pp90^{RSK}- and protein kinase C-dependent pathway regulates p42/44^{MAPK}-induced LDL receptor transcription in HepG2 cells

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Abstract **We have previously shown that different extracellular stimuli require signaling through the Raf/MEK/p42/44^{MAPK} cascade to induce LDL receptor expression. The present studies were designed to delineate the molecular mechanisms underlying p42/44^{MAPK}-induced LDL receptor transcription in HepG2-ΔRaf-1:ER cells, a modified HepG2 cell line in which the Raf-1/MEK/p42/44^{MAPK} cascade can be specifically activated by anti-estradiol ICI182,780 in an agonist-specific manner. Using these cells, we show that: *a*) LDL receptor induction was reduced in reporter constructs containing mutation in either Sp1 or sterol-regulatory element-1 (SRE-1) sites, whereas inactivation of both sites abolished the induction; *b*) E1A, which inhibits CREB binding protein (CBP), a common activator of SRE-1 binding protein and Sp1, strongly repressed the induction; *c*) intracellular inhibition of the 90 kDa ribosomal S6 kinase (pp90^{RSK}) cascade reduced LDL receptor induction; *d*) highly selective protein kinase C (PKC) inhibitors effectively abrogated the induction without affecting activation of pp90^{RSK}; and *e*) overexpression of PKCβ significantly induced LDL receptor promoter activity. Taken together, these results demonstrate that pp90^{RSK} and PKCβ are downstream effectors and Sp1, SRE-1 binding protein, and CBP are part of the transcriptional complex resulting in induction of LDL receptor expression in response to activation of the Raf/MEK/p42/44^{MAPK} cascade. These findings identify for the first time a role for PKCβ in determining the specificity of p42/44^{MAPK} signaling by participating with pp90^{RSK} in regulating gene expression.**—Kapoor, G. S., C. Golden, B. Atkins, and K. D. Mehta. **pp90^{RSK}- and protein kinase C-dependent pathway regulates p42/44^{MAPK}-induced LDL receptor transcription in HepG2 cells.** ***J. Lipid Res.*** **2003.** 44: **584–593.**

Supplementary key words 90 kDa ribosomal S6 kinase • extracellular signal-regulated kinase • CREB binding protein

Mitogen-activated protein kinases (MAPKs) are the central mediators that propagate extracellular signals from the cell membrane to the nucleus. These serine/threonine kinases are present in all cell types and play a critical role in regulation of a wide variety of biological response mechanisms. The p42/44^{MAPK} group of MAPKs (also called ERK1/ERK2) is a central component of signal transduction pathways activated by diverse extracellular stimuli, including mitogens, growth factors, and cytokines (1–3). The p42/44^{MAPK} group itself is activated by phosphorylation on both threonine and tyrosine residues by two dual-specificity MAPK kinases (also known as MEKs). MEKs are in turn activated by serine phosphorylation catalyzed by a number of MAPK kinase kinases, which include Raf-1, B-Raf, Mos, and MEK kinase-1. Upon activation by MEK via the Raf/MEK/p42/44^{MAPK} cascade, phosphorylated p42/44^{MAPK} induces pleiotropic effects ranging from phosphorylation of cytoplasmic proteins to activation of nuclear factors (1–3). Among the first substrates of p42/44^{MAPK} is the family of 90 kDa ribosomal S6 kinases (pp90^{RSK}s, also known as MAPKAP-K1) (4). This kinase family is activated via phosphorylation, and its members have been shown to be ubiquitous and versatile mediators of signal transduction (5–7). Diverse substrates have been identified, including transcription factors such as the cAMP response element binding protein (CREB) (8), estrogen receptor-α (9), IκBα/NF-κB, and c-Fos (10); the transcriptional coactivators CREB binding protein (CBP)

Abbreviations: CBP, CREB binding protein; CREB, cAMP response element binding protein; HepG2-ΔRaf-1:ER, HepG2 cells stably expressing ΔRaf-1:ER; ICI182,780, (7α-[9-[(4,4,5,5,5,-pentafluoropentyl) sulfinyl]nonyl]-estra-1,3,5(10)-triene-3,17β-diol; LPDS, lipoprotein-deficient serum; MAPK, mitogen-activated protein kinase; p42/44^{MAPK}, extracellular signal-regulated kinase; PKC, protein kinase C; pp90^{RSK}, 90 kDa ribosomal S6 kinase; SRE, sterol regulatory element; SREBP, SRE binding protein; WT, wild type.

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and p300 (11, 12); several proteins in the ribosomal complex (13); glycogen synthase kinase-3 (14); the neutral cell adhesion molecule L1 CAM (15); the Ras GTP-GDP exchange factor Sos (16); and the p34 cdc2 inhibitory kinase Myt 1 (17). The diversity of these substrates suggests that pp90^{RSK} is involved in the regulation of a wide range of cellular functions (4).

For the past several years, our laboratory has focused on the molecular mechanisms underlying regulation of LDL receptor induction by a variety of transcriptional modulators. To investigate the role of the p42/44^{MAPK} signaling cascade in regulating LDL receptor expression, we have previously utilized a specific cell-permeable noncompetitive inhibitor of MEK-1/2, PD98059 (2-(2′-amino-3′-methoxyphenyl)-oxanaphthalene-4-one). Our studies demonstrated for the first time requirement of the Raf-1/MEK/p42/44^{MAPK} cascade for the induction of LDL receptor transcription in human hepatoma HepG2 cells by a variety of agents, including phorbol esters, hepatocyte growth factor, interleukin-1β, and anisomycin (reviewed in ref. 18). Investigations by other laboratories also supported the requirement of this signaling cascade for insulin- and oncostatin-induced LDL receptor expression (19, 20). Taken together, these studies showed that different extracellular stimuli require signaling through p42/44^{MAPK} to induce LDL receptor expression in hepatic cells. Recently, insulin- and oncostatin-induced LDL receptor transcription have been related to phosphorylation of two different nuclear factors via p42/44^{MAPK}. Kotzka et al. (19) showed that modification of SRE binding protein (SREBP) is responsible for insulin-induced LDL receptor transcription, whereas Liu et al. (20) have shown data supporting the involvement of a novel nuclear factor interacting within the TATA box region to induce LDL receptor expression. Given the complexities of insulin and oncostatin signaling resulting in the activation of multiple pathways, it is not possible to define the sole contribution of the p42/44^{MAPK} signaling cascade in the induction of LDL receptor promoter activity.

To determine the mechanism responsible for p42/44^{MAPK}-induced LDL receptor transcription, we have generated a HepG2-derived cell line (designated HepG2-ΔRaf-1:ER) that stably expresses an inducible form of ΔRaf-1:ER, a fusion protein consisting of an oncogenic form of human Raf-1 kinase linked to the hormone binding domain of the human estrogen receptor (21). These cells were used to demonstrate that activation of the Raf-1/MEK/p42/44^{MAPK} cascade alone by anti-estradiol ICI182,780 (7α-[9-[(4,4,5,5,5,-pentafluoropentyl) sulfinyl]nonyl]-estra-1, 3,5(10)-triene-3,17β-diol) is sufficient to induce LDL receptor transcription to levels observed using cytokines or growth factors in HepG2 cells (22).

In the present work, we explored how the multiresponsive LDL receptor promoter may be upregulated by activation of p42/44^{MAPK} and determined the underlying mechanism for promoter stimulation at the transcription factor and cofactor levels. Using HepG2-ΔRaf-1:ER cells, we showed involvement of sterol regulatory element-1 (SRE-1) and an adjacent Sp1 site, together with the requirement of CBP for transactivation of LDL receptor promoter in response to p42/44^{MAPK} activation. Further examination demonstrated that inhibition of protein kinase C (PKC) blocks p42/44^{MAPK}-induced LDL receptor transcription without affecting activation of p42/44^{MAPK} or pp90^{RSK}, suggesting the requirement of a Raf-1-dependent and PKC-mediated pathway in the induction process. Transfection and biochemical studies support requirement of the PKCβ isoform in the induction process. The above results predict the likely existence of multiple signaling cascades that are differentially coupled to the pp90^{RSK} signaling pathway to regulate specific gene expression in human cells.

MATERIALS AND METHODS

Materials

TRIzol and tissue culture supplies were purchased from Life Technologies (Invitrogen Corp.). Fetal bovine lipoprotein-deficient serum (LPDS) was obtained from PerImmune, Inc. Zeta-Probe blotting membrane and the protein assay reagent were purchased from Bio-Rad Laboratories. PD98059, calphostin C, bisindolylmaleimide I, and Go6976 were purchased from Calbiochem-Novabiochem Corp. ICI182,780 was purchased from Tocris. The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham-Pharmacia Biotech. Purified recombinant human PKC isoforms were obtained from Calbiochem.

Antibodies and plasmids

Phospho-specific antibodies to the activated forms of p42/44^{MAPK} (Thr202/Tyr204), and phospho-pp90^{RSK} (Ser259) were purchased from New England Biolabs. Antibodies to Raf-1 kinase, p42/44^{MAPK} and MAPK phosphatase-1 (MKP-1) were obtained from Santa Cruz Biotechnology, Inc. ΔRaf-1:ER plasmid, which expresses a fusion protein consisting of an oncogenic form of human Raf-1 kinase (amino acids 305 to 648, which encode all of the kinase domain contained in conserved region 3 but none of conserved regions 1 or 2) and the hormone binding domain of the human estrogen receptor, was kindly provided by Dr. Martin McMahon (University of California, San Francisco) (21). The human wild-type (WT) LDL receptor promoter-luciferase reporter construct B (−173/+35) and the pSV-β-galactosidase (pSV-β-Gal) vectors have been described previously (23). The pp90^{RSK} WT cDNA was graciously provided by Dr. J. L. Maller (University of Colorado School of Medicine) (24). The Gal4-CBP chimera constructs have been described previously; Gal4 (1-147), Gal4-CBP (full-length), and a reporter plasmid, 5 × Gal4-luciferase, were obtained from Dr. John C. Chrivia (St. Louis University School of Medicine) (25). The expression vectors for WT E1A, and a deletion mutant (Δ2-45) were obtained from Dr. Robert J. Rooney (Duke University Medical Center) (26). The human LDL receptor promoter reporter plasmid containing C/EBP and cAMP response element (CREB) mutants, designated CREMU1 and TATA2a (20), respectively, were constructed by standard PCR-based mutagenesis and confirmed by DNA sequence analysis. The construction of plasmids containing single-point mutations in Sp1 (C to A), SRE-1 (A to C), and FP1 (G to T) have been described earlier (27). Plasmid DNA was prepared by purification through columns, as instructed by the manufacturer (Qiagen, Inc.). The constitutively active PKC isoforms constructs were a gift from Dr. Peter Parker (Imperial Cancer Research Fund, London) (28).

Cell culture

The human hepatoma cell line HepG2 and its derivative HepG2-ΔRaf-1:ER cell line that stably expresses the ΔRaf-1:ER chimera were maintained as monolayer cultures in Eagle's minimum essential medium (MEM) (BioWhittaker, Inc.) supplemented with 10% FBS (Life Technologies). Cells were grown at 37°C in a humidified 5% carbon dioxide-95% air atmosphere.

Transient transfection

Transient transfections were performed by cationic lipid-mediated DNA transfection using LipofectAMINE (GIBCO-BRL, Inc). In brief, 3×10^5 HepG2-ΔRaf-1:ER cells were seeded in a 35 mm dish and cultured for about 48 h, when the cells reached 50–70% confluency. The cells were washed with serum/antibiotics-free MEM medium prior to transfection. A total of 1.0 μg of plasmid DNA and 3 μl of LipofectAMINE reagent diluted in 100 μl of serum/antibiotic-free DMEM medium was placed onto the cells. The transfection was performed at 37°C in the incubator with 5% CO_2 for 5 h. After transfection, the lipid-DNA complex was replaced with fresh culture medium with or without the reagents, as indicated for designed treatments to the cells. The cells were then cultured for another 12 h in the absence or presence of 1 μM ICI182,780, harvested, and assayed for luciferase activity as described previously (29). Luciferase activity was normalized by the protein content of the lysate, determined by Bradford assay using γ-globulin as a standard. To measure the effect of sterols on LDL receptor promoter activity, constitutively active β-galactosidase expression vector pSV-β-Gal (0.2 μg) was included in each transfection reaction, so that luciferase activity could be normalized for differences in transfection efficiency. β-Galactosidase activity was assayed with a luminescent substrate (Galacton Plus) according to the manufacturer's instructions (TROPIX, Inc.).

Western blot analysis

Cells were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium vanadate, and 10 mM sodium fluoride. Cell extracts (10–20 μg) were resolved on a 10% acrylamide separating gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane. Membrane blocking, washing, antibody incubation, and detection by ECL were performed as previously described (29). Quantitative analysis of protein levels was performed by densitometric scanning of the autoradiograms and represented three or more independent experiments. To quantify the signals, membranes were scanned by a Storm 860 phosphoimager (Molecular Dynamics), and image and quantification analyses were carried out with ImageQuant 5.0 software (Molecular Dynamics). All values are reported as normalized to control, which was set to 1.

RNA isolation and Northern blot analysis

Total cellular RNA was isolated from treated and untreated HepG2-ΔRaf-1:ER cells using TRIzol Reagent (Life Technologies) as described earlier (29). A total of 10 μg of RNA was fractionated on a 1% formaldehyde agarose gel and transferred onto Zeta-Probe membrane by capillary blotting. RNA blots were hybridized in 7% SDS, 1% BSA, 100 μg/ml salmon sperm DNA, and 0.25 M sodium phosphate (pH 7.2), with human LDL receptor- or human squalene synthetase (SS)-specific single-stranded M13 probe labeled with [α-^{32}P]dCTP. In most cases, the same blot was rehybridized with ^{32}P-labeled single-stranded M13 probe specific for β-actin. After hybridization for 16 h, the membranes were washed twice for 30 min in 1 × SSC/0.1% SDS at 58°C. Hybridized filters were washed and exposed to Kodak X-ray film. The relative intensities of specific bands were determined densitometrically within the linear range of the film on a Model 300A laser densitometer (Molecular Dynamics) with ImageQuant software. LDL receptor mRNA was normalized to β-actin mRNA level, and data for each point were plotted as the percentage of LDL receptor mRNA compared with controls.

RESULTS

Induction of LDL receptor expression follows pp90^{RSK} activation in HepG2-ΔRaf-1:ER cells

We wished to assess the rapidity of ICI182,780-mediated activation of Raf-1 kinase, p42/44^{MAPK}, and pp90^{RSK}, as well as induction of LDL receptor transcription in HepG2-ΔRaf-1:ER cells. Cells were stimulated with 1 μM ICI182,780 for varying periods from 1 to 16 h, and changes in the phosphorylation levels of Raf-1 kinase, p42/44^{MAPK}, and pp90^{RSK}, and expression of LDL receptor gene were measured before and after the treatment. As shown in **Fig. 1A**, phosphorylation of Raf-1 kinase, p42/44^{MAPK}, and pp90^{RSK} were clearly detected after 1 h of ICI182,780 treatment, with maximal responses achieved between 2 h and 4 h. The increase in p42/44^{MAPK} phosphorylation was not accompanied by any changes in the expression of either p42/44^{MAPK} or MAP kinase phosphatase-1 after ICI182,780 treatment of HepG2-ΔRaf-1:ER cells. It is important to note that phosphorylation of the above kinases is followed by full (approximately 8-fold) induction of LDL receptor expression within 3 to 4 h of ICI182,780 treatment. On the other hand, expression of SS, another gene of the cholesterol biosynthetic pathway, was slightly induced (approximately 2-fold), and no significant effect on actin gene expression was observed under identical conditions (Fig. 1B). Comparison of the kinetics of activation of MAPKs with LDL receptor induction suggests that the induction of receptor expression possibly follows sequential activation of the Raf-1-p42/44^{MAPK}-pp90^{RSK} cascade (Fig. 1C).

If the sequential Raf-1-p42/44^{MAPK}-pp90^{RSK} pathway plays a role in ICI182,780-induced LDL receptor expression, pretreatment with PD98059, a specific inhibitor of MEK that prevents the activation of p42/44^{MAPK} and, subsequently, the activation of pp90^{RSK}, would lead to prevention of the above pathway and subsequent induction of LDL receptor expression. When ICI182,780-stimulated activation of pp90^{RSK} was prevented by pretreatment with PD98059, ICI182,780-dependent induction of LDL receptor expression was also blocked (22; also shown later in Fig. 5), indicating that the activation of protein kinases that are downstream of MEK-1/2 mediates ICI182,780-induced LDL receptor expression.

Mutation of either the Sp1 site or the SRE-1 site reduced p42/44^{MAPK}-induced LDL receptor transcription

To evaluate the contribution of already known regulatory elements in ICI182,780-induced LDL receptor transcription, transient transfections were performed with WT

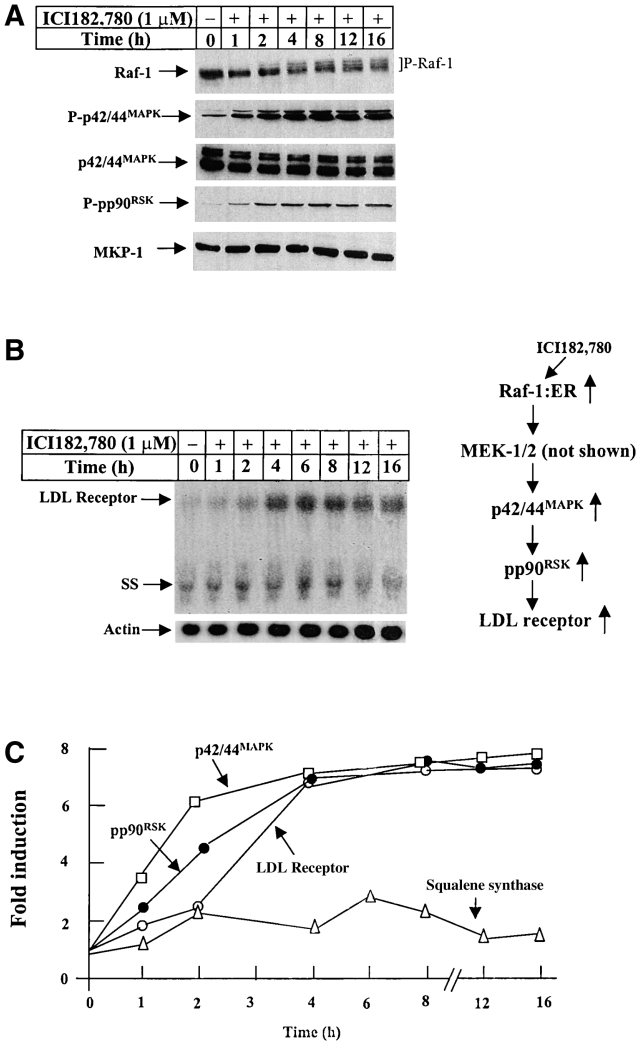

Fig. 1. Time-dependent phosphorylation of Raf-1 kinase, p42/44^{MAPK}, and 90 kDa ribosomal S6 kinase (pp90^{RSK}), and induction of LDL receptor expression by ICI182,780 in HepG2-ΔRaf-1:ER cells. A: HepG2-ΔRaf-1:ER cells (5×10^5) were seeded into 100 mm dishes. After culturing for 24 h, the media were replaced with 0.5% FBS/MEM 4 h before exposure to ICI182,780 (1 μM). At the indicated times, the treated cells were lysed in SDS sample buffer, and Raf-1 kinase, p42/44^{MAPK}, and pp90^{RSK} phosphorylation levels were determined by immunoblotting with phosphorylation-specific antibodies. MKP-1- and phosphorylation-independent p42/44^{MAPK}-specific antibodies were used to examine their expression levels in the above extracts. Results shown are indicative of four different experiments. B: On day 0, 1×10^6 cells were plated. On day 2, cells were re-fed with fresh medium. On day 4, cells were either left untreated (0) or treated for the indicated times with ICI182,780 (1 μM) in medium containing 0.5% FBS. Total RNA was isolated at the indicated times and subjected to Northern blotting to determine amounts of LDL receptor, squalene synthetase (SS), and actin mRNAs. Results shown are indicative of three separate experiments. C: Results are expressed as the relative-fold induction by ICI182,780 compared with untreated cells (given an arbitrary value of 1). Autoradiographs were quantitated densitometrically. LDL receptor and SS mRNA levels were normalized by comparison with levels of actin. The numbers for relative-fold induction of p42/44^{MAPK} and pp90^{RSK} were derived by quantitating intensity of bands and are approximate representations. Values shown are the mean of two different experiments.

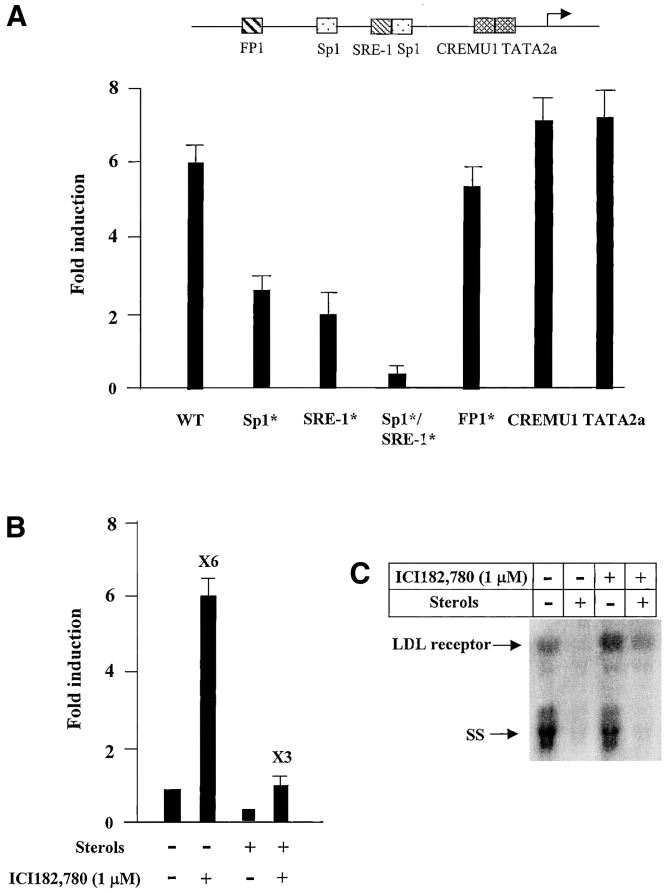

Fig. 2. The p42/44^{MAPK}-dependent increase in human LDL receptor expression is mediated at the transcriptional levels through sterol regulatory element-1 (SRE-1) and the neighboring Sp1 site. A: HepG2-ΔRaf-1:ER cells were transfected with the indicated reporter constructs (0.5 μg/well). After transfection, cells were cultured in medium containing 0.5% FBS/MEM for 20 h and then stimulated with 1 μM ICI182,780 for 12 h prior to lysis. The relative-fold induction represents an increase in luciferase activity of constructs stimulated by ICI182,780 relative to unstimulated constructs. The results for each of the mutants are averages of at least five independent experiments and are presented as means ± SE; each transfection was performed in duplicate. B: Schematic representation of the effects of sterols on p42/44^{MAPK}-induced LDL receptor promoter activity in HepG2-ΔRaf-1:ER cells. Cells were transiently transfected with the human LDL receptor promoter reporter (0.5 μg/well) together with pSV-β-Gal (0.2 μg/well). After treatment for 12 h with 1 μM ICI182,780 in the absence [10% lipoprotein-deficient serum (LPDS)] or presence of sterols (10% LPDS plus 10 μg/ ml cholesterol and 2 μg/ml 25-hydroxycholesterol), the cells were harvested for duplicate measurements of luciferase and β-galactosidase activities. Corrected luciferase activities were calculated as described earlier (23). Relative-fold induction is the ratio of corrected luciferase activity in the presence of ICI182,780 divided by the corrected luciferase activity in the absence of ICI182,780 and is indicated on the top of each bar. C: ICI182,780 induces endogenous LDL receptor expression even in the presence of sterols. HepG2-ΔRaf-1:ER cells were either untreated or treated with ICI182,780 (1 μM) in the absence or presence of sterols. Sterols were added 4 h prior to the addition of ICI182,780. After an additional 4 h, total RNA was isolated and subjected to Northern blotting. Similar results were obtained in three different experiments.

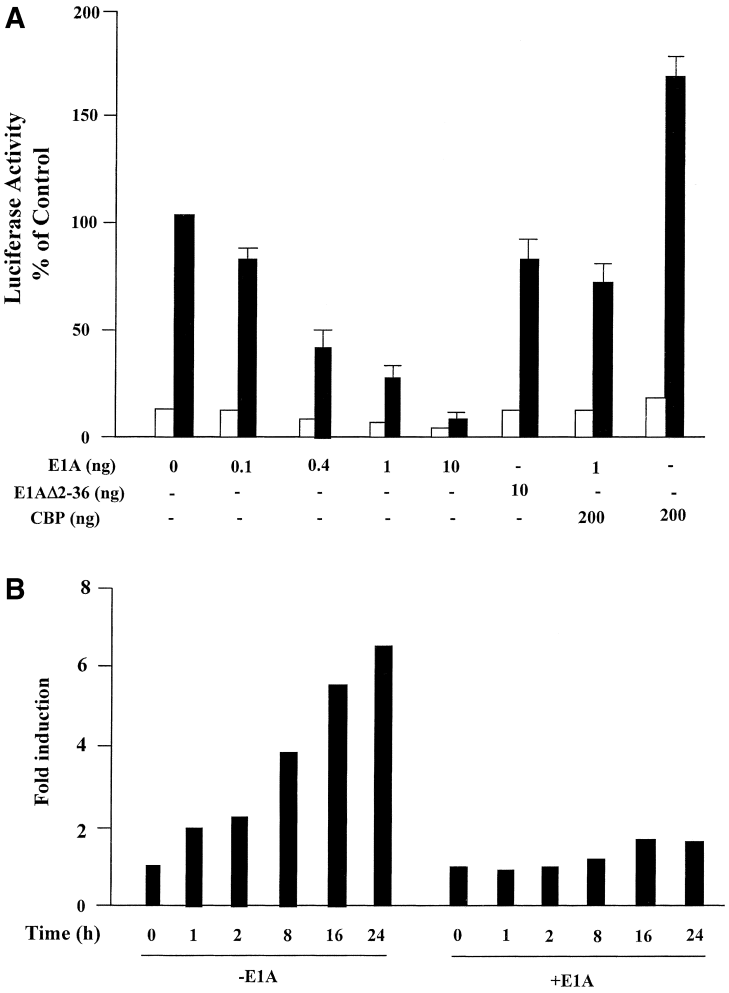

Fig. 3. Transactivation mediated by p42/44^{MAPK} is strongly inhibited by E1A, demonstrating the requirement of CREB binding protein (CBP). A: Human LDL receptor promoter reporter was transiently transfected into HepG2-ΔRaf-1:ER cells with or without expression plasmid encoding E1A or the blank vector pcDNA3. The graph shows the relative luciferase activities, where the luciferase activity of reporter construct transfected without the E1A expression vector was set at 1. Luciferase activity was normalized by the protein concentration of the lysate. Normalized luciferase activities are given from six different experiments and are presented as means ± SE. Open rectangle, absence of ICI182,780; filled rectangle, presence of ICI182,780. B: Time-dependent induction of human LDL receptor promoter reporter by ICI182,780 in HepG2-ΔRaf-1:ER cells in the absence or presence of E1A expression plasmid. The human LDL receptor promoter reporter (0.5 μg) was transiently transfected into HepG2-ΔRaf-1:ER cells with or without expression plasmid encoding E1A (10 ng), and luciferase activity was measured at the indicated times. Luciferase activities were normalized by the protein concentration of the lysates. Values are expressed relative to unstimulated HepG2-ΔRaf-1:ER cells (set at 1) and are the means of two different experiments; each transfection was performed in triplicate.

and different point-mutated LDL receptor promoter variants described previously for their responsivity to p42/44^{MAPK} activation (20, 27). The human LDL receptor promoter constructs containing the WT and mutant −173/+35 bp sequences fused upstream of the luciferase reporter gene were used for transfecting HepG2-ΔRaf-1:ER cells for functional analyses. As shown in **Fig. 2A**, the presence of exogenous ICI182,780 resulted in a dramatic increase of luciferase activity with the WT construct. None of the point mutations in FP1, CRE, and C/EBP sites significantly affected ICI182,780-dependent transactivation, whereas mutation of either SRE-1 or Sp1 significantly reduced transactivation, thus suggesting their role in the induction process. Importantly, the construct with the double mutation of both Sp1 and SRE-1 sites abolished the ICI182,780-stimulated transactivation to activities comparable to that of empty vector. The above data suggest involvement of both Sp1 and SRE-1 sites for ICI182,780-dependent increases in LDL receptor promoter activity.

We also used a different approach to investigate the role of SRE-1 in the induction process. Considering that SRE-1 does not contribute to LDL receptor expression in the presence of sterols, we performed induction experiments in their presence. We observed reduced induction of LDL receptor promoter activity (3-fold) (Fig. 2B) and of endogenous LDL receptor gene (2-fold) (Fig. 2C) even when sterols were present. Since SRE-1 is nonfunctional at high concentrations of sterols due to lack of SREBPs in the nucleus, LDL receptor induction under these conditions could be attributed to Sp1.

Requirement of an E1A-sensitive factor (CBP) in p42/44^{MAPK}-induced LDL receptor transcription

It is possible that the requirement of these sites is indirect. Factor(s) binding to both Sp1 and SREBP-2 may be critical for transactivation, and therefore recruitment of CBP is important for induction by the p42/44^{MAPK} cascade (30–32). To test the functional significance of CBP in the induction process, we used adenoviral E1A protein, an inhibitor of CBP (33), in the transient transfection assays. As shown in **Fig. 3A**, when a DNA construct expressing the WT E1A 12S RNA was cotransfected with the human LDL receptor promoter-driven reporter gene into HepG2-ΔRaf-1:ER cells, induction of the LDL receptor promoter was inhibited in a concentration- (Fig. 3A) and time-dependent manner (Fig. 3B). Thus, it is very likely that the inhibitory effect of E1A on LDL receptor induction is due to the inhibition of CBP.

Although E1A is known to inhibit CBP, it can have multiple effects on cell function that are independent of CBP

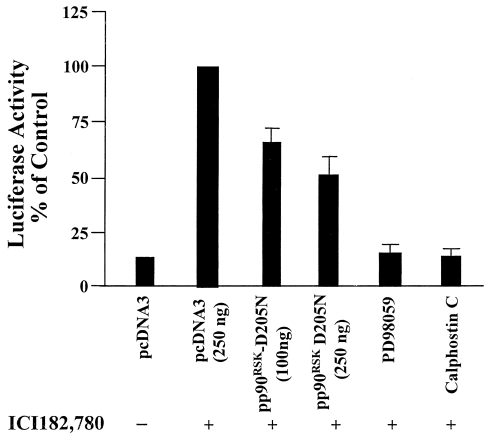

Fig. 4. Inhibition of pp90^{RSK} reduced p42/44^{MAPK}-induced transactivation of LDL receptor promoter activity. Expression of a kinase-defective pp90^{RSK} (D205N) mutant, and treatment with PD98059 or calphostin C significantly reduced increase in LDL receptor promoter activity upon ICI182,780 treatment. Human LDL receptor promoter reporter plasmid was cotransfected with or without the indicated amounts/well of control plasmid pcDNA3 or expression plasmid encoding pp90^{RSK} (D205N) kinase-defective mutant into HepG2-ΔRaf-1:ER cells. Transfected cells were assayed for luciferase activity after treatment for 12 h with 1 μM ICI182,780. Luciferase activities were normalized by the protein concentration of the lysate. Values are expressed relative to luciferase activity obtained with wild-type (WT) human LDL receptor promoter reporter alone upon ICI182,780 treatment (100%). Results are presented as means ± SE. Calphostin C (0.25 μM) and 50 μM PD98059 were used in the above studies.

inhibition (34). To rule out involvement of other interactions, we tested E1A mutants defective in CBP binding to assess the importance of this interaction for inhibition of LDL receptor promoter activity. An N-terminal truncation mutant, E1AΔ2-36, has been shown previously to be defective in CBP binding (35, 36). Expression of this mutant in HepG2-ΔRaf-1:ER cells had little effect under conditions in which WT E1A nearly abolished LDL receptor induction (Fig. 3A). Taken together, these results support a model in which CBP is crucial for ICI182,780-mediated transactivation of the LDL receptor promoter.

Involvement of pp90^{RSK} in p42/44^{MAPK}-induced LDL receptor expression

If pp90^{RSK} mediates LDL receptor induction, then inhibition of pp90^{RSK} should lead to blocking of LDL receptor induction. To address this point, we performed cotransfection experiments using the human LDL receptor promoter reporter (WT) together with the expression vector for a kinase-defective pp90^{RSK} (D205N), or the empty vector control pcDNA3. Consistent with the role of pp90^{RSK}, inhibition of the endogenous pp90^{RSK} activity by transfecting kinase-negative pp90^{RSK} (D205N) mutant, or treatment with PD98059 to inhibit endogenous MEK-1/2, resulted in reduction of the human LDL receptor promoter activity (**Fig. 4**). Unlike PD98059, we cannot expect to see dramatic differences in luciferase expression levels by pp90^{RSK} (D205N) mutant because of low transfection efficiency (10% to 30%) in transient transfections. Therefore, pp90^{RSK} is most likely a critical kinase responsible for activating LDL receptor promoter activity. From these results, it can be concluded that sequential activation of the Raf/MEK/p42/44^{MAPK}/pp90^{RSK} cascade possibly mediates LDL receptor induction.

Requirement of a second PKC-dependent signaling pathway for p42/44^{MAPK}-induced LDL receptor expression

Inhibition of p42/44^{MAPK}-induced LDL receptor promoter activity by calphostin C (Fig. 4) prompted us to investigate the role of PKC in the induction process. In order to examine the role of this kinase, HepG2-ΔRaf-1:ER cells were incubated for 30 min with various selective PKC inhibitors before the addition of ICI182,780. After 4 h, changes in the phosphorylation levels of p42/44^{MAPK} and pp90^{RSK} as well as LDL receptor expression were examined. As shown in **Fig. 5**, none of the PKC inhibitors used in this study had any effect on activation of either p42/44^{MAPK} or pp90^{RSK} in response to ICI182,780. Nevertheless, all these PKC inhibitors significantly suppressed ICI182780-dependent LDL receptor expression. Unlike bisindolylmaleimide I, both calphostin C and Go6976 were most effective in blocking ICI182,780-induced LDL receptor expression (Fig. 5A). The above results suggest that pp90^{RSK} activation by ICI182780 occurs by a PKC-independent mechanism and that both PKC and pp90^{RSK} signaling pathways are essential for ICI182780-induced LDL receptor expression.

To test the effects of inhibiting both PKC and pp90^{RSK} at the same time, HepG2-ΔRaf-1:ER cells were pretreated with Ro-31-8220, and LDL receptor expression was examined after ICI182,780 treatment. Interestingly, Ro-31-8220, which specifically inhibits both PKC and pp90^{RSK} at the concentrations employed (37), not only completely blocked ICI182,780-induced LDL receptor expression but also reduced receptor levels below the basal levels (Fig. 5C). These results further reinforce the requirement of both PKC and pp90^{RSK} signaling cascades for maximal induction of LDL receptor expression by ICI182,780.

Effects of overexpression of PKCβ on p42/44^{MAPK}-induced LDL receptor expression: PKCβ is sensitive to inhibition by sterols

Because of specific inhibition of classical PKC isoforms by Go6976, the potential candidates involved in the induction process could be PKCα and PKCβ, as PKCγ is not present in HepG2 cells. To resolve which of the PKC isoforms may be involved in mediating induction of the LDL receptor induction, we selectively overexpressed PKCα or PKCβ in HepG2 cells and measured the effect on LDL receptor promoter activity. Interestingly, overexpression of constitutively active PKCβ significantly increased LDL receptor promoter activity (approximately 5- to 6-fold), whereas under similar conditions, constitutively active PKCα did not appreciably alter luciferase expression relative to the level determined with the control empty vector (**Fig. 6**). To further examine the role of this isoform, we depleted the endogenous PKCβ isoform by using the antisense oligonucleotides, and found that, unlike sense-

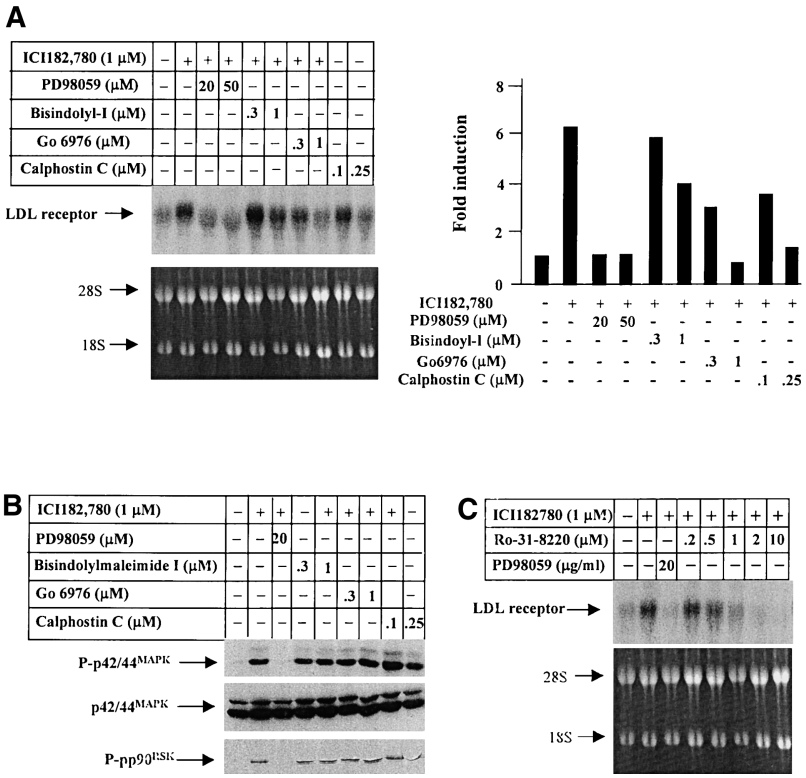

Fig. 5. P42/44^{MAPK}-induced LDL receptor transcription requires a protein kinase C (PKC)-dependent pathway, and the requirement is independent of pp90^{RSK} activation. A: The induction of endogenous LDL receptor expression is blocked by PKC inhibitors. HepG2-ΔRaf-1:ER cells were treated with 1 μM ICI182,780 for 4 h as described in Fig. 1 after pretreatment with indicated concentrations of PKC inhibitors for 30 min. Total RNA was subjected to Northern blot analyses, and the filter was hybridized with a ^{32}P-labeled LDL receptor probe. Shown is an ethidium bromide-stained RNA gel before blotting onto nitrocellulose to demonstrate approximately equal loading of RNA in all lanes. A representative autoradiogram is shown on the left side, and the results of the densitometric analysis of LDL receptor mRNA levels normalized to the stained RNA gel are shown in the side panel [fold induction over untreated cells (zero time point)]. B: Typical Western blot showing that the ICI182,780-dependent increase in phosphorylation of p42/44^{MAPK} or pp90^{RSK} is independent of PKC inhibition. Total cellular protein extracts were prepared under parallel conditions, and Western blot analyses were performed with 30 μg of protein using the antibodies specific for total protein or activated phosphorylated forms of p42/44^{MAPK} or pp90^{RSK}. The figure shown is representative of at least six different experiments. C: Pretreatment with Ro-31-8220 completely blocked ICI182,780-dependent induction of endogenous LDL receptor expression in HepG2-ΔRaf-1:ER cells. Cells were either untreated or treated with ICI182,780 (1 μM) for 4 h in the absence or presence of indicated concentrations of Ro-31-8220 added 30 min prior to the ICI182,780 addition. Total RNA was subjected to Northern blot analyses, and the filter was hybridized with a ^{32}P-labeled LDL receptor probe. Shown is an ethidium bromide-stained RNA gel before blotting onto nitrocellulose to demonstrate equal loading of RNA in all lanes. Results shown are indicative of three separate experiments.

strand control oligonucleotides, antisense oligonucleotides to PKCβ partially reduced the induction process (results not shown). Taken together, the above studies strongly support the involvement of PKCβ in p42/44^{MAPK}-induced LDL receptor transcription.

DISCUSSION

In the present study, we examined the functional relationship between p42/44^{MAPK} activation and induction of LDL receptor transcription in HepG2-ΔRaf-1:ER cells in an attempt to identify the underlying mechanisms that control LDL receptor promoter activity. We found that PKCβ and pp90^{RSK} mediate largely independent signaling pathways, and that the participation of both pathways is necessary to achieve maximal p42/44^{MAPK}-induced LDL receptor transcription.

A role for PKC in the signaling pathway responsible for p42/44^{MAPK}-induced LDL receptor transcription has been implicated for the first time by our studies. Four lines of evidence support a functional role of PKCβ in the induction process. First, inhibition of ICI182,780-induced LDL receptor transcription by calphostin C, a highly selective PKC inhibitor that interacts with the regulatory domain of PKC and competes with the phorbolester binding site located in the C1 domain (38), indicates possible involvement of conventional PKC isoforms in the induction process. Second, another highly selective PKC inhibitor, Go6976 (39), which preferentially inhibits PKCα, PKCβ, and PKCγ, profoundly blocked the induction, further supporting involvement of these isoforms in the p42/44^{MAPK}-induced LDL receptor transcription. Third, overexpression of PKCβ, and not of PKCα, dramatically induced LDL receptor promoter activity. Finally, depletion of endogenous PKCβ using an antisense approach partially in-

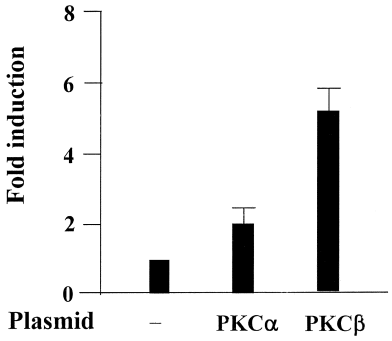

Fig. 6. Overexpression of PKCβ induces LDL receptor transcription. HepG2-ΔRaf-1:ER cells were cotransfected with the human LDL receptor promoter reporter and a vector control plasmid or expression vector for each of the constitutively active PKC isoforms tested. After 24 h, cells were incubated in medium containing 0.5% FBS, and cells were harvested after further incubation for 12 h. Luciferase activity was determined and normalized to the protein content of each extract (29). Luciferase activity expressed by cells transfected with empty vector was given an arbitrary value of 1. The amounts of DNA used were as follows: LDL receptor-luciferase reporter, 0.6 μg/well; and expression vector, 0.3 μg/well. The results shown are means ± SE of three independent experiments.

hibited the induction process. Using this approach, various laboratories have earlier established a specific role for PKC isoforms in various cellular processes (40–42). Taken together, these results support involvement of a PKCβ-dependent pathway in the induction of LDL receptor transcription by p42/44^{MAPK}. The common ability of calphostin C and PD98059 to block LDL receptor induction may suggest that PKC and the p42/44^{MAPK} cascade are proximal and distal components, respectively, of a contiguous LDL receptor-regulatory signaling pathway. Earlier work in HepG2 cells has shown that PKC and pp42/44^{MAPK} may participate in such a contiguous pathway in response to activation of LDL receptor expression by phorbolesters (43). However, our present observation that PKC inhibitors could not prevent pp90^{RSK} activation by ICI182,780 indicates that the PKC-mediated mechanism is not responsible for the activation of pp90^{RSK}.

Another important finding of these studies is the integration of the Raf-1/MEK/p42/44^{MAPK}/pp90^{RSK} cascade, possibly through CBP, in the induction of LDL receptor transcription. The parallel changes observed in the phosphorylation of pp90^{RSK} and LDL receptor expression in response to ICI182,780 stimulation, in the absence or presence of MEK-1/2 and PKC inhibitors, are consistent with an effector role for pp90^{RSK} in the ICI182,780-mediated regulation of LDL receptor transcription. Furthermore, expression of the CBP inhibitor E1A completely blocked ICI182,780-induced LDL receptor transcription, thus mimicking the effect of deleting both Sp1 and SRE-1 sites, whereas expression of an E1A mutant (Δ2-36) defective in CBP binding had little effect on the induction of transcription. The addition of exogenous CBP to HepG2-ΔRaf-1:ER cells stimulated LDL receptor promoter activity in the absence or presence of E1A (Fig. 3A). Although the potentiation of LDL receptor expression by CBP was consistently greater, the stimulation was only moderate. Two considerations are relevant at this point: (1) HepG2 cells already express high levels of CBP (33, 36); thus, CBP may not be limiting in this system; and (2) CBP is likely to function in a large heteromeric complex, and the transient expression of a single component may not effectively generate functional complexes. This may also explain the requirement for an additional signaling cascade, as supported by our present study, to control the induction process. In this regard, it is interesting to note that the activated forms of pp90^{RSK} have already been implicated in the regulation of various nuclear transcriptions, and a functionally relevant pp90^{RSK}-CBP complex has been shown to be required for the induction of growth factor-responsive genes (4, 11, 12). Thus, it is possible that the Raf-1 kinase cascade regulates CBP activity, possibly through stimulating its transactivation by activated pp90^{RSK} in these cells. In fact, targeting of CBP by the Raf-1 kinase cascade could potentially explain the involvement of its interacting partners, the SREBP and CREB nuclear factors, in insulin-induced and oncostatin-induced LDL receptor expression (19, 20), respectively, via the Raf-1/MEK/p42/44^{MAPK} cascade.

How is the induction of LDL receptor transcription mediated through pp90^{RSK} and PKCβ? It is reasonable to assume, on the basis of earlier studies (30–32), that CBP regulates LDL receptor transcription via interaction with Sp1 and SREBP-2 proteins (Figs. 2 and 3). It is possible that both nuclear factors interact with distinct domains of CBP, resulting in a positive cross-talk. This scenario may be analogous to the situation in which thyroid hormone receptor and NF-E2 interact with different domains of CBP to increase transactivation (44). Similarly, it has been shown that NF-E2 interacts with the N-terminal domain of CBP while GATA-1 interacts with its carboxy-terminal domain (45). We speculate that modification of CBP may trigger induction by facilitating protein-protein interactions between Sp1, SREBP-1, and other transcription factors. Thus, CBP-Sp1-SREBP-1 interaction not only is crucial to holding together the functional coactivation complex, but also can serve as a platform for the assembly of further cofactors, resulting in the formation of higher-order hetero-oligomers that generate unique surfaces possibly required for protein-protein interactions with additional transcriptional factors. E1A binding to CBP alters the interaction between the components of the transcriptional complex in such a way that it prevents the recruitment of the coactivation complex. Targeting of CBP by pp90^{RSK} and PKCβ pathways could occur by two distinct but not necessarily exclusive mechanisms. Although unproven, it is intriguing to consider the possibility that phosphorylation of CBP at one site by PKCβ is essential to facilitate sequential modification by pp90^{RSK}. Alternatively, PKCβ is not involved in CBP phosphorylation, and phosphorylation of Sp1, SREBP, or some other nuclear factor is required for the assembly of larger complexes consisting of additional transcriptional cofactors. Compatible with this possibility is our earlier demonstration that PKC partially regulates SREBP-dependent transcription of

LDL receptor gene (46). It remains to be seen whether the convergence of these protein kinases on the same or different nuclear factors promotes formation of a competent transcriptional complex to allow maximal induction of the LDL receptor promoter in response to p42/44^{MAPK} activation.

In conclusion, pp90^{RSK} and PKCβ appear to be important downstream effectors of the Raf-1 signaling cascade, and interaction between these pathways is necessary to regulate the induction of LDL receptor expression by the Raf-1/MEK/p42/44^{MAPK} pathway. The involvement of a PKCβ-dependent pathway and its interaction with Raf/MEK/p42/44^{MAPK}/pp90^{RSK} is novel and provides an additional step of control in regulating LDL receptor transcription. Considering that the cross-talk between various signaling pathways involving protein phosphorylation is an emerging theme in gene regulation, it is very likely that cooperative interactions of the pp90^{RSK} signaling cascade with several other pathways may be discovered in the future.

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