Modulation of LDL receptor mRNA stability by phorbol esters in human liver cell culture models

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Abstract In the human hepatocarcinoma cell lines HepG2 and Hep3B, low density lipoprotein receptor (LDLR) mRNA levels were rapidly and transiently induced after treatment with phorbol-12-myristate-13-acetate (PMA), increasing by approximately 50-fold and 8-fold, respectively, within 4 h before returning to near basal levels by 24 h. The difference in magnitude of mRNA accumulation between these cell lines is at least partly due to a rapid 2- to 2.5-fold stabilization of LDLR mRNA in HepG2 cells after PMA treatment. Stabilization of LDLR mRNA in response to PMA was also observed in HH01 cells, a human hepatocyte coculture system derived from normal human liver. In both HepG2 and HH01 cells, PMA treatment induced a rapid morphological change with characteristics of cytoskeletal reorganization. The changes in morphology and stabilization of LDLR mRNA by PMA were coincident in the cell lines tested and were independent of de novo gene expression. Subcellular fractionation studies indicated that LDLR polysomes may be associated with the cytoskeleton in HepG2 cells. Disruption of the actin cytoskeleton but not microtubules abrogated stabilization of LDLR mRNA by PMA. These data suggest that components of the actin cytoskeleton are involved in the regulated decay of LDLR mRNA in some human liver cell culture systems.—**Wilson, G. M., E. A. Roberts, and R. G. Deeley.** Modulation of LDL receptor mRNA stability by phorbol esters in human liver cell culture models. *J. Lipid Res.* 1997. **38:** 437–446.

Supplementary key words colchicine • cytochalasin D • cytoskeleton • polyribosomes • posttranscriptional regulation

Low density lipoprotein (LDL) is the most abundant cholesterol-carrying lipoprotein in human plasma. It enters into cells by interaction with the LDL receptor (LDLR), an integral plasma membrane glycoprotein expressed in a wide variety of cell types, but most abundantly in liver. Liver is the sole organ in which cholesterol removed from the circulation may be excreted via conversion to bile salts. As serum LDL-cholesterol levels remain a major prognostic indicator of atherosclerotic risk, it is important that the mechanisms regulating hepatic expression of LDLR be understood (1).

The highly differentiated human hepatocarcinoma cell lines HepG2 and Hep3B are the most commonly used hepatic cell culture models of cholesterol and lipoprotein metabolism (2–6). These lines have retained the ability to synthesize and secrete a wide variety of serum proteins produced by normal liver tissue (7). Recently, human cell lines have been described that are derived directly from normal liver. One such line, termed HH01, was established as a coculture of pediatric human hepatocytes and rat liver endothelial cells (8), in which the hepatocytes remain highly differentiated and comprise >95% of the established cell line. It was anticipated that the HH01 line would be useful for testing whether regulatory events described in HepG2 or Hep3B cells might be applicable to normal human liver.

Repression of LDLR expression has been extensively studied in HepG2 cells in response to cholesterol and lipoprotein loading (3, 5, 6, 9–11). Recent studies have also demonstrated that transcription of the LDLR gene in HepG2 may be induced by activation of several signal transduction pathways, including the cyclic AMP, inositol 1,4,5-trisphosphate-Ca^{2+}, and diacylglycerol-protein kinase C (PKC) systems (12, 13). Activation of PKC by treatment with phorbol esters has been shown to increase LDL-binding and catabolism in HepG2 cells (14). These studies suggest that, in addition to intracellular cholesterol levels, expression of LDLR in HepG2 is influenced by extracellular signals activating selected signal transduction pathways.

In this report, we demonstrate that LDLR mRNA is rapidly and transiently induced in HepG2 and Hep3B cells in response to treatment with the phorbol ester

Abbreviations: BSA, bovine serum albumin; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LDLR, low density lipoprotein receptor; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate.

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phorbol-12-myristate-13-acetate (PMA). In HepG2 cells, this induction involves increased transcription of the LDLR gene and transient stabilization of LDLR mRNA that is independent of de novo gene expression. Treatment with PMA also induces a rapid change in cellular morphology in HepG2 cells which is consistent with reorganization of the cytoskeleton. Although neither modulation of LDLR mRNA stability nor changes in morphology occur with Hep3B cells they are observed in HH01 cells in response to PMA, demonstrating that these mechanisms are not restricted to HepG2 cells. Finally, we provide evidence that LDLR mRNA may associate with components of the cytoskeleton in HepG2 cells, and that the integrity of the actin cytoskeleton influences the rate of LDLR mRNA degradation.

MATERIALS AND METHODS

Materials

[^{32}P-α]dATP (3000 Ci/mmol) was purchased from NEN (Mississauga, ON) and [^{32}P-γ]ATP (3000 Ci/mmol) from ICN Canada (Montreal, PQ). Cytochalasin D was purchased from Calbiochem (La Jolla, CA). Phorbol-12-myristate-13-acetate (PMA), colchicine, insulin, hydrocortisone, thyrotropin releasing hormone, linoleic acid, BSA, and selinous acid were purchased from Sigma (St. Louis, MO). Tissue culture media, penicillin, steptomycin, and fetal bovine serum were from Gibco/BRL (Burlington, ON). Iron-supplemented calf serum was from HyClone (Logan, UT). Actinomycin C_1 was purchased from Boehringer Mannheim Canada (Laval, PQ).

Cell culture

The hepatocarcinoma cell lines HepG2 and Hep3B were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and maintained in minimum essential medium (MEM) supplemented with 10% iron-supplemented calf serum. Prior to harvesting, these cultures were incubated in media containing 10% fetal calf serum (FCS) for at least 4 days. The hepatocyte coculture cell line HH01, derived from normal human liver (8), was maintained in αMEM supplemented with 10% FCS, insulin (20 ng/mL), hydrocortisone (8 nM), L-glutamine (1 mM), thyrotropin releasing hormone (2 nM), linoleic acid:BSA (1 ng/mL:1 μg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), and selinous acid (0.2 nM).

Preparation of RNA and northern analyses

Total RNA was isolated using TRIzol reagent (Gibco/BRL) according to the manufacturer's instructions. The polyA(+) RNA fraction was subsequently prepared using the polyATtract system (Promega, Madison, WI) according to the manufacturer's instructions. PolyA(+) RNA was fractionated on 1.2% formaldehyde agarose gels and transferred to nylon membranes (MagnaCharge: MSI, Westborough, MA) by capillary blotting in 10 × SSC [1 × SSC: 150 mM NaCl, 15 mM Na_3citrate (pH 7.0)]. The membrane was then washed with 5 × SSPE [1 × SSPE: 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA (pH 7.7)] at 60°C for 5 min and vacuum baked at 80°C for 1 h.

RNA blots were probed with either *i*) cDNA fragments that had been radiolabeled by random priming with [^{32}P-α]dATP (15), or *ii*) oligonucleotides labeled with [^{32}P-γ]ATP using polynucleotide kinase (15). The cDNA fragments included an *Eco*RI + *Bgl*II coding region fragment of LDLR cDNA from pLDLR3 (ATCC), an *Xba*I + *Pst*I fragment of human GAPDH cDNA from pHcGAP (ATCC), and a 300 bp fragment of the human β-actin coding region generated by polymerase chain reaction, generously donated by Dr. S-P. Tam (Queen's University). Blots probed with random-primed cDNA fragments were pre-hybridized for 4 h at 42°C in 5 × SSPE containing formamide (50%), 4 × Denhardt's solution (16), SDS (0.5%), and sheared, denatured herring testes DNA (200 μg/mL). After addition of the denatured DNA probe, incubation continued for 16–24 h. The blot was washed briefly in 6 × SSC at room temperature followed by four 15-min washes in 0.1 × SSC/0.1% SDS at 52°C prior to autoradiography.

An antisense oligonucleotide complementary to bases +1956 to +1975 (relative to the start of translation) was used to probe for human serum albumin mRNA. Blots probed with oligonucleotides were pre-hybridized for 4 h at 47°C in 6 × SSC containing 0.2% SDS, 0.2% Ficoll, 0.2% polyvinylpyrollidone, and 0.1% pyrophosphate. After addition of labeled oligonucleotides, blots were incubated for 16–24 h and washed four times for 10 min in 6 × SSC/0.2% SDS at 47°C.

Assays of mRNA decay

The half-lives of specific mRNAs expressed in the tested cell lines were determined by actinomycin time course assays. After addition of 5 μg/mL actinomycin C_1 to freshly confluent cultures, cells were lysed in TRIzol reagent at selected time points and polyA(+) RNA was isolated, fractionated, and blotted as described above. Levels of LDLR mRNA were determined by hybridization of radiolabeled probes and subsequent autoradiography. Blots were also probed for GAPDH mRNA to provide a loading control as well as a basis for comparison of mRNA decay rates. Autoradiograph density measurements were made on appropriately ex-

posed films with a Molecular Dynamics Computing Densitometer (Molecular Dynamics, Sunnyvale, CA).

Analyses of mRNA decay data

For any given mRNA, a linear plot of $\ln([\text{mRNA}]_t/[\text{GAPDH}]_t)$ versus time (t) confirms first-order decay kinetics. $[\text{mRNA}]_t$ and $[\text{GAPDH}]_t$ represent the remaining fraction of tested and GAPDH mRNAs, respectively, measured after t hours of actinomycin treatment. Tested mRNA half-life was then calculated as

$$t_{1/2(\text{mRNA})} = \ln(0.5)\left[\frac{dy}{dt} + \frac{\ln(0.5)}{t_{1/2(\text{GAPDH})}}\right]^{-1}$$

where $y = \ln([\text{mRNA}]_t/[\text{GAPDH}]_t)$. The half-life of the endogenous GAPDH mRNA was taken to be 40 h, as has been reported for 28S rRNA in subconfluent and freshly confluent contact-inhibited cell culture systems (17–19). Comparison of actinomycin time course blots of total RNA probed for both 28S rRNA and GAPDH mRNA demonstrated no significant difference in their decay kinetics (data not shown). Furthermore, varying values of GAPDH mRNA half-life from 20 h to ∞^+ has little effect on the calculated half-lives of unstable mRNAs ($t_{1/2} < 2$ h), as the proportion of GAPDH mRNA degraded over short time intervals becomes negligible.

An estimate of the standard deviation of regression slope was calculated as

$$s_r = \sqrt{\frac{\Sigma(y_c - y_o)^2}{n-2}}$$

where y_c represents the value of $\ln([\text{mRNA}]_t/[\text{GAPDH}]_t)$ for any given t calculated from the regression function, while y_o represents the experimentally observed value (20). Assessment of significance between regression slopes was performed using the paired Student's t test at $n - 2$ degrees of freedom. Significant differences were considered to be those with $P < 0.05$.

Fractionation of polysomes

Polysomes from HepG2 cells were fractionated using a modification of a protocol by Northemann, Schmelzer, and Heinrich (21). Cells from five 150-mm plates were washed twice with PBS containing 0.5 mM EDTA and scraped into a total of 5 mL 0.25 M sucrose in buffer A [buffer A: 200 mM HEPES/NaOH (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 25 mM dithiolthreitol] containing 0.15 mg/mL heparin and 0.25% diethylpyrocarbonate as inhibitors of endogenous RNases. The suspended cells were broken with 100 strokes of a Dounce homogenizer (tight pestle) on ice and made to 10 mL final with buffer A. Unlysed cells and nuclei were removed by centrifugation at 6000 g for 10 min at 4°C. The supernatant was then layered in 5-mL aliquots onto discontinuous gradients of sucrose in buffer A (2 mL each of 1.5 M, 1.75 M, and 2.2 M sucrose), and centrifuged at 113 000 g for 25 h at 4°C. The supernatant (above 1.5 M sucrose) was removed and stored as free (non-polysomal) ribonucleoproteins (mRNPs), while polysomes localized between the 1.5 M and 2.2 M sucrose layers were retained as membrane-associated. RNA was recovered from each of these fractions by precipitation with isopropanol and resuspension in TRIzol reagent. Material pelleted below the sucrose gradient was resuspended directly into TRIzol reagent, and was comprised of free and cytoskeletal-associated polysomes. PolyA(+) RNA was isolated, size fractionated, and blotted from each of these fractions as described above.

RESULTS

LDLR mRNA is differentially induced in HepG2 and Hep3B cells in response to PMA treatment

Treatment of HepG2 and Hep3B cell cultures with 160 nM PMA rapidly induced expression of LDLR mRNA (**Fig. 1**). LDLR mRNA levels increased approximately 50- and 8-fold in HepG2 and Hep3B cells, respectively, reaching maximum levels within 4 h of PMA treatment. This response was transient and levels in both cell lines returned to basal states within 24 h. No significant variation in GAPDH mRNA levels was observed in either cell line in response to PMA treatment.

LDLR mRNA is transiently stabilized in HepG2 and HH01 cells, but not Hep3B cells, in response to PMA treatment

Although the time-frames of LDLR mRNA accumulation in HepG2 and Hep3B cells are similar after PMA induction, the magnitude of the response in HepG2 is significantly greater. To test for variability in LDLR mRNA stability between the two cell lines, mRNA half-lives were determined by actinomycin time course assays of both untreated and PMA-induced cultures. After addition of PMA, the half-life of LDLR mRNA in HepG2 cells was found to increase rapidly from 45 to 102 min (**Fig. 2**). Induction of LDLR mRNA also occurred more quickly in HepG2 cells, increasing 10-fold within the first hour of PMA treatment (Fig. 1). The enhanced rate of LDLR mRNA accumulation after PMA induction in HepG2 cells when compared with Hep3B cells is consistent with rapid stabilization of LDLR mRNA in the HepG2 line. The stability of the mRNA in HepG2 de-

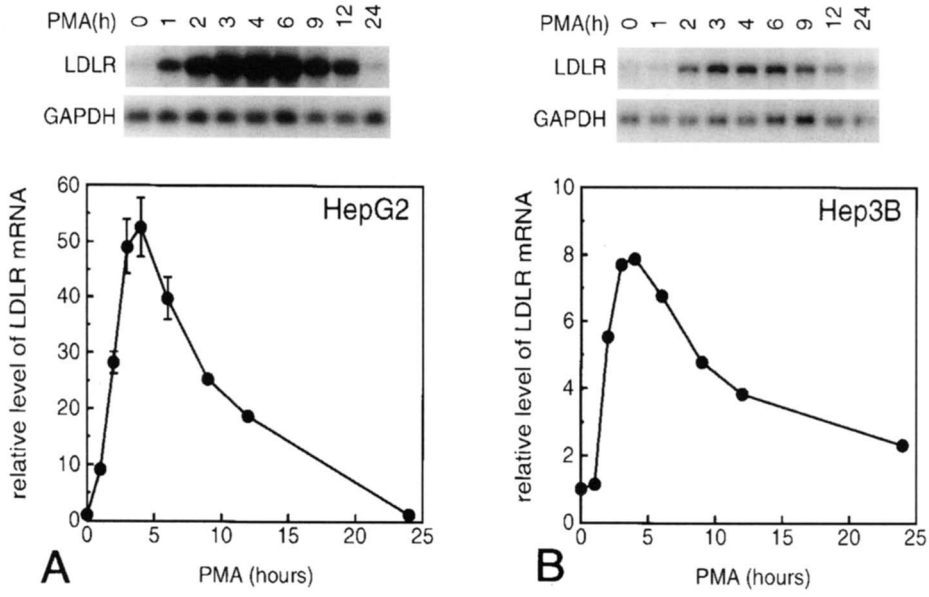

Fig. 1. Induction of LDLR mRNA by PMA in HepG2 and Hep3B cells. Northern blot analysis of LDLR mRNA levels in HepG2 (A) and Hep3B (B) cells at selected time points after addition of 160 nM PMA. Eight μg polyA(+) RNA isolated from each sample was used per lane and probed for LDLR and GAPDH mRNAs as described in Materials and Methods. The graphs denote LDLR mRNA levels at each time point as a function of uninduced levels. All data values were normalized to GAPDH mRNA levels. Due to the range of LDLR mRNA levels observed in HepG2 cells, the results from several autoradiograph exposures were quantified. The error bars reflect the numerical spread obtained for each sample by comparison with other samples within the quantitative range of the film.

creased steadily with the time of PMA incubation and returned to the uninduced half-life by 24 h. Based on the duration of the accumulation phase of the PMA response (4 h), and the half-lives of LDLR mRNA in the absence (45 min) and presence (~90 min) of PMA, we estimate that at peak induction stabilization may account for approximately one-third of the accumulated mRNA. The return to basal mRNA levels observed over the next 20 h indicates that during this period transcription of the LDLR gene must also return to pre-induction levels.

In Hep3B cells, no significant variation from a half-life of 30–40 min was detected for LDLR mRNA after PMA treatment. To test whether this stabilization phenomenon was a peculiarity of HepG2 cells, similar mRNA decay experiments were performed on HH01 cultures. In these cells, the half-life of LDLR mRNA was also significantly increased by PMA treatment, to a degree similar to that seen in HepG2 (Fig. 2).

To determine whether stabilization of LDLR mRNA by PMA in HepG2 cells required de novo gene expression, HepG2 and Hep3B cells were treated with actinomycin C_1 prior to PMA addition (**Fig. 3**). Pretreatment of HepG2 cells with actinomycin did not inhibit subsequent stabilization of LDLR mRNA by PMA, demonstrating that the mechanism involved was independent of de novo gene expression. In Hep3B cells, no significant change in LDLR mRNA decay kinetics was observed after PMA addition, consistent with the lack of LDLR mRNA stabilization in this cell line.

PMA induces rapid morphological changes in HepG2 cells consistent with re-organization of the cytoskeleton

Treatment of HepG2 cells with 160 nM PMA rapidly induced major changes in cellular morphology, with cells becoming flattened and polygonal in contrast to the more compact, irregular shapes in which HepG2 cells typically grow (**Fig. 4**). Similar changes in morphology were observed in HH01 cells, but not in Hep3B cultures. These changes in morphology were obvious within 20 min of PMA treatment, but were less distinct 24 h after addition of PMA (data not shown). In HepG2 and HH01 cells, these changes in morphology were observed even in the presence of actinomycin C_1, demonstrating that de novo gene expression was not required (data not shown). In addition, a rapid 3- to 5-fold induction of β-actin mRNA levels was observed in HepG2 and HH01 cells in response to PMA (**Fig. 5**). β-Actin mRNA levels were only minimally affected by PMA treatment in Hep3B cells. The rapidity of the morphological changes, the lack of requirement for ongoing transcrip-

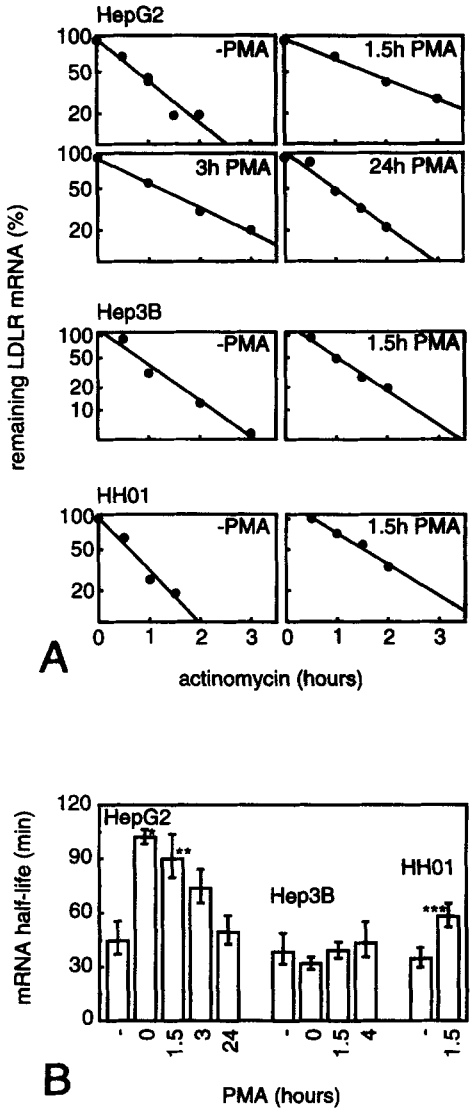

Fig. 2. Transient stabilization of LDLR mRNA induced by PMA in HepG2 and HH01 cells but not in Hep3B. (A) LDLR mRNA stability was determined by actinomycin C_1 time course assays of HepG2, Hep3B, and HH01 cultures pretreated with 160 nM PMA for the times indicated, prior to addition of the transcription inhibitor. Dimethylsulfoxide (DMSO) was added at 0.02% to samples not containing PMA as a vehicle control. At indicated actinomycin time points, polyA(+) RNA was isolated from each sample, and LDLR and GAPDH mRNAs were quantified by Northern analysis as described in Materials and Methods. Data at each actinomycin time point were plotted as the percentage of LDLR mRNA remaining normalized to GAPDH mRNA levels. (B) Summary of calculated half-lives of LDLR mRNA in each cell line at different times post-PMA treatment. Values shown on the horizontal axis indicate the time of exposure to PMA prior to actinomycin treatment. Bars labeled (−) indicate mRNA half-life in the absence of PMA, while the half-life of LDLR mRNA after 0 h PMA in both HepG2 and Hep3B cells was determined by linear regression of $\ln([\text{LDLR mRNA}]_t/[\text{GAPDH mRNA}]_t)$ versus t for the post-PMA data points shown in Fig. 3. This allowed the stability of LDLR mRNA to be determined immediately after PMA addition. All half-life values are shown ± s_e. The errors associated with half-life determinations are not uniformly distributed due to the exponential nature of first order decay kinetics. ($^*P < 0.005$ versus −PMA, $^{**}P < 0.01$, $^{***}P < 0.05$).

tion, and the induction of β-actin gene expression suggested that organization of the cytoskeleton in HepG2 and HH01 cells, but not Hep3B cells, was sensitive to PMA. Furthermore, the coincidence of morphological changes and stabilization of LDLR mRNA in response to PMA in the cell lines tested suggested that components of the cytoskeleton may be involved in the regulation of LDLR mRNA stability.

Polysomes containing LDLR mRNA may be associated with the cytoskeleton

Discontinuous sucrose gradients were used to separate polysomes from HepG2 cells into three distinct pools: free mRNPs (non-polysomal), membrane-associated polysomes, and free/cytoskeletal-associated polysomes. Northern analyses of polyA(+) RNA isolated from each of these fractions allowed the distribution of individual mRNAs to be determined (**Fig. 6**). β-Actin mRNA was almost exclusively present in the fraction containing free and cytoskeletal-associated polysomes, consistent with the subcellular distribution reported by Hesketh and Pryme (22). Serum albumin polysomes were largely recovered in the membrane-associated fraction, as expected for mRNAs encoding secreted protein products, and in agreement with the reported distribution of serum albumin polysomes in chicken liver (23). In HepG2 cells, approximately 70% of LDLR polysomes was recovered in the free and cytoskeletal-associated fraction (Fig. 6). A similar distribution was observed in PMA-treated HepG2 cells, indicating that modulation of LDLR mRNA stability by PMA was not the result of changes in polysome localization.

Integrity of the actin cytoskeleton is required for the short constitutive half-life and PMA-induced stabilization of LDLR mRNA

To investigate whether cytoskeletal components might be involved in regulating the stability of LDLR mRNA in HepG2 cells, assays of mRNA decay were performed on cultures pretreated for 24 h with cytochalasin D, to disrupt actin micro- and intermediate-filaments, or colchicine, to inhibit microtubule assembly. Each reagent was added at 5 μM, a concentration similar to that shown to induce morphological changes in other cultured cell systems (24, 25). Pretreatment with cytochalasin D resulted in a significant increase in constitutive LDLR mRNA half-life as compared with untreated cells ($P < 0.01$), approaching the mRNA stability seen in control PMA-treated HepG2 cells (**Fig. 7**). This stabilization event contributes to the approximately 20-fold increase in LDLR mRNA steady-state levels seen after cytochalasin D treatment of HepG2 cells (Fig. 7D). Transcription of the LDLR gene must also be induced by cytochalasin D to account for the magni-

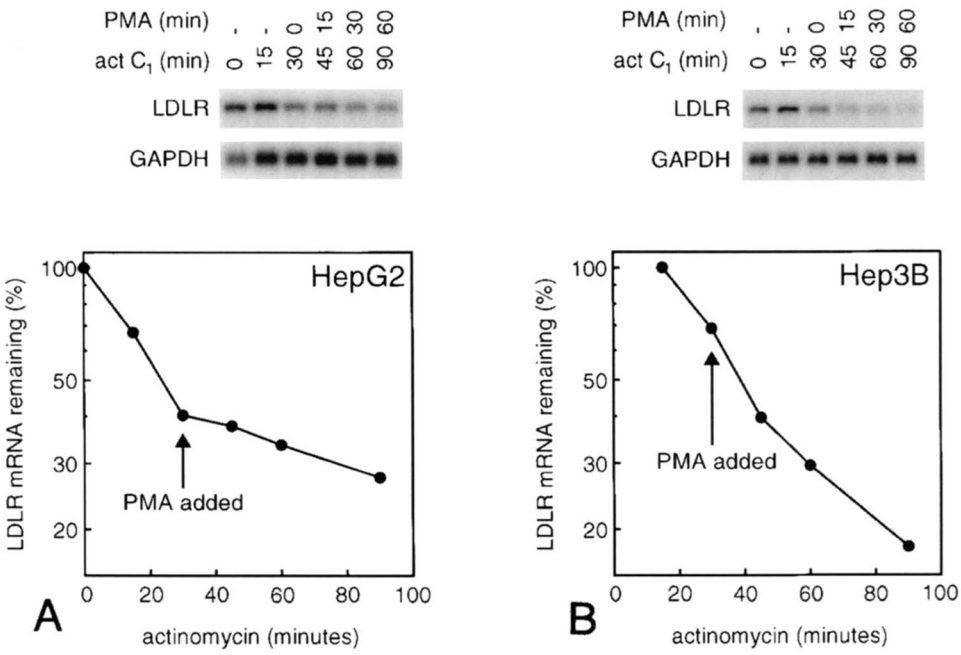

Fig. 3. Stabilization of LDLR mRNA by PMA in HepG2 cells pretreated with actinomycin. HepG2 (A) and Hep3B (B) cells were treated with 5 μg/mL actinomycin C_1 for 30 min prior to addition of 160 nM PMA. PolyA(+) RNA was harvested from samples at selected time points and probed for LDLR and GAPDH mRNA levels as described in Materials and Methods. Data at each time point were plotted as the percentage of LDLR mRNA remaining normalized to GAPDH mRNA levels.

tude of this response. Addition of PMA to cytochalasin-pretreated cells, however, did not further increase LDLR mRNA stability. Pretreatment with colchicine had little effect on the constitutive stability of LDLR mRNA in HepG2 as compared to untreated control cells, and did not inhibit PMA-inducible stabilization ($P < 0.01$). These results indicate that integrity of the actin cytoskeleton is required for the short constitutive half-life exhibited by LDLR mRNA. Furthermore, treatment with PMA may inhibit this cytoskeleton-dependent mRNA decay mechanism without mRNA dissociation.

DISCUSSION

The transient induction of LDLR mRNA observed in HepG2 cells in response to PMA treatment is consistent with the increase in LDL binding and catabolism reported by Kamps and van Berkel (14). They noted, however, that LDL binding was not induced in cultured primary rat or human hepatocytes, and postulated that modulation of LDLR expression by PMA was specific for HepG2 cells. Our studies have shown that the induction of LDLR mRNA by PMA was also attenuated in Hep3B relative to HepG2 cells, due in part to the lack of mRNA stabilization in Hep3B cells. To determine whether some unique property of HepG2 cells was responsible for the alterations in LDLR mRNA stability seen after PMA treatment, similar studies were performed using a cell line derived from normal human liver cells, HH01. The HH01 line is maintained in a highly differentiated state, as evidenced by the constitutive expression of several proteins characteristic of hepatocytes including glucose-6-phosphatase, serum albumin, α-fetoprotein, and transferrin (8). In addition, the drug-metabolizing activities of several cytochrome P-450 1A subfamily members were shown to be preserved in the HH01 line. Our data noting both transcriptional and posttranscriptional regulation of LDLR expression by PMA in HepG2 and HH01 cells demonstrate that these induction mechanisms are not specific for HepG2 cells. It is quite possible, however, that these regulatory mechanisms operate in actively growing and dividing cells or that they are lost under the conditions used to establish short term primary cultures.

The morphological changes observed in HepG2 cells in response to PMA were first reported by Duronio, Huber, and Jacobs (26). Using [^{3}H]thymidine incorporation assays, they demonstrated that DNA synthesis was transiently repressed in HepG2 cells after treatment with phorbol-12,13-dibutyrate, reaching a nadir between 18 and 24 h. Our studies revealed a similar tran-

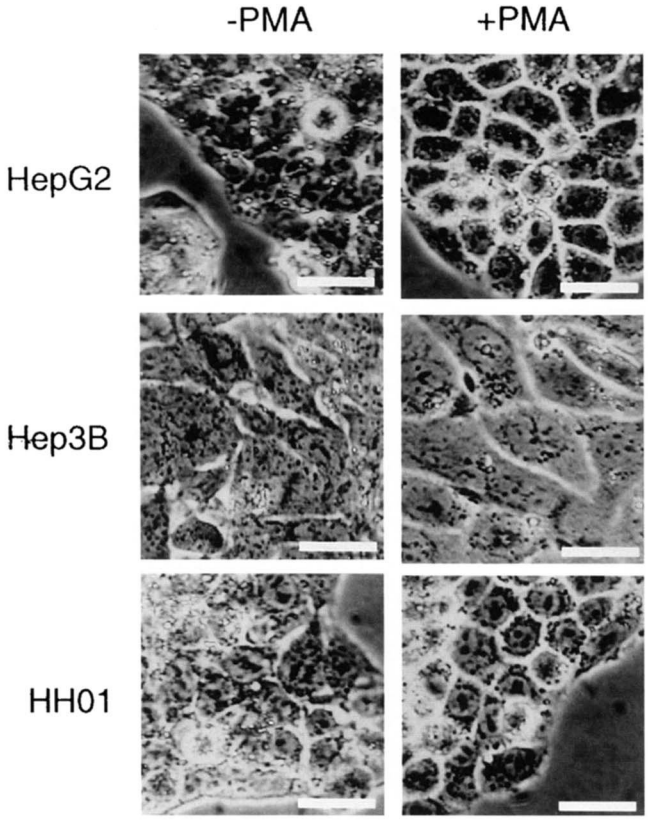

Fig. 4. Morphological effects of PMA treatment on HepG2, Hep3B, and HH01 cells. Phase-contrast photomicrographs of HepG2, Hep3B, and HH01 cells treated with 0.1% DMSO or 160 nM PMA for 1.5 h. The white bars indicate 25 μm.

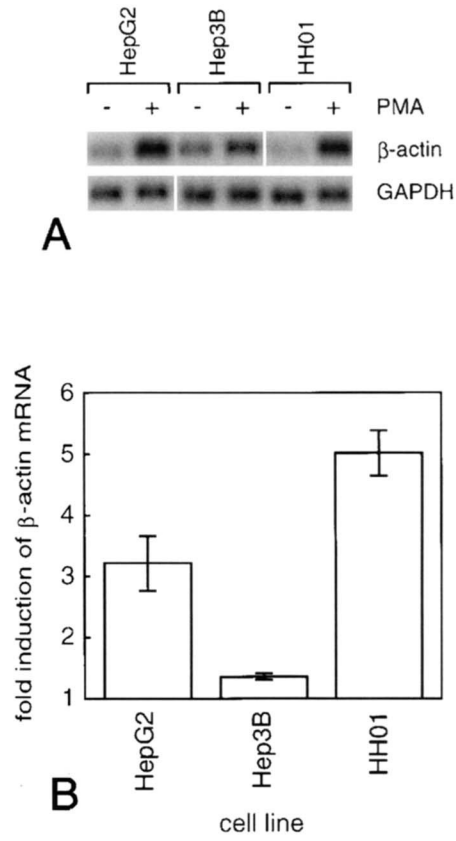

Fig. 5. Induction of β-actin mRNA by PMA in HepG2, Hep3B, and HH01 cells. (A) HepG2, Hep3B, and HH01 cells were treated with 0.02% DMSO (−) or 160 nM PMA (+) for 1.5 h. PolyA(+) RNA was isolated, Northern blotted, and probed for β-actin and GAPDH mRNAs as described in Materials and Methods. (B) Relative induction of β-actin mRNA in HepG2, Hep3B, and HH01 cells by PMA, normalized to GAPDH mRNA levels. The bars represent the mean ± spread of duplicate samples.

sient repression of [^{3}H]thymidine incorporation in HH01 after PMA treatment (data not shown). Furthermore, flow cytometry of propidium iodide-stained HepG2 nuclei revealed a substantial decrease in the population of cells in S phase, and a slight decrease in the G_2/M population after 24-h PMA treatment (data not shown). These data suggest that the cell cycle is transiently arrested at the G_1/S switch after PMA treatment of HepG2 cells, in contrast with the mitogenic response reported in some other cell lines (27–29).

Phorbol esters have also been reported to induce (30, 31) or inhibit (32, 33) differentiation in some cell lines. Duronio et al. (26) reported that levels of serum albumin, α-fetoprotein, and c-*fos* mRNAs were not affected by phorbol ester treatment of HepG2 cells, suggesting that the differentiated state of these cells was not significantly altered. Furthermore, our observations that the morphological changes induced by PMA in HepG2 and HH01 cells are very rapid and do not require ongoing transcription are also inconsistent with a substantial change in the cellular differentiated state.

Phorbol ester treatment of some cell types has been shown to have a marked effect on organization of the cytoskeleton and the extracellular matrix. In renal epithelium, treatment with phorbol esters induced cell rounding and loss of substratum attachment (34, 35). Chicken sternal chondrocytes displayed the reverse phenomenon, attaching and spreading out on culture dishes after introduction of PMA (36). In chicken chondrocytes and the human leukemic cell line K562 (37), cytoskeletal alterations in response to phorbol ester treatment were accompanied by an increase in β-actin expression. In HepG2 and HH01 cells, a similar combination of morphological change and β-actin mRNA induction was observed in response to PMA treatment. These effects were minimized in Hep3B cells, suggesting that the observed changes in HepG2 and HH01 morphology in response to PMA are likely the result of cytoskeletal reorganization. Variations observed in the responses of Hep3B and HepG2/HH01 cells to PMA may be a function of differences in PKC signalling path-

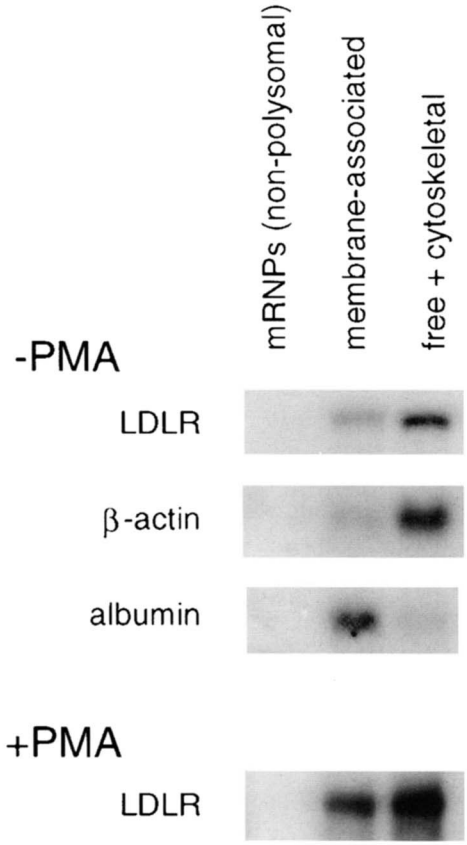

Fig. 6. Recovery of LDLR polysomes in cytoskeletal-associated polysome fractions in HepG2 cells. Polysomes were fractionated from HepG2 cells treated with 0.02% DMSO (−PMA) or 160 nM PMA (+PMA) for 1.5 h. The purified polyA(+) RNA from each fraction was isolated and Northern blotted as described in Materials and Methods. The resulting blots were probed for specific mRNAs as shown.

ways. Several responses are differentially modulated in these lines, including LDLR mRNA stabilization, β-actin mRNA induction, and changes in morphology. It is possible that an event early in the PKC signalling pathway functions differently in Hep3B cells, leading to attenuation of selected downstream events.

The polysome fractionation studies from HepG2 cells showed that polysomes containing LDLR mRNA were largely present in the fraction containing free and cytoskeletal-associated polysomes. Specific association of polysomal mRNAs with components of the cytoskeleton has been well established for some mRNAs including β-actin (22, 38), c-*myc* (39, 40), major histocompatibility complex, class HLA-B7 (41), and some histone mRNAs (41). However, polysomes encoding integral plasma membrane proteins are expected to be associated with the endoplasmic reticulum (ER) for proper protein trafficking. Thus, for the increased levels of LDLR mRNA to be contributing to induction of functional LDLR activity in PMA-treated HepG2 cells as observed by Kamps and van Berkel (14), polysomes containing LDLR mRNA must be associated with membrane components. It appears likely that a more complex arrangement exists for LDLR mRNA involving both membrane- and cytoskeletal-attachment sites. With the polysome fractionation procedure used, the high density of the cytoskeletal matrix may be sufficient to pull attached polysomes to the bottom of the gradient, even if bound to membrane components. A similar model has been proposed for polysomes containing HLA-B7 mRNA, which have been reported to associate with both membrane and cytoskeletal components (41). Our data provide four lines of evidence that indicate components of the cytoskeleton are involved in the modulation of LDLR mRNA stability: *1*] LDLR mRNA stabilization was accompanied by apparent re-organization of the cytoskeleton in HepG2 and HH01 cells: *2*] both cytoskeletal re-organization and mRNA stabilization were independent of de novo gene expression; *3*] LDLR polysomes largely co-purified with the cytoskeletal-associated polysome pool; and *4*] disruption of the actin cytoskeleton with cytochalasin D increased the constitutive stability of LDLR mRNA but abrogated further stabilization by PMA. Gross perturbations of the cytoskeleton have also been shown to alter the stability of some other mRNA species (42, 43).

The data presented here are consistent with a model of regulated LDLR mRNA decay in which a PMA-modulated element within the mRNA encodes a constitutive destabilizing function. The function of this element is dependent on the integrity of the actin cytoskeleton, likely via association of LDLR mRNA with cytoskeletal components, and is transiently inhibited after PMA treatment in HepG2 cells. When the integrity of the actin cytoskeleton is compromised, this element does not function to destabilize the mRNA, and subsequent treatment with PMA has no effect. This requirement for cytoskeletal integrity in rapid mRNA decay suggests that components of the RNA degradative machinery may also be localized to cytoskeletal structures.

The authors wish to thank Dr. J.G. Johnson for his expert assistance during the flow cytometry studies, and Dr. B.E. Elliott for his assistance with the photomicroscopy experiments. G.M.W. is the recipient of an E.G. Bauman Fellowship (Queen's University) and a Research Traineeship from the Heart and Stroke Foundation of Canada. This work was supported by a grant (R.G.D.) from the Heart and Stroke Foundation of Ontario.

Manuscript received 20 September 1996 and in revised form 10 December 1996.

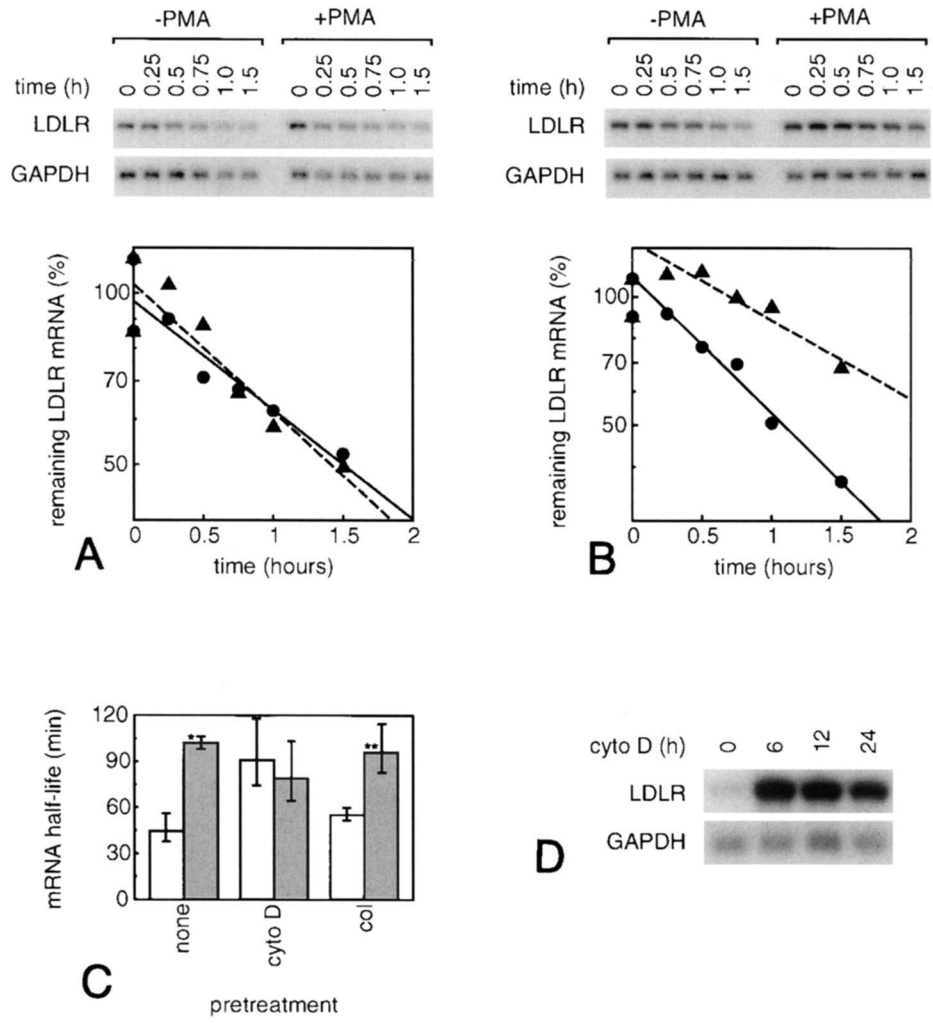

Fig. 7. Abrogation of PMA-induced stabilization of LDLR mRNA in HepG2 cells by pretreatment with cytochalasin D but not colchicine. HepG2 cells were treated with (A) 5 μM cytochalasin D or (B) 5 μM colchicine for 24 h. Actinomycin C_1 was then added at $t = -0.5$ hours, and 0.02% DMSO (circles, solid line) or 160 nM PMA (triangles, dashed line) at $t = 0$ h. PolyA(+) RNA was isolated at time points thereafter and analyzed for LDLR mRNA and GAPDH mRNA levels as described in Materials and Methods. Data at each time point were plotted as the percentage of LDLR mRNA remaining normalized to GAPDH mRNA levels. (C) Calculated LDLR mRNA half-lives in HepG2 cells treated with 0.02% DMSO (open bars) or 160 nM PMA (shaded bars) after 24 h cytochalasin D (cyto D) or colchicine (col) pretreatments. Half-life values are shown ± s_r (*$P < 0.005$ versus −PMA, **$P < 0.01$). (D) Northern analysis of LDLR mRNA levels in HepG2 cells treated with 5 μM cytochalasin D for the times indicated. From each sample, 8 μg polyA(+) RNA was gel fractionated, blotted, and probed for LDLR and GAPDH mRNAs as described in Materials and Methods.

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