Phorbol ester promotes histone H3-Ser10 phosphorylation at the LDL receptor promoter in a protein kinase C-dependent manner

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Abstract **Histone modification is emerging as a major regulatory mechanism for modulating gene expression by altering the accessibility of transcription factors to DNA. This study unravels the relationship between histone H3 modifications and LDL receptor induction, focusing also on routes by which phosphorylation is mediated in human hepatoma HepG2 cells. We show that while histone H3 is constitutively acetylated at LDL receptor chromatin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) causes rapid hyperphosphorylation of histone H3 on serine 10 (histone H3-Ser10), despite global reduction in its phosphorylation levels. Ser10 hyperphosphorylation precedes LDL receptor induction and is independent of the p42/44^{MAPK}, p38^{MAPK}, pp90^{RSK}, or MSK-1 cascade. Interestingly, inhibition of protein kinase C (PKC) blocks Ser10 hyperphosphorylation and also compromises LDL receptor induction by TPA. Consistent with its role, recombinant purified PKC phosphorylate purified histone H3-Ser10. Collectively, our findings highlight a novel role for PKC in regulating histone H3-Ser10 phosphorylation and suggest that histone modification provides numerous regulatory opportunities to set the overall range of control attainable for LDL receptor gene induction.**—Huang, W., V. Mishra, S. Batra, I. Dillon, and K. D. Mehta. **Phorbol ester promotes histone H3-Ser10 phosphorylation at the LDL receptor promoter in a protein kinase C-dependent manner. *J. Lipid Res.* 2004.** 45: **1519–1527.**

Supplementary key words low density lipoprotein receptor induction • histone modification • histone H3 on serine 10 • mitogen-activated protein kinase • protein kinase C

Mechanisms for regulating gene expression in response to extracellular stimuli have been a focus of major research efforts for many years. It is now apparent that this is achieved by a variety of different signal transduction mechanisms, which have the net result of modifying and regulating transcription machinery and the chromatin environment at particular target genes. Two of the most extensively studied mechanisms of signaling into the nucleus involve mitogen-activated protein kinase (MAPK) cascades and protein kinase C (PKC). At present there are at least three main pathways that are defined according to the MAPK that is activated: *i*) the p42/44^{MAPK} (also known as ERK-1/2) pathway; *ii*) the p46/54^{JNK} (also known as JNK) pathway; and *iii*) the p38^{MAPK} pathway (1–3). Mitogenic stimuli, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), activate p42/44^{MAPK} very rapidly and strongly and elicit weaker activation of p46/54^{JNK} and p38^{MAPK}, whereas various stress stimuli and anisomycin activate p46/54^{JNK} and p38^{MAPK} pathways very strongly but produce little or no p42/44^{MAPK} activation. The signaling networks leading to the activation of MAPKs themselves and some of their downstream targets have been extensively studied. Activation of MAPK pathways leads ultimately to the phosphorylation of transcription factors bound to their regulatory elements and/or histones associated with the promoters of target genes. Likewise, PKCs are activated by many extracellular and intracellular signals, including TPA, and have been implicated in a multitude of physiological functions in the cell (4, 5). PKCs also constitute a large family of isoforms, each with distinct properties. Twelve distinct members have been discovered to date in mammalian cells and have been subdivided into three distinct subfamilies: conventional PKCs, including α, β1 and the splice variant βII, and γ; the novel PKCs δ, ε, η, and θ; and the atypical PKCs ζ and ι/λ.

Manuscript received 2 March 2004 and in revised form 11 May 2004.

Published, JLR Papers in Press, May 16, 2004.
DOI 10.1194/jlr.M400088-JLR200

Abbreviations: ECL, enhanced chemiluminescence; histone H3-Ser10, histone H3 on serine 10; Lys14, lysine 14; MAPK, mitogen-activated protein kinase; MEK-1/2, mitogen/extracellular-regulated protein kinase kinase-1 and -2; PD98059, 2-(2′-amino-3′-methoxyphenyl) oxanaphthalene-4-one; PKC, protein kinase C; SB202190, 4-(4-fluorophenyl)-2-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl) 1H-imidazole; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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Histones are ubiquitous proteins that organize DNA into nucleosomes and chromatin. Each nucleosome consists of an inner core of two copies each of the four histones, H2A, H2B, H3, and H4, surrounded by 146 bp of double-stranded DNA. Histone H1 binds to the outer surface of the DNA that surrounds the core histones and to the stretches of linker DNA that connect nucleosomes. Of the core histones, histone H3 modification is believed to play an important role in modulating the transcriptional activity of specific genes (6–8). There is a growing evidence that transcription-associated serine 10 phosphorylation and/or lysine 14 (Lys14) acetylation is unique to a small set of genes that are rapidly turned on and off in response to extracellular signals in mammalian cells (9–14). Recent studies have outlined the possibility that histone H3 on serine 10 (histone H3-Ser10) phosphorylation alone may have a role in the regulation of transcription by acting as a signal for subsequent Lys14 acetylation (10, 11, 15–17). Various stimuli, including TPA and stresses such as UV light, induce global phosphorylation of histone H3-Ser10 in starved fibroblast and epidermal cells, respectively (14, 18). It is becoming clear that multiple kinases can phosphorylate histone H3 in a cell type-specific manner (6, 9). Among the three related MAPK families identified to date, $p42/44^{MAPK}$ and its downstream kinase $pp90^{RSK}$, as well as $p38^{MAPK}$ and MSK-1 (downstream of both $p42/44^{MAPK}$ and $p38^{MAPK}$), have been implicated as histone H3-Ser10 kinase in human and mouse fibroblast cells (6, 19). The role for $pp90^{RSK}$ is not yet clear, although recent knockout studies have established that MSKs are the major kinases for histone H3-Ser10 phosphorylation in response to mitogenic and stress stimuli in fibroblasts (12). On the other hand, arsenite-induced histone H3-Ser10 phosphorylation is mediated by Akt, $p42/44^{MAPK}$, and $pp90^{RSK}$ but not by MSK-1 in JB6C1 41 cells (20). Studies with granulosa cells have established a role for protein kinase A in follicle-stimulating hormone-induced histone H3-Ser10 phosphorylation (21, 22).

At present, our knowledge of the identity of genes regulated by this response is limited, as is our knowledge of the role of MAPKs in histone H3-Ser10 phosphorylation at other loci. Also, the kinase involved in liver cells, as well as the importance of histone H3 modifications in the context of other TPA-regulated genes, is not clear. In the present report, we have addressed some of these issues by analyzing histone modifications that occur at the LDL receptor chromatin in response to TPA in human hepatoma HepG2 cells. TPA is a well-known activator of PKC and $p42/44^{MAPK}$ and dramatically induces LDL receptor expression at the transcriptional level in human hepatoma HepG2 cells (23). We have earlier established these signaling pathways as critical mediators of LDL receptor induction in HepG2 cells by a variety of transcriptional modulators, including TPA (23–25). Based on the suggested role of $p42/44^{MAPK}$ cascade in regulating histone H3-Ser10 phosphorylation, the involvement of this kinase raised an interesting possibility that this histone modification may play an important role in LDL receptor induction by TPA. In this study, we have investigated the relationship among histone posttranslational modifications, the signaling pathway involved, and LDL receptor induction by TPA in HepG2 cells. Our findings demonstrate key roles for PKC in controlling LDL receptor induction, likely by modulating Ser10 phosphorylation in TPA-stimulated HepG2 cells.

EXPERIMENTAL PROCEDURES

Materials

TPA, glycogen (molecular biology grade), and proteinase K (molecular biology grade) were purchased from Sigma Chemical Co. PKC inhibitor calphostin C (26, 27), mitogen/extracellular-regulated protein kinase kinase-1 and -2 (MEK-1/2) inhibitor 2-(2′-amino-3′-methoxyphenyl)oxanaphthalene-4-one (PD98059) (28), $p38^{MAPK}$ inhibitor 4-(4-fluorophenyl)-2-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole (SB202190) (29), and $pp90^{RSK}$ inhibitor Ro-31-8220 (30) were obtained from Calbiochem. Phospho-Ser10-, acetyl-Lys9-, and acetyl-Lys14-histone H3 antibodies and anti-acetylated histone H4 were purchased from Upstate Biotechnology. Phospho-specific antibodies to the activated forms of $p42/44^{MAPK}$ (Thr202/Thr204), $p38^{MAPK}$ (Thr180/Tyr182), MEK-1/2 (Ser217/Ser221), MSK-1 (Ser376), and $pp90^{RSK}$ (Ser380) were purchased from Cell Signaling Technology. Antibodies to unphosphorylated PKC, $p42/44^{MAPK}$, and histone H3 were purchased from Santa Cruz Biotechnology. Recombinant purified histone H3, PKCβII, and PKCε were purchased from Upstate Biotechnology. Culture supplies were from Becton Dickinson Labware and BioWhitaker, Inc. TRIzol, protein A agarose, salmon sperm DNA solution, and fetal bovine serum were from Invitrogen Corp. Nitrocellulose blotting membrane and protein assay reagent were purchased from Bio-Rad. The enhanced chemiluminescence (ECL) detection kit and [^{32}P]dCTP were purchased from Amersham Pharmacia Biotechnology. AmpliTaq GoldDNA polymerase and supplies for PCR were from Applied Biosystems.

Cell culture

The human hepatoma HepG2 and modified HepG2-ΔRaf-1:ER cells (31) were maintained as monolayer cultures in Eagle's minimal essential medium supplemented with 10% fetal bovine serum and antibiotics. Cells were grown at 37°C in a humidified 5% carbon dioxide/95% air atmosphere.

Western blot analysis

Cells were seeded on six-well plates at a density of 1×10^5 cells/well in medium supplemented with 10% FBS. After 48 h, cells were refed with fresh medium and grown for another 24 h and treated accordingly. Whole cell extracts were prepared by and probed with the required antibody. Immunoreactive peptides on the filters were detected by ECL as described earlier (23–25). 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 PhosphorImager (Molecular Dynamics), and image and quantification analyses were carried out with ImageQuant 5.0 software. All values are reported as normalized to control, which was set to 1.

Northern blot analysis

Total RNA was isolated from cultured cells using TRIzol, and Northern blotting was done essentially as described earlier (23–

25). Briefly, 10 μg of total cellular RNA was fractionated on a 1% formaldehyde agarose gel and transferred to a Zeta-Probe membrane by capillary blotting. RNA blots were hybridized with LDL receptor-specific single-stranded M13 probe labeled with [α-^{32}P]dCTP. 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 stained RNA gels, and data for each point were plotted as the percentage of LDL receptor mRNA compared with controls.

Chromatin immunoprecipitation assays

We used a modification of the technique described by Thomson et al. (13). Briefly, formaldehyde was added directly to the cell culture medium at a final concentration of 1% for 10 min (37°C) to cross-link DNA and its associated proteins. Cells were washed once in phosphate-buffered saline before scraping in phosphate-buffered saline containing protease inhibitors (1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). Cells were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM sodium butyrate, 100 mM sodium orthovanadate, and protease inhibitors) and left on ice for 10 min. Cells were centrifuged at 3,000 rpm for 4 min at 4°C. Nuclei were resuspended in wash buffer (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM sodium butyrate, 20 mM β-glycerophosphate, 100 mM sodium orthovanadate, and protease inhibitors), then centrifuged as above. Nuclear pellets were resuspended in sonication buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, 10 mM sodium butyrate, 20 mM β-glycerophosphate, 100 μM sodium orthovanadate, and protease inhibitors) and sonicated using a Sonic Vibracell 150 W sonicator. The resulting supernatant contained ~200–1,000 bp DNA fragments. SDS was added to a final concentration of 1%, and the chromatin solutions were rotated at room temperature for 1 h. Insoluble material was removed by centrifugation at full speed in a micrcentrifuge at room temperature for 15–20 min. Five microliters of the supernatant was saved as input DNA, and the remainder was diluted 1:10 in chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 16.7 mM Tris-HCl) containing protease inhibitors. Samples were then processed immediately or stored at −80°C for later ChIP analysis. For immunoprecipitation, the chromatin solution was cleared with a salmon sperm DNA/protein A agarose 50% gel slurry (Upstate Biotechnology) for 30 min before overnight incubation (4°C) with 5 μg of histone modification-specific antibody. Nonimmune rabbit serum and addition of no antibody were used for negative controls. After immunoprecipitation, the chromatin antibody/protein A agarose complexes were washed sequentially three times each (3 min on a rocker plate) in low-salt, high-salt, lithium chloride, and Tris/EDTA buffers, followed by two treatments with freshly made elution buffer (1% SDS and 50 mM $NaHCO_3$). The eluates were pooled, NaCl was added to a 10 mM final concentration, and the mixture was heated at 65°C for 4 h to reverse the formaldehyde cross-links. Additionally, the DNA input sample cross-links were reversed in a similar manner. The samples were digested with proteinase K for 1 h at 45°C. DNA from the samples was obtained by phenol-chloroform extraction and ethanol precipitation. DNA pellets were then resuspended in 20 μl of sterile water, and 1 μl aliquots were used in the PCRs. ^{32}P-labeled dCTP was incorporated into the PCR products for visualization and quantitation. All PCR procedures were performed using a Perkin-Elmer Gene Amp 2400 thermal cycler and AmpliTaq Gold DNA polymerase. The sequences of the primers used in this study for the LDL receptor promoter region (amplified region, −295 to +1) are as follows: 5′-TGTTAACAGTTAAACATCGAGAA-3′ and 5′-CCCGCGATTGCACTCGGGGC-3′. The linear range of primers was determined empirically using different amounts of HepG2 genomic DNA. Subsequent PCR analyses were carried out using the optimum cycle number determined for each primer set. Genomic DNA control reactions were always carried out alongside the immunoprecipitated DNA samples. PCR products were resolved on 5% polyacrylamide-TBE gels, dried, visualized by autoradiography, and quantified using a PhosphorImager.

RESULTS

MAPKs do not regulate global histone H3-Ser10 phosphorylation

To understand the contribution of histones to post-translational modifications in TPA-induced LDL receptor expression, we first asked whether TPA affects posttranslational modification of bulk histones by probing with a panel of modification-specific antibodies. As shown in **Fig. 1**, TPA markedly reduced steady-state levels of Ser10 phosphorylation (and, to some extent, Lys9 acetylation) of histone H3 in a time-dependent manner, whereas global acetylation of Lys14 remained largely unaffected. Likewise, global acetylation of histone H4 did not change by TPA treatment.

Although global or inducible phosphorylation of histone H3 has been shown to be mediated via p42/44^{MAPK}, p38^{MAPK}, and/or their downstream kinases in serum-starved human fibroblast cells (6, 9), the role of these kinases in HepG2 cells remains to be established. To determine whether dephosphorylation of histone H3-Ser10 is related to changes in the activity of these MAPKs, the kinetics of their phosphorylation/activation by TPA was also examined in the above cell extracts. As shown in Fig. 1, even though TPA potently activated p42/44^{MAPK} and p38^{MAPK} (lanes 1–8), Ser10 phosphorylation showed reduction in a time-dependent manner (lanes 1–8), indicating a lack of correlation between activation of the above kinases and histone H3-Ser10 phosphorylation levels.

To further examine the relationship between MAPKs and histone H3-Ser10 phosphorylation, various specific inhibitors were used, and the results are also shown in Fig. 1. Our studies show the following: *i*) Global histone modifications, including histone H3-Ser10 phosphorylation, were unaffected by PD98059 and SB202190 either alone or together in the absence of TPA (lanes 14–16); likewise, pretreatment with calphostin C had no effect on global histone H3-Ser10 phosphorylation (lanes 17 and 18). *ii*) PD98059 significantly blocked TPA-dependent activation of p42/44^{MAPK} and pp90^{RSK} without affecting the TPA-dependent dephosphorylation of histone H3-Ser10 (lane 9), thus ruling out the involvement of this signaling pathway. *iii*) SB202190 alone did not affect the TPA-dependent

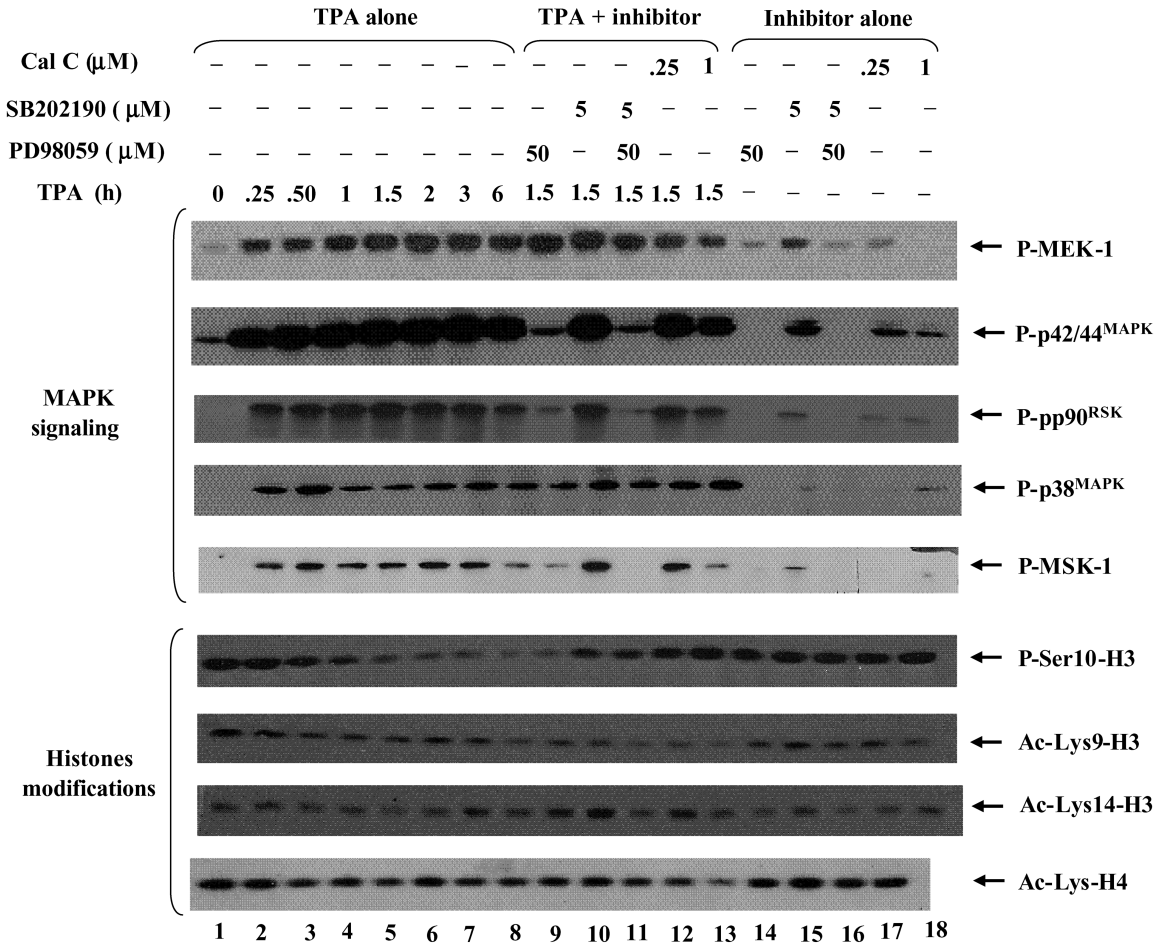

Fig. 1. Kinetics of mitogen-activated protein kinase (MAPK) activation and changes in global histone posttranslational modifications by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in the absence or presence of the indicated inhibitors in HepG2 cells. A total of 1×10^5 cells were grown on six-well dishes and treated with 100 nM TPA. After the indicated times, cells were washed with 1× PBS at 37°C and lysed in 1× SDS sample buffer. Equal amounts of whole cell lysates were separated by SDS-PAGE on 12% gels, and phosphorylation levels of MAPKs were analyzed by Western blotting using phospho-specific antibodies. Western blotting to assess the levels of nonphosphorylated p42/44^{MAPK} revealed that protein loading was comparable among all samples (data not shown). Changes in histone modifications were also examined in the above samples using anti-acetyl lysine 9 (Lys9), anti-acetyl Lys14, anti-phospho-serine 10 (Ser10) histone H3, or anti-acetylated histone H4. The experiment was repeated three times with similar results. For all studies with inhibitors, they were added 30 min before TPA treatment for 1.5 h. Cal C, calphostin C; MEK-1, mitogen/extracellular-regulated protein kinase kinase-1; PD98059, 2-(2′-amino-3′-methoxyphenyl)oxanaphthalene-4-one; SB202190, 4-(4-fluorophenyl)-2-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole.

activation of pp90^{RSK} and MSK-1 (lane 10) but blocked TPA-dependent Ser10 dephosphorylation. *iv*) SB202190 plus PD98059 completely blocked the activation of pp90^{RSK} and MSK-1 (lane 11) and also blocked Ser10 dephosphorylation. *v*) Calphostin C alone completely blocked dephosphorylation of Ser10 by TPA, independent of its effects on MAPKs (lanes 12 and 13). The effect of calphostin C is to be expected because TPA is a specific activator of PKC, whereas the slight effect of SB202190 on TPA-dependent dephosphorylation (Fig. 1, lane 5 vs. lane 10) indicates possible involvement of p38^{MAPK}-dependent pathway in the dephosphorylation process. From these studies, it is safe to conclude that TPA-dependent global dephosphorylation of histone H3-Ser10 is independent of the p42/44^{MAPK}/pp90^{RSK} pathway and is mediated by PKC and p38^{MAPK}.

Hyperphosphorylation of histone H3-Ser10 correlates with LDL receptor induction by TPA

To discover whether TPA treatment affected the phosphorylation and/or acetylation of histone H3 on nucleosomes associated with the LDL receptor promoter, ChIP assays were used. The specificity and accuracy of these assays were monitored by performing mock ChIP reactions in the absence of antibody and by carrying out PCR assays in the linear range of amplification. TPA did not significantly modulate histone H3 acetylation on Lys14 but had a strikingly positive effect on Ser10 phosphorylation of histone H3 (**Fig. 2A**). An increase in Ser10 phosphorylation was detected within 0.5 h of exposure to TPA, phosphorylation reached a peak at 1.5 h, and returned to the basal level at 3 h. As opposed to the rapid and transient increase in Ser10 phosphorylation, TPA caused slightly

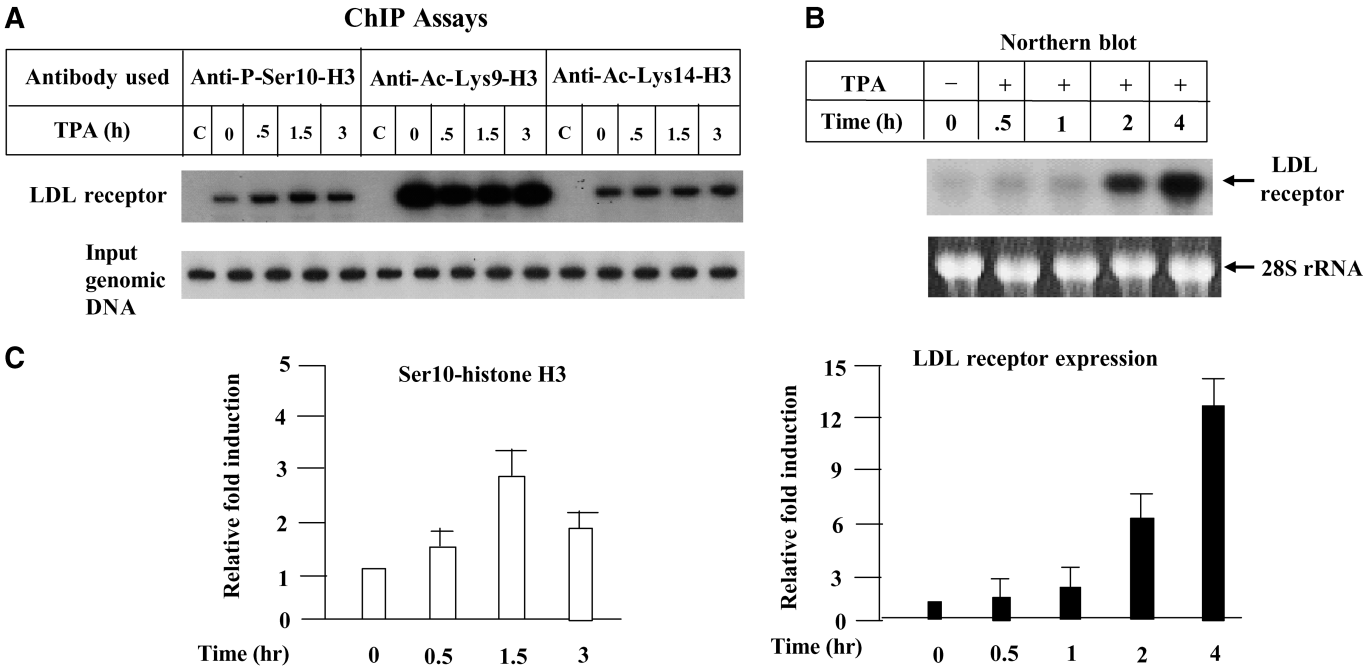

Fig. 2. Effects of TPA on the phosphorylation and acetylation of histone H3 associated with the LDL receptor chromatin, and effects of TPA on its expression in HepG2 cells. A: Chromatin immunoprecipitation (ChIP) analysis of the LDL receptor promoter in response to TPA treatments. HepG2 cells were treated with 100 nM TPA for the indicated times or left untreated (0), whereupon chromatin was prepared after cross-linking. ChIP assays were performed using either normal rabbit IgG (control) or an antibody specific for phospho-Ser10, acetyl-Lys9, or acetyl-Lys14 histone H3. Aliquots of the chromatin before immunoprecipitation (input, 1:80 of released chromatin) along with one-tenth of immunoprecipitated DNAs were amplified with primers to the LDL receptor. Input genomic DNAs were used to ensure equal amounts of DNA in each sample. Results shown are representative of three different experiments. B: TPA-dependent changes in LDL receptor expression were measured under similar conditions by Northern blotting. A total of 5×10^5 cells were plated on day 0. On day 2, cells were refed with fresh medium. On day 3, cells were either left untreated (0) or treated for the indicated times with 100 nM TPA in medium containing 10% FBS. Total RNA was isolated and subjected to Northern blotting to determine the amounts of LDL receptor. Ethidium bromide staining of the RNA gel before blotting onto nitrocellulose is shown to demonstrate equal loading of RNA in all lanes. C: Comparison of TPA-induced changes in histone H3 on serine 10 (histone H3-Ser10) phosphorylation at the LDL receptor promoter, along with changes in its expression. Relative histone modification levels (fold of phosphorylation) were determined by quantitation of PCR products and by correction with the input data. Similarly, Northern blots were quantified by correction with the stained RNA gels. Results are expressed as fold induction by TPA compared with untreated cells (set at 1). Similar results were obtained in at least three experiments. Results are mean ± SE of three experiments.

delayed and prolonged induction of LDL receptor expression (Fig. 2B). Weak induction was observed at 2 h, maximal induction was observed 4 h after stimulation, and the LDL receptor remained increased at least until 8 h (data not shown), the maximum point measured. In short, Ser10 phosphorylation preceded LDL receptor induction (Fig. 2C). In contrast, Lys9 acetylation showed a slight and transient decrease under the same conditions.

Phosphorylation of histone H3-Ser10 in vivo and in vitro by PKC

To test whether the phosphorylation of histone H3-Ser10 associated with the LDL receptor chromatin is linked to the activation state, we characterized the effect of PD98059, SB202190, or calphostin C on histone H3-Ser10 phosphorylation. Cells pretreated with inhibitors for 30 min, as described in the legend of Fig. 2A, were then treated with TPA for 1.5 h. Even though PD98059 completely inhibited all detectable increases in $p42/44^{MAPK}$ phosphorylation (Fig. 1, lane 9), it had no effect on Ser10 phosphorylation at the LDL receptor chromatin (**Fig. 3A**, lane 3). Similarly, pretreatment with the $pp90^{RSK}$ inhibitor Ro-31-8220 (Fig. 3A, lane 7) and the $p38^{MAPK}$ inhibitor SB202190 (Fig. 3A, lane 4) had no effect on Ser10 phosphorylation at the LDL receptor promoter. In contrast, calphostin C, a selective PKC inhibitor, completely blocked histone H3 hyperphosphorylation (Fig. 3A, lanes 5 and 6). These results indicate that histone H3-Ser10 hyperphosphorylation at the LDL receptor chromatin by TPA is not downstream of $p42/44^{MAPK}$, $pp90^{RSK}$, MSK-1, or $p38^{MAPK}$ and is mediated directly by PKC in HepG2 cells.

If PKC mediates TPA-induced hyperphosphorylation of histone H3-Ser10, then active PKC should phosphorylate this protein in vitro. We have earlier shown the involvement of PKCε and PKCβ in LDL receptor induction by a variety of transcriptional modulators (17–19). To test their role, we incubated purified histone H3 protein with either purified recombinant PKCε or PKCβ in the presence of Mg-ATP. The phosphorylation level of histone H3-Ser10 was detected with Ser10-phospho-specific antibodies by Western blot analysis. These results show that active PKC

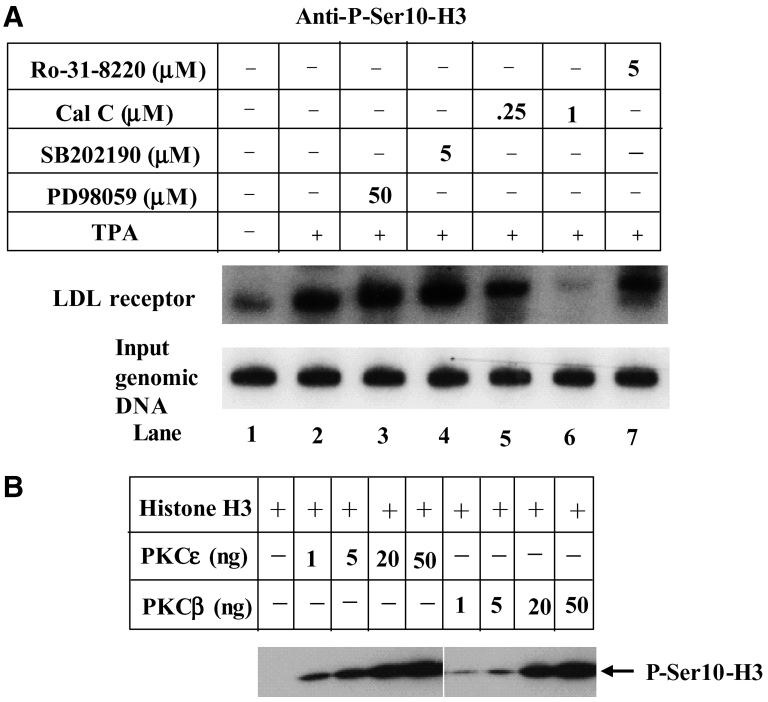

Fig. 3. Phosphorylation of histone H3-Ser10 in vivo and in vitro by active protein kinase C (PKC). A: Hyperphosphorylation of histone H3-Ser10 at the LDL receptor chromatin is mediated via PKC. ChIP analysis of histone H3-Ser10 phosphorylation in response to 100 nM TPA for 1.5 h was performed in the absence or presence of inhibitor for PKC or MAPK cascades added 30 min before TPA addition. Similar results were obtained in three separate experiments. The control lane represents PCR products obtained using normal rabbit IgG. B: Purified recombinant PKCβ or PKCε directly phosphorylate purified histone H3-Ser10. Phosphorylation of histone H3-Ser10 was carried out at 30°C for 45 min in the presence of purified histone H3 (250 ng/tube), kinase buffer, 200 μM ATP, and the indicated amount of one of the PKC isoforms. The phosphorylated protein was detected by Western blotting with phospho-Ser10-histone H3 antibody. Results shown are representative of at least three different experiments.

isoforms can directly phosphorylate histone H3-Ser10 in vitro (Fig. 3B).

To further investigate the interaction between histone H3 and PKC in vivo, we used anti-phospho-Ser10 histone H3 or anti-PKC to coimmunoprecipitate the complex of histone H3 with PKC from HepG2 cells after TPA stimulation. No histone H3-PKC complex was found (data not shown).

p42/44^{MAPK}-induced LDL receptor expression is not accompanied by hyperphosphorylation of Ser10

Substantial inhibition of the TPA-dependent activation of p42/44^{MAPK} and pp90^{RSK} at higher concentrations of calphostin C (Fig. 1, lane 13) suggested the possibility that the effect of calphostin C on Ser10 phosphorylation is mediated through the p42/44^{MAPK} cascade, as PKC is known to activate p42/44^{MAPK} in HepG2 cells (23). To rule out this possibility, we took advantage of the HepG2-ΔRaf-1:ER cell line that stably expresses an inducible form of human Raf-1 kinase (amino acids 305–648, which encode all of the kinase domain contained in the conserved region 3) fused to the hormone binding domain of the human estrogen receptor. We previously used this cell line to show that activation of the Raf-1/MEK/p42/44^{MAPK} cascade alone is sufficient to induce LDL receptor expression in HepG2 cells (31). Cells were stimulated with 1 μM ICI182,780 for varying periods from 1 to 8 h, and changes in p42/44^{MAPK} phosphorylation, LDL receptor expression, and Ser10 phosphorylation were measured before and after treatments. Consistent with our earlier observations, ICI182,780 treatment increased p42/44^{MAPK} phosphorylation and LDL receptor expression in a time-dependent manner (**Fig. 4A**). However, the increase in p42/44^{MAPK} activation was not accompanied by any changes in Ser10 phosphorylation at the global level or at the LDL receptor promoter chromatin by ChIP assays (Fig. 4B), thus ruling out any involvement of this pathway in TPA-induced hyperphosphorylation of Ser10 at the LDL receptor promoter.

DISCUSSION

We have earlier established PKC and p42/44^{MAPK} as critical mediators of TPA-dependent superinduction of LDL receptor transcription in HepG2 cells (23). We have now begun to delineate nuclear events downstream of the above kinases and their roles in the induction process. In this paper, we show a novel role for PKC in histone H3-Ser10 phosphorylation and also link hyperphosphorylation of this serine with the LDL receptor induction by TPA. Four independent observations led us to conclude that PKC-mediated histone H3-Ser10 phosphorylation is possibly associated with LDL receptor induction. First, TPA caused rapid hyperphosphorylation of histone H3-Ser10 at the LDL receptor chromatin without significantly affecting acetylation of histone H3-Lys9 and/or -Lys14. TPA-dependent increase in Ser10 phosphorylation reached a peak at 1.5 h and returned to the basal level at 3 h, whereas maximal induction of the LDL receptor was observed at 4 h after stimulation, suggesting that phosphorylation is required to initiate but not to maintain stimulated transcription. Similar to our results, inducible histone H3-Ser10 phosphorylation, without any changes in histone H3 acetylation, has recently been linked to the induction of retinoic acid receptor expression by retinoids (16). Although increased histone H3 acetylation has been

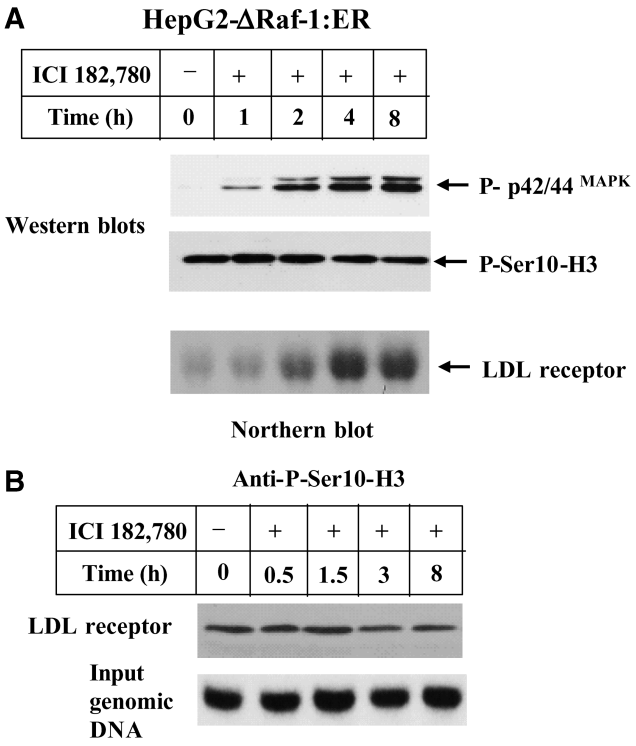

Fig. 4. Activation of p42/44^{MAPK} alone induces LDL receptor expression without hyperphosphorylating histone H3-Ser10. On day 1, 1 × 10^6 cells were plated for RNA analysis. On day 2, cells were refed with fresh medium. On day 4, cells were either left untreated or treated for the indicated times with 1 μM ICI182,780 in medium containing 0.5% FBS. Total RNA was isolated at the indicated times and subjected to Northern blotting to determine the amounts of LDL receptor and actin mRNAs. Results shown are representative of at least five different experiments. For Western blot analysis, 2 × 10^5 cells were seeded onto 100 mm dishes. After culturing for 24 h, the media were replaced with 0.5% FBS at 4 h before exposure to 1 μM ICI182,780. At the indicated time, cells were lysed in SDS sample buffer and probed for phospho-p42/44^{MAPK} and phospho-histone H3-Ser10. A: ICI182,780 (1 μM) treatment of HepG2-ΔRaf-1:ER cells resulted in p42/44^{MAPK} activation and LDL receptor induction in a time-dependent manner. B: ChIP analysis of the LDL receptor promoter in HepG2-ΔRaf-1:ER cells. Cells were treated with 1 μM ICI182,780, and chromatin was immunoprecipitated and analyzed as described for Fig. 2. Input DNA was done to ensure equal amounts of DNA in the samples.

shown earlier at the LDL receptor chromatin in response to depletion of sterols (32), the role of this modification in the induction process was not studied. Another point that should be noted is that Tjian and colleagues (33) earlier demonstrated that CREB-binding protein-associated histone acetyltransferase activity is not critical for the synergistic activation of the LDL receptor promoter by sterol-regulatory element binding protein-1a/Sp1 on chromatin templates, which argues against the involvement of histone acetylation in the induction process. In addition, there are reports demonstrating that correlation between histone acetylation levels and transcriptional activity is not universal (34, 35). Thus, an increase in Ser10 phosphorylation during LDL receptor induction by TPA is consistent with a role of this modification in promoting transcription. Second, calphostin C blocked histone H3-Ser10 hyperphosphorylation at the LDL receptor chromatin and also compromised the transcriptional induction of LDL receptor expression by TPA. Third, the ability of PKC to phosphorylate histone H3-Ser10 directly in an in vitro assay with purified recombinant proteins suggests that the mechanism of phosphorylation is most likely through PKC. Lastly, activation of p42/44^{MAPK} alone in HepG2-ΔRaf-1:ER cells induced LDL receptor expression, but the induction was not accompanied by any significant hyperphosphorylation of histone H3-Ser10 at the LDL receptor promoter, suggesting that hyperphosphorylation of histone H3-Ser10 is possibly independent of this kinase in HepG2 cells. The tight correlation between histone H3-Ser10 phosphorylation and LDL receptor induction supports a functional link between these two events. At present, the precise mechanism by which the phosphorylation of histone H3-Ser10 participates in LDL receptor induction is not clear. There are at least two potential mechanisms by which this phosphorylation could influence LDL receptor transcription. First, phosphoepitope on histone H3-Ser10 provides binding sites for the recruitment of coactivators or chromatin-remodeling complexes, thus facilitating the assembly of active transcription complexes. Alternatively, histone phosphorylation contributes to the destabilization of nucleosomes and chromatin structure, resulting in the access of selected promoter regions to transcription factors and coactivators, ultimately leading to enhanced transcription. The second possible scenario is supported by an earlier in vivo footprinting study at the human LDL receptor promoter in response to TPA, which showed no change in the occupancy of the regulatory sites but possible reorganization of the promoter into a relatively more accessible configuration specifically (36). We thus favor the second mechanism, in which chromatin structure at the LDL receptor promoter possibly inhibits transcription and PKC-dependent hyperphosphorylation of histone H3-Ser10 may be required to initiate LDL receptor induction by TPA.

Another important finding is that TPA stimulated phosphorylation of histone H3-Ser10 at the LDL receptor chromatin, whereas it actually decreased global phosphorylation of this residue, demonstrating a clear dissociation between transcriptional response per se and the general alteration of the phosphorylation state of cellular histone H3-Ser10. Similar to our results, experiments performed in *Drosophila* have also shown that the heat shock of salivary gland dramatically reduced global histone H3-Ser10 phosphorylation while it substantially increased phosphorylation of this serine on a few loci containing genes encoding heat shock proteins (17). Because histone H3-Ser10 phosphorylation correlates with the transcription status of many genes, global reduction in its phosphorylation by TPA may represent a cellular defense mechanism to halt the transcription of nonessential genes.

The nature of the cellular kinase that is involved in either loci-specific or global histone H3-Ser10 phosphorylation in various cell types is unclear. A number of kinases have been reported to phosphorylate histone H3-Ser10,

including p42/44^{MAPK}, p38^{MAPK}, pp90^{RSK}, MSK-1/2, and/or PKA. In the case of HepG2 cells, our results support the involvement of PKC in regulating the phosphorylation of histone H3-Ser10 associated with the LDL receptor promoter. Interestingly, PKCs are known to translocate into the nucleus of mammalian cells in response to TPA to phosphorylate nuclear proteins for the regulation of gene expression (4, 5). Our finding raises an interesting possibility that some of the transcriptional effects of PKC may reflect histone H3-Ser10 phosphorylation and chromatin reorganization rather than, or in addition to, direct phosphorylation of transcription factors. Although we have been unsuccessful in our attempts to cross-link histone H3 and PKC, the inability to demonstrate a physical interaction of these two proteins most likely reflects the transient nature of this catalytic reaction.

The emerging picture from our work is that TPA sends a signal to the LDL receptor promoter directly through the PKC-mediated hyperphosphorylation of histone H3-Ser10. Consequently, one of the challenges of future investigations is to fully define the role of this modification in LDL receptor induction by TPA. Additionally, the above findings also raise an interesting possibility that Ser10 phosphorylation may act downstream of PKC as an integral component of the transforming pathway elicited by a large number of oncogene-initiated pathways converging at PKC to promote aberrant cell proliferation.

The authors thank Dr. Gurpreet Kapoor for performing some of the experiments described in this paper. These studies were supported by grants from the National Heart, Lung, and Blood Institute (HL-RO165540) and by an American Heart Association Established Investigator grant (9940163N).

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