assay

The glutathione-S-transferase (GST)-SREBP-1a-NT and GST-SREBP-2-NT fusion proteins were expressed in *E. coli* strain BL21 and purified according to the manufacturer's recommendations (Pharmacia Biotech). Protein phosphorylations by MAP kinases ERK1 or ERK2 (Upstate Biotechnology) were performed with 10 μ g GST-SREBP-1a-NT, 10 μ g GST-SREBP-2-NT fusion protein or 0.5 μ g MBP (myelin basic protein) and activated GST-MAP kinase ERK1 (400 ng/assay) or ERK2 (100 ng assay) fusion proteins in kinase buffer (5 mm MOPS (pH 7.2), 6.25 mm β -glycerophosphate, 1.25 mm EGTA, 0.25 mm sodium-orthovanadate, 0.25 mm DTT). The reaction was initiated by addition of 50 μ M [γ - 32 P]ATP (10 Ci/mmol) in a final volume of 40 μ l kinase buffer. The reaction was terminated after 15 min at 25°C by addition of Laemmli sample buffer. The phosphorylations of GST-SREBP-1a-NT and GST-SREBP-2-NT were analyzed after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by autoradiography.

RESULTS

SREBPs are cellular sensors of lipid metabolites and might also integrate endocrine signals.

SREBPs are mediators of insulin and PDGF action

As previously reported (19, 20), SREBP-binding sterol regulatory *cis* element of the LDL receptor (LDLR) gene promoter *sre-1* is also the *cis* element for insulin as well as

insulin-like growth factor (IGF-1) and is the main regulatory element mediating the effects of PDGF as well as EGF. To evaluate a possible direct role of SREBPs in the signal transduction of insulin and PDGF on LDL receptor promoter activity, stable HepG2 cell lines were used having reduced intracellular levels of SREBP-1 (19) or SREBP-2 (Fig. 1A). In SREBP-1-deficient cells, SREBP-2 was moderately reduced, while SREBP-2-deficient cells have unaltered SREBP-1 expression (data not shown). Both cell lines showed reduced expression of the endogenous LDLR, determined by RT-PCR (Fig. 1B). Promotor reporter gene analyses using the wild-type LDLR promoter construct pHLDL4 showed reduced basal LDLR promoter activity in both SREBP-deficient cell lines (Fig. 1C). Transient transfections of these cell lines showed that the effects of insulin and PDGF on LDL receptor promoter activity were significantly reduced in SREBP-1- (Fig. 2) as well as in SREBP-2-deficient cells (Fig. 3). Accordingly, the insulin and PDGF action on LDL receptor promoter activity could be reconstituted by overexpression of the transcriptional active (mature) domain of SREBP-1a or SREBP-2 in the corresponding SREBP-deficient cell lines.

SREBPs are linked to the MAP kinase cascade

As SREBPs mediate the effects of insulin and PDGF on LDL receptor promoter activity, the next series of experiments were designed to identify the intracellular signaling pathway coupling SREBPs to the receptor-associated tyrosine kinases of insulin and PDGF at the cell surface. Major intracellular signaling steps of insulin and PDGF are activation of phosphatidylinositol (PI) 3-kinase or mitogen-activated protein kinases. To elucidate whether one of these

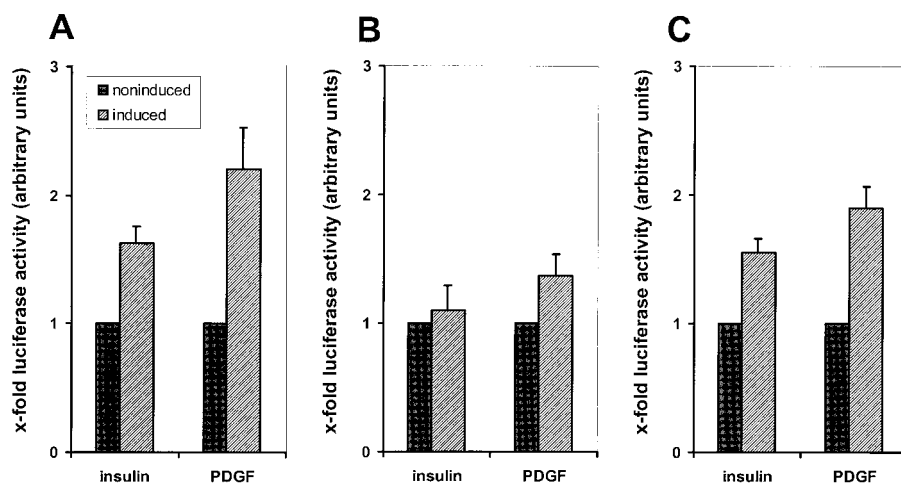


Fig. 2. Role of SREBP-1a in hormonal activation of LDLR promoter activity. HepG2/pcDNA3 cells (A) and SREBP-1(-) cells without (B) or with ectopically expressed SREBP-1a-NT (0.05 μ g/well) (C) were transiently transfected with LDLR promoter construct pHLDL4 (1 μ g/well). Before harvesting as described under Materials and Methods, cells were incubated with insulin (1×10^{-7} m) or PDGF (3.3×10^{-9} m) for 4 h. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 μ g/well). Results are given as means (\pm SD) of five independent experiments, each performed in triplicate. Insulin and PDGF stimulation was reduced in SREBP-1(-) cells (B vs. A) and significantly reconstituted (B vs. C), the lowest level of significance was $P < 0.01$. Black bars: noninduced cells; hatched bars: cells induced by insulin or PDGF as indicated. Ectopic expression of SREBP-1a in SREBP-1(-) cells increased basal promoter activity 6-fold.

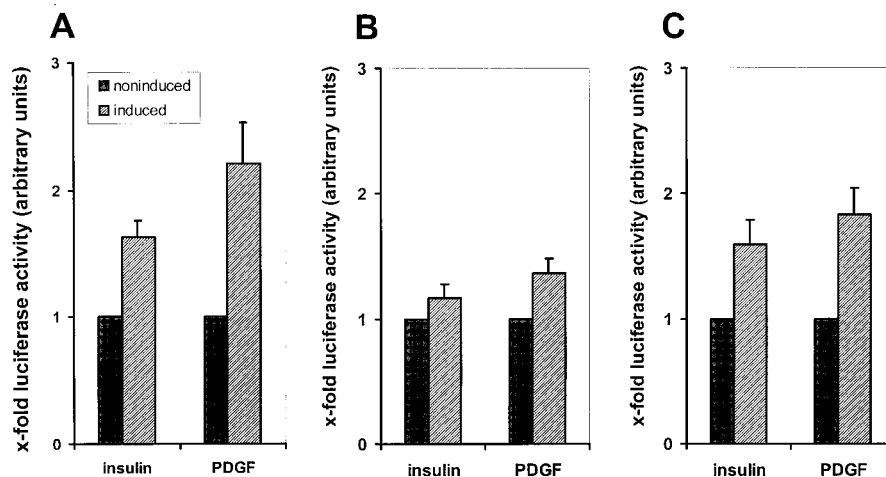


Fig. 3. Role of SREBP-2 in hormonal activation of LDLR promoter activity. HepG2/pcDNA3 cells (A) and SREBP-2(-) cells without (B) or with ectopically expressed SREBP-2-NT (0.05 $\mu\text{g}/\text{well}$) (C) were transiently transfected with LDLR promoter construct phLDL4 (1 $\mu\text{g}/\text{well}$). Before harvesting as described under Materials and Methods, cells were incubated with insulin (1 $\times 10^{-7}$ m) or PDGF (3.3 $\times 10^{-9}$ m) for 4 h. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 $\mu\text{g}/\text{well}$). Results are given as means (\pm SD) of five independent experiments, each performed in triplicate. Insulin and PDGF stimulation was reduced in SREBP-2(-) cells (B vs. A) and significantly reconstituted (B vs. C), the lowest level of significance was $P < 0.01$. Black bars: noninduced cells; hatched bars: cells induced by insulin or PDGF as indicated. Ectopic expression of SREBP-2 in SREBP-2(-) cells increased basal promotor activity 3-fold.

pathways mediates the effects of insulin and PDGF on LDL receptor promoter activity, HepG2 cells were preincubated with selective inhibitors (Fig. 4). Wortmannin blocked PI 3-kinase activity under these experimental conditions (25 nm, data not shown), but had no effect on insulin and PDGF-stimulated LDL receptor promoter activity. However, incubation of cells with 75 μm of the MAP-kinase kinase (MEK) inhibitor PD 98059 completely abolished the effects of insulin and PDGF. To further support that the MAP kinase cascade might mediate the effects of insulin and PDGF on LDL receptor promoter activity, endogenous upstream activators of the MAP kinase pathway, i.e., MEKK1 or MEK1, were ec-

topically expressed. Figure 5 shows that overexpression of MEKK1 and MEK1 stimulated LDL receptor promoter activity (phLDL4) several-fold. These effects were greatly reduced by using LDL receptor promoter constructs in which the SREBP-binding *cis*-element *sre-1* was inactivated by point mutation (phLDL7) or deletion (phLDL10). Cotransfection of HepG2 cells with vectors containing the nucleotide sequences coding either for the transcriptional active N-terminal domain of SREBP-1a (Fig. 6) or SREBP-2 (Fig. 7) enhanced the effects of constitutively active MEKK1 or MEK1 almost 10-fold. To evaluate whether SREBPs were directly activated by MAP kinases in intact cells, we used the hetero-

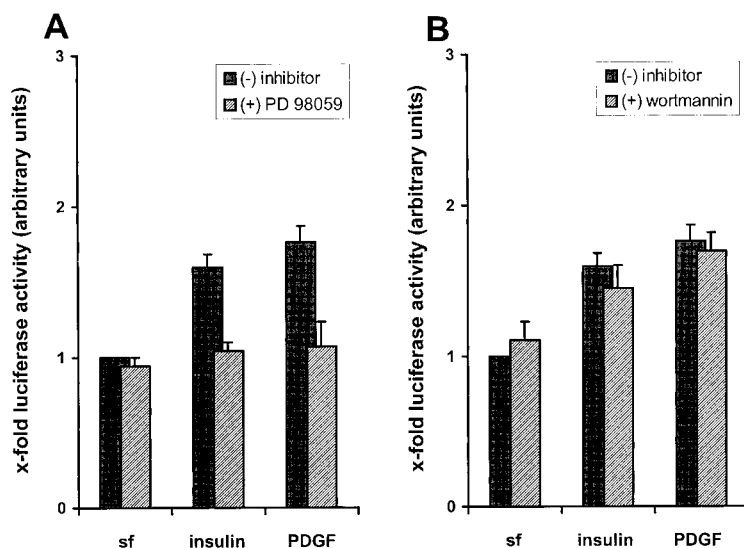


Fig. 4. Effects of MAP kinase pathway inhibitor PD98059 and the PI-3 kinase inhibitor wortmannin on insulin or PDGF induced LDLR promoter activity. HepG2 cells were transiently transfected with LDLR promoter construct phLDL4 (1 $\mu\text{g}/\text{well}$) and cultured in 0.5% LPDS medium for 16 h. Before harvesting, cells were incubated with insulin (10⁻⁷ m) or PDGF (3.3 $\times 10^{-9}$ m) for 4 h either in absence or presence of 75 μm PD 98059 (A) or 25 nm wortmannin (B) added 45 min prior insulin or PDGF treatment. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 $\mu\text{g}/\text{well}$). All transfection experiments were carried out in triplicate. Results are given as a means (\pm SD) of four independent experiments, each performed in triplicate.

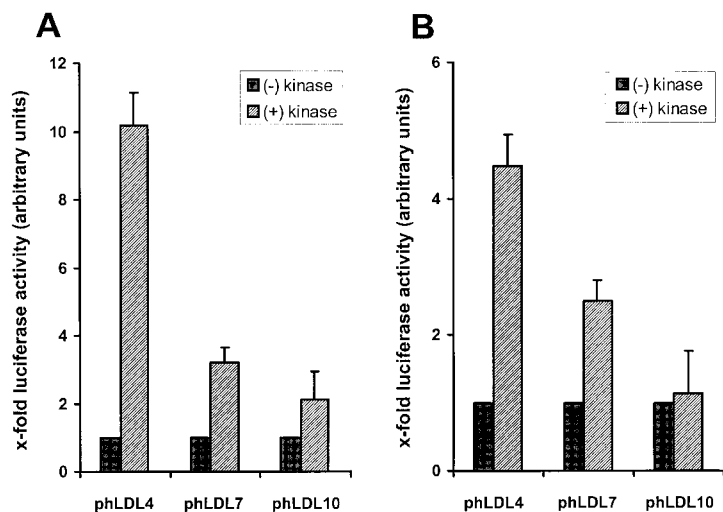


Fig. 5. Effects of MEKK1 and MEK1 converge on *sre1* in the promoter of LDLR gene. HepG2 cells were either transiently transfected with LDLR promoter construct phLDL4 (1 μ g/well), phLDL7 (1 μ g/well), or phLDL10 (1 μ g/well) along with expression vectors (0.05 μ g/well) without (black bars) or containing (grey bars) (A) dominant active MEKK1 or (B) activated MEK1, respectively. Conditions and promoter constructs are described under Materials and Methods. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 μ g/well). Results are given as a means (\pm SD) of three independent experiments, each performed in triplicate.

logues Gal4 system. Cotransfection of HepG2 cells with a vector containing either N-terminal domain of SREBP-1a or SREBP-2 coupled to the DNA binding domain of yeast Gal4 revealed that ectopic expression of constitutively active MEKK1 or MEK1 stimulated the transcriptional activities of SREBP-1a and SREBP-2 (**Fig. 8**). The stimulatory effect of upstream MAP kinase pathway activators on transcriptional activity of SREBPs was about 2-fold by MEK1 and about 7- to 10-fold by MEKK1.

SREBPs are substrates of MAP kinases in vitro

To study whether SREBPs might be direct substrates of MAP kinases, we produced and purified recombinant

GST fusion proteins of SREBP-1a and SREBP-2. **Figure 9** shows that incubation of the N-terminal transcriptional active protein domains of SREBP-1a and SREBP-2 with activated recombinant ERK1 or ERK2 led to a significant phosphorylation in vitro. Phosphoamino acid analysis revealed phosphorylation exclusively on serine residues, which was reversible by alkaline phosphatase treatment (data not shown).

DISCUSSION

SREBPs have been initially identified as transcription factors that modulate the expression rate of the LDL re-

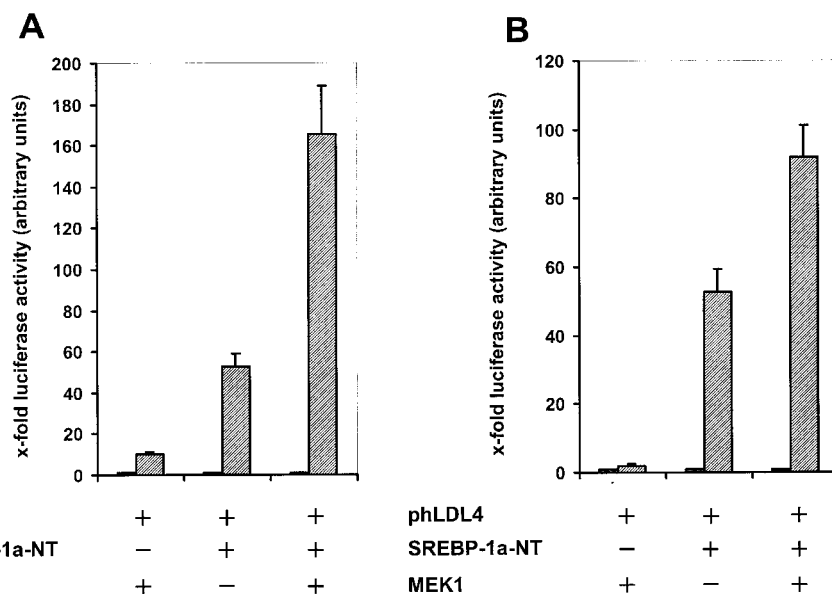


Fig. 6. MEKK1 and MEK1 enhance transcriptional activation of LDLR gene by SREBP-1a. HepG2 cells were transiently transfected with LDLR promoter construct phLDL4 (1 μ g/well) along with expression vectors (0.05 μ g/well) without (black bars) or containing (grey bars) (A) SREBP-1a-NT and dominant active MEKK1 or (B) SREBP-1a-NT and activated MEK1, respectively. Conditions and vectors are described under Materials and Methods. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 μ g/well). Results are given as a means (\pm SD) of five independent experiments, each performed in triplicate.

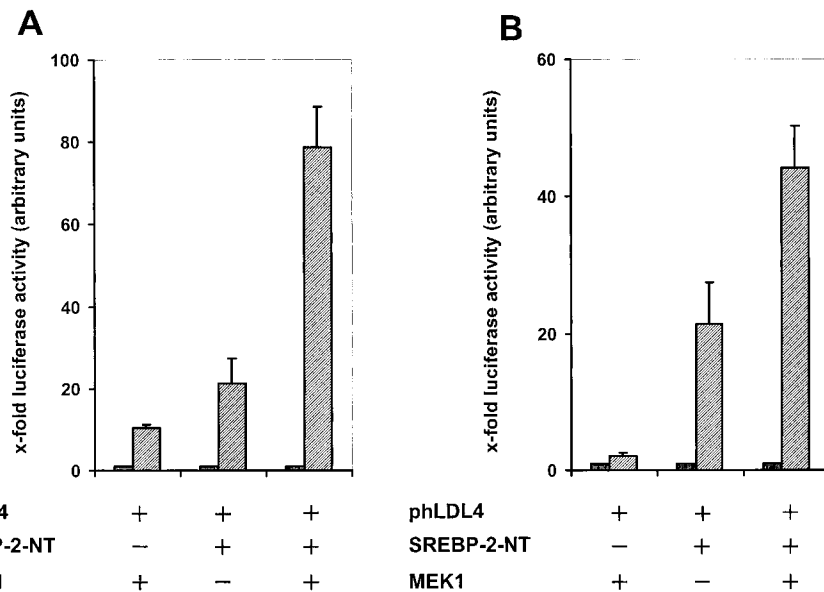


Fig. 7. MEKK1 and MEK1 enhance transcriptional activation of LDLR gene by SREBP-2. HepG2 cells were transiently transfected with LDLR promoter construct phLDL4 (1 μ g/well) along with expression vectors (0.05 μ g/well) without (black bars) or containing (grey bars) (A) SREBP-2-NT and dominant active MEKK1 or (B) SREBP-2-NT and activated MEK1, respectively. Conditions are described under Materials and Methods. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 μ g/well). Results are given as a means (\pm SD) of five independent experiments, each performed in triplicate.

ceptor gene, and are regulated by the intracellular levels of sterols. Until now, there appear to exist three SREBPs, SREBP-1a, SREBP-1c, and SREBP-2. Here we demonstrate that SREBPs 1) are a convergence point also for signaling

induced by insulin and PDGF, 2) are modulated by MAP kinase-related signaling, e.g., increasing their transcriptional activity, and 3) are phosphorylated by MAP kinases in vitro. In this study we have used the LDL receptor pro-

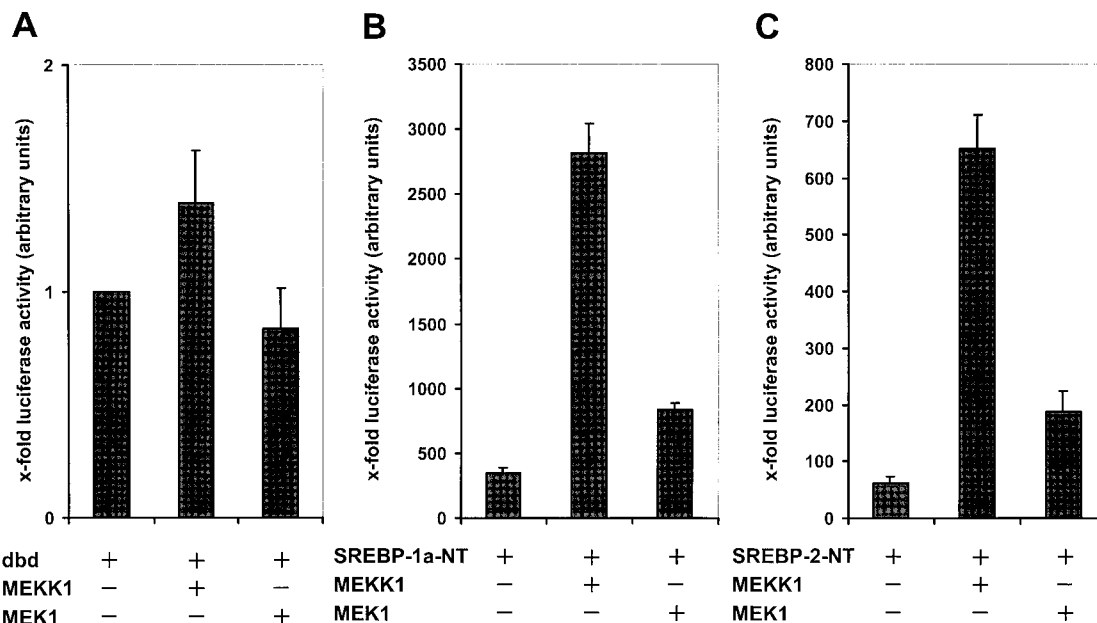


Fig. 8. Kinases of MAP kinase cascade enhance transcriptional activity of SREBPs. HepG2 cells were transiently transfected with reporter plasmid pFR-Luc (1 μ g/well) and an expression vector for Gal4 fusion protein (0.05 μ g/well) coding for the binding domain of Gal4 alone (dbd) (A), or the latter fused either to SREBP-1a-NT (B) or SREBP-2-NT (C) along with expression vectors (0.05 μ g/well) without or containing dominant active MEKK1 or activated MEK1 as indicated. Conditions are described under Materials and Methods. Transfection efficiency was monitored and normalized by cotransfecting cells with pSV β -galactosidase vector (1 μ g/well). Results are given as means (\pm SD) of five independent experiments, each performed in triplicate.

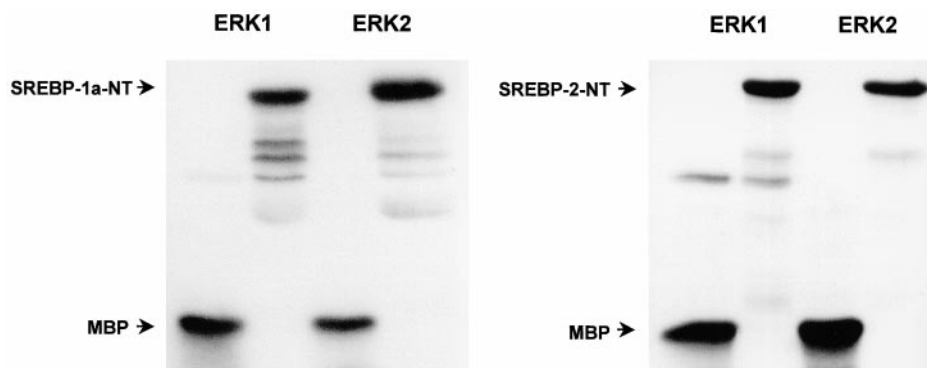


Fig. 9. Phosphorylation of SREBP-1a and SREBP-2 by MAP kinases ERK1 and ERK2 in vitro. Bacterial-synthesized glutathione-S-transferase (GST)-SREBP-1a-NT or (GST)-SREBP-2-NT fusion protein was treated with active GST-MAP kinase ERK1 or ERK2 fusion proteins and separated by SDS-PAGE followed by autoradiography. Conditions are described under Materials and Methods. A representative result from five independent experiments is shown. Myelin basic protein (MBP) served as an internal standard to monitor kinase activity.

motor, because this promoter contains the classic SREBP-binding 10 base pair *cis* element *sre-1*. However, it must be further investigated whether the transcriptional regulatory activity of SREBPs is similar for other genes also containing *sre-1* or *sre-1*-like element.

SREBPs: a convergence point of metabolic and endocrine signaling

The LDL receptor gene is not only regulated by cholesterol but also by drugs, growth factors, and hormones. It has been shown that insulin and PDGF can induce the number of LDL receptors at the cell surface in hepatic and non-hepatic cells and can elevate the LDL receptor mRNA levels (23–26). Furthermore, the insulin-induced effect on LDL receptor mRNA levels can even be seen in the presence of LDL cholesterol levels (250 $\mu\text{g}/\text{ml}$) that completely suppress LDL receptor gene expression (19). Experiments using various 5'-deleted and in vitro mutated LDL receptor promoter constructs showed that the insulin-sensitive *cis* element in the LDL receptor promoter is identical to the sterol-sensitive and SREBP-binding *cis* element *sre-1* (19). Similar experiments have been obtained for PDGF, although in PDGF action the *sre-1* flanking SP1 sites are also involved in the gene regulation of the LDL receptor (data not shown). Further direct evidence that SREBPs are involved not only in cholesterol-regulated events, but also in mediating the effects of growth factors and hormones like insulin, was obtained using SREBP-1-deficient cell lines in which the effect of insulin is greatly reduced while there is no alteration of the SREBP-1 cleavage rate (19). To investigate this in more detail, SREBP-1 and SREBP-2-deficient cell lines were generated (Fig. 1). The observation that SREBP-1-deficient cells also have a moderate reduction of SREBP-2 mRNA levels is expected, as the promoter region of SREBP-2 contains a functional *sre-1* element (18). It is interesting to note that the effects of insulin and PDGF were not only decreased in SREBP-1-deficient cells, but also in SREBP-2-deficient HepG2 cells. Accordingly, the effects of insulin and PDGF could be reconstituted by overexpressing or reintroducing SREBP-1a

or SREBP-2 into the corresponding cell lines. These data prove that SREBPs mediate the effects of insulin and PDGF on the LDL receptor promoter and that both transcription factors are needed.

SREBPs are modulated by MAP kinase-related signaling

Major signaling pathways of insulin and PDGF or receptor-associated tyrosine kinases are the PI 3-kinase and MAP kinase cascade. Activation of the PI 3-kinase (27–29) appears to play an essential role in insulin-mediated action on the translocation of glucose transporters and might have some gene regulatory effects by affecting the activity of the S6⁷⁰ kinase. However, many gene regulatory events induced by receptor-associated kinases are mediated by the MAP kinase cascade (30, 31). There are different families of MAP kinases, but the effects of insulin and PDGF are mostly linked to the ERK-family. MAP kinases ERK1 and ERK2 phosphorylate different transcription factors possibly thereby modulating their transcriptional activity, e.g., Elk-1 (32) or PPAR- γ 2 (33). One hypothesis in gene regulation by insulin is that this hormone might modulate preexisting transcriptional complexes of cells and thereby exhibit a pleiotropic hormonal effect on gene regulation depending on the pattern of preexisting transcription factors in a given cell. The fact that the inhibition of the MAP kinase cascade, but not of the PI-3 kinase cascade, completely abolishes the effects of insulin and PDGF on LDL receptor promoter activity indicates that this intracellular signaling pathway might be linked to the SREBPs. This evidence was further substantiated by overexpressing upstream activators of the MAP kinase cascade, like MEKK1 or MEK1. An ectopic expression of these kinases stimulated LDL receptor promoter activity several-fold and these effects were greatly reduced by point mutated or deleted LDL receptor promoter constructs abolishing the ability of the promoter to bind SREBPs. The residual promoter activity observed in MEKK1- or MEK1-activated cells transfected with LDL receptor promoter constructs containing a functionally inactivated *sre-1* might (20) indicate that other regulatory

promoter elements are involved in activation as it also has been observed for PDGF (20). Furthermore, MEK1, which is a relatively selective activator of the ERK-family of MAP kinases, appears to have always a lower stimulatory effect on LDL receptor promoter activity (see Figs. 5 to 7) than MEKK1, which as an upstream activator can also stimulate other MAP kinase families, like p38 kinase (34). Recently, evidence has been obtained that modulation of the p38 kinase pathway might be involved in LDL receptor gene regulation by cytokines (35). Direct evidence that endogenous activation of the MAP kinase cascade stimulates the transcriptional activity of the N-terminal bHLH-LZ-containing domain of SREBPs was obtained by using the Gal4 system (see Fig. 8). This system is of special value to investigate a possible direct regulatory effect on a given transcription factor independent of its DNA-binding domain or cellular background. The experiments indicate that activation of the MAP kinase cascade increases the transcriptional activity of SREBP-1a and SREBP-2 to a similar degree. Enhancement of transcriptional activity of SREBPs might be caused either by a direct modulation of the protein structure and thereby DNA-binding activity or by regulating possible protein-protein interactions. We believe that this MAP kinase cascade-related mechanism might be one, which is cholesterol-independent and affects the transcriptional activity of SREBPs directly.

SREBPs are substrates of MAP kinases

The most likely regulatory mechanism by which MAP kinase cascade-related signaling affects transcriptional activity of SREBPs is phosphorylation. The first evidence that MAP-kinase cascade might be directly linked to the SREBPs was proven by the ability of ERK1 and ERK2 to phosphorylate SREBP-1a and SREBP-2 in vitro. Using different protein domains of SREBPs, we could show that only the N-terminal (Fig. 9) transcriptional active and not the C-terminal domain (data not shown) of SREBPs was phosphorylated. Potential phosphorylation sites for MAP kinases are threonine or serine residues, which are followed by a proline (36). Considering this sequence motive, there are 17 potential sites in the N-terminal domain of SREBP-1a and 11 sites in SREBP-2. Future studies should identify the phosphorylation sites and analyze their functional relevance by in vitro mutagenesis.

Taken together, we provide evidence that SREBPs are not only regulated by intracellular sterol levels, but also by phosphorylation. Therefore, there might be signals of metabolic and endocrine parameters converging at transcription factors like SREBPs. This might be of importance not only for the regulation of the LDL receptor promoter, but also for all other genes affected by SREBPs. ■

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