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Research Article

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Proliferation of Hepatic Stellate Cells Is Inhibited by Phosphorylation of CREB on Serine 133

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Abstract

Proliferating, activated, hepatic stellate cells have a high level of collagen type I expression. Therefore, stellate cell proliferation is a critical step in hepatic fibrosis. Here we show that proliferation of activated primary rat stellate cells was blocked by elevation of cAMP with 8 Br-cAMP or isomethylbutyl xanthine, a phosphodiesterase inhibitor, and by stimulation of Ca²⁺ fluxes with the Ca²⁺ ionophore A-23187. Because phosphorylation of CREB on Ser133 is an important mediator of cAMP-protein kinase (PKA) and Ca²⁺-calmodulin kinase II (CAMK-II) activation, we tested whether CREB-PSer133 was essential for stellate cell quiescence. Nuclear extracts from quiescent, but not from activated, stellate cells contained CREB-PSer133. Moreover, the phosphorylation of CREB on Ser133 was stimulated in activated cells by inducing the activity of PKA or CAMK-II. In addition, coexpression of CREB and either a constitutively active PKA or a constitutively active CAMK-II inhibited the proliferation of activated stellate cells. In contrast, expression of CREB alone, PKA or CAMK-II alone, CREB-Ala 133 (which lacks the Ser133 phosphoacceptor) with PKA or CAMK-II, or CREB with inactive PKA or CAMK-II mutants did not affect stellate cell proliferation, suggesting that CREB-PSer133 is necessary for blocking the stellate cell cycle. Conversely, expression of a *trans*-dominant negative CREB-Ala 133 mutant (which competes with CREB/CREB-PSer133 for cognate DNA binding sites and presumably for protein interactions) induced a greater than fivefold entry into S-phase of quiescent stellate cells, compared with control cells expressing either β -galactosidase or wt CREB, indicating that CREB-PSer133 may be indispensable for the quiescent stellate cell phenotype. This study suggests that PKA and CAMK-II play an essential role on stellate cell activation through the induction of CREB phosphorylation on Ser133, and provides potential approaches for the treatment of hepatic fibrogenesis in patients with chronic liver diseases. (*J. Clin. Invest.* 1997. 99:1322–1328.) Key words: hepatic stellate cell proliferation • liver fibrogenesis • CREB phosphorylation • protein kinase A • calcium calmodulin kinase-II

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Introduction

Collagen type I is excessively deposited in the extracellular matrix protein in hepatic fibrosis (1, 2), which in turn contributes to the morbidity and mortality of patients with chronic liver diseases (3). Hepatic stellate cells play a key role in the pathogenesis of hepatic fibrosis (4, 5). Although we (6) and others (7, 8) reported that quiescent stellate cells produce little collagen type I, proliferating, activated (myofibroblastic) stellate cells display a high level of collagen $\alpha_1(I)$ gene expression. In addition, the degree of fibrogenesis in liver diseases is most likely affected by the increased population of stellate cells, which results from their proliferation (9).

Therefore, stellate cell proliferation is a critical step in hepatic fibrogenesis. Studies with primary cultures of adult rat stellate cells have provided evidence that cell type-specific mechanisms modulate their proliferation (10–12). For example, the induction of stellate cell proliferation by TGF α or collagen type I matrix is mediated by oxidative stress through *c-myc* and probably NF κ B (11). Conversely, endothelin-1 inhibits the growth of stellate cells (12). However, little is known about the signal transduction pathways and the nuclear factors involved in either the normal, quiescent or the activated, proliferating phenotype of hepatic stellate cells. Because stimulation of the cyclic adenosine 3',5' monophosphate (cAMP)-dependent protein kinase (PKA)¹ inhibits proliferation of some tumoral cell lines (13–15), we analyzed the potential role of this signal transduction pathway on stellate cell proliferation.

In this study, we found that stimulation of the cAMP/PKA signal transduction pathway (16), or its parallel pathway Ca²⁺/calmodulin kinase-II (CAMK-II) (17, 18), inhibits proliferation of activated stellate cells. Moreover, our results suggest that activation of either PKA or CAMK-II induces phosphorylation of CREB on Ser 133, which is an important modulator of the stellate cell cycle.

Methods

Cell cultures. Stellate cells were prepared from male Sprague-Dawley rats (400–500 g) by in situ perfusion and single-step density Nycodenz gradient (Accurate Chemical & Scientific Corp., Westbury, NY), as described previously (6, 19). Cells were plated on collagen type I, EHS matrix (Matrigel), or plastic (according to the experimental design) tissue culture dishes, with the initial seeding of fat-storing cells at a density of $2 \times 10^5/cm^2$. Matrigel's (Collaborative Biomedical Products, Bedford, MA) major components are laminin, collagen IV, proteoglycans, entactin, and nidogen. It also contains

1. Abbreviations used in this paper: CAMK-II, calmodulin kinase-II; CBP, CREB binding protein; MDA, malondialdehyde; PCNA, proliferating cell nuclear antigen; PKA, cAMP-dependent protein kinase; RARs, retinoic acid receptors.

TGF β , fibroblast growth factor, and tissue plasminogen activator. Medium was changed every 48 h for all conditions. Stellate cells were identified by their typical autofluorescence at 328 nm excitation wavelength, staining of lipid droplets by oil red, and immunohistochemistry with a monoclonal antibody against desmin (20). Greater than 95% of the cells were stellate cells. Freshly isolated stellate cells were transfected with the mammalian vectors expressing the protein of interest using lipofectin (GIBCO BRL, Gaithersburg, MD) as described by the manufacturer. To increase the transfectability of activated cells, a transfection-enhancing reagent (Life Technologies Inc., Gaithersburg, MD) was added in conjunction with lipofectamine as recommended by the manufacturer. The total amount of transfected DNA was 2.5 μ g. The transfection efficiency was 44 \pm 10% for day 0 quiescent cells and 28 \pm 8% for activated cells growing on a collagen type I matrix. Cells were fixed at 48 or 120 h after transfection for passage 1 and passage 0, respectively. In some experiments, cells were labeled with 2 μ Ci [3 H]thymidine (70–80 Ci/mmol; Amersham Corp., Arlington Heights, IL). After 3 h of labeling, cells were harvested and [3 H]thymidine incorporation into DNA was determined as described (21).

Nuclear extract preparation. Nuclei were prepared by a modification of the procedure described previously (21–23). Cells were homogenized in 1 ml of 100 mM KCl, 10 mM Tris, pH 7.6, 1 mM EDTA, 0.5% NP-40, 10 mM NaF, and 10 mM Na pyrophosphate with a glass Dounce homogenizer with a loose fitting pestle. The homogenized cells were placed above a cushion consisting of 2 M sucrose. The nuclei were precipitated by a 4,000 g centrifugation at 4°C for 30 min and frozen at –70°C.

Immunohistochemistry. Cells were fixed with acetone/methanol (50:50) at –20°C for 20 min and immunostained as described previously (20, 21). Antibodies directed against β -galactosidase, CREB, CREB-P $\text{Ser}133$, or proliferating cell nuclear antigen (PCNA) were obtained from 5 Prime–3 Prime, Inc. (Boulder, CO), New England Biolabs Inc. (Beverly, MA), Upstate Biotechnology, Inc. (Lake Placid, NY), and Novocastra (Burlingame, CA). Fluorescent labels were visualized using a dual channel Zeiss microscope as described previously (21). Cytochromes used were FITC and Texas red (Vector Labs, Inc., Burlingame, CA). The number of PCNA(+) cells was expressed as a percentage of total transfected cells (β -galactosidase [+]). At least 200 cells were analyzed per each experimental point, and a minimum of two observers analyzed each immunohistochemical experiment as described previously (21). Negative control samples were processed in parallel under the same conditions, but with omission of the first antibody. Detection of CREB and CREB-P $\text{Ser}133$ in nuclear extracts from rat stellate cells in primary culture or freshly isolated from rat livers was performed by Western blot following the chemiluminescence protocol (DuPont, Wilmington, DE), using antibodies against CREB (Santa Cruz Biotechnologies, Santa Cruz, CA) or CREB-P $\text{Ser}133$ (Upstate Biotechnology, Inc.) as described (22, 23).

Statistical analysis. Results were expressed as mean of at least three independent experiments. Both the ANOVA and Bonferroni tests were used for analysis of variance, with a *P* value of < 0.05 as significant.

Results

First, we examined the role of cAMP on the proliferation of activated primary rat stellate cells. Inhibition of stellate cell proliferation was attempted in passage 0 cells activated on collagen type I for 6 d or in passage 1 cells activated on plastic for 11 d (7, 8, 11). These cells exhibited a high entry into S-phase (> 80%) in agreement with previous studies (11). Cell proliferation was determined by incorporation of [3 H]thymidine into DNA, which occurs in S-phase, as described previously (21). As shown in Fig. 1, proliferation of stellate cells whether activated by collagen type I or plastic was blocked by elevation of cAMP with 8Br-cAMP (100 μ M), a cAMP isomer resistant

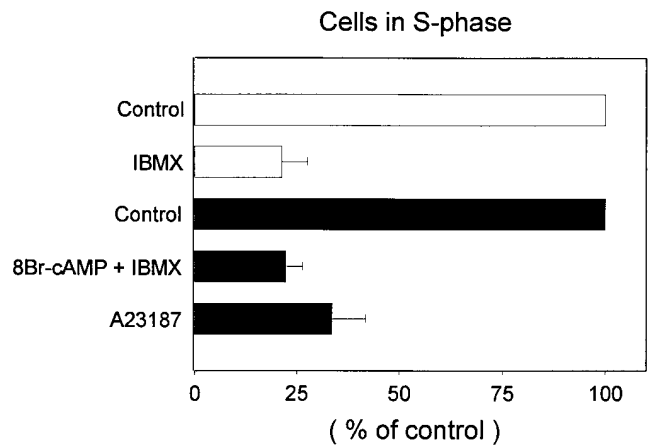


Figure 1. cAMP and Ca $^{2+}$ fluxes inhibit hepatic stellate cell proliferation. The incorporation of [3 H]thymidine into DNA was analyzed in primary stellate cells. Cells were cultured on collagen type I (closed bars) or on plastic (open bars) in media with 10% fetal calf serum. Cells received no treatment (control) or were treated for 24 h with 8Br-cAMP (100 μ M), IBMX (500 μ M), or A-23187 (1 μ M) as described in Methods. [3 H]Thymidine (2 μ Ci) was added for the last 3 h of the incubations. Values represent the percentage of [3 H]thymidine incorporation of control cells; *P* < 0.05 for IBMX, 8Br-cAMP + IBMX, and A-23187 compared with controls.

to hydrolysis, and/or isomethylbutyl xanthine (500 μ M), a phosphodiesterase inhibitor.

Because intracellular Ca $^{2+}$ fluxes modulate many cellular functions in parallel to cAMP (16–18), we analyzed whether A-23187 (1 μ M), an inducer of Ca $^{2+}$ fluxes (24), had similar effects to cAMP on stellate cell proliferation. We found that treatment of activated stellate cells with A-23187 also inhibited cell entry into S-phase (Fig. 1).

Many of the cellular effects resulting from the activation of cAMP/PKA pathway are mediated by the phosphorylation of CREB on Ser 133 (25, 26). Therefore, we assessed the role of CREB-P $\text{Ser}133$ on stellate cell cycle. Nuclei were obtained from quiescent and activated stellate cells through a sucrose gradient in the presence of protease and phosphatase inhibitors as described (23, 27). Equal amounts of nuclear protein from quiescent and activated stellate cells were analyzed by SDS-PAGE and visualized by Coomassie blue staining (Fig. 2). Using specific antibodies in a protein immunoblot, we detected CREB as 43-kD monomers in nuclear extracts from quiescent cells. The amount of CREB was decreased in nuclear extracts from proliferating, activated stellate cells (Fig. 2). In nuclear extracts from quiescent cells, CREB was phosphorylated on Ser 133 (Fig. 2). In addition to recognizing CREB in protein immunoblots, anti-CREB-P $\text{Ser}133$ detected small quantities of two other proteins after longer exposures. These are most likely members of the CREB-ATF family, ATF1 or CREB β (38 kD) and CREM (30 kD), that have phosphoacceptor sequences similar to that of CREB-P $\text{Ser}133$ (28). The phosphorylation of ATF-1 and CREM can be induced by cAMP and Ca $^{2+}$ (18). Nuclear extracts from activated stellate cells contained negligible amounts of CREB-P $\text{Ser}133$ (Fig. 2), suggesting a role for CREB/CREB-P $\text{Ser}133$ on the stellate cell cycle. As expected, PCNA, an index of S-phase (29), was present in nuclear extracts from activated

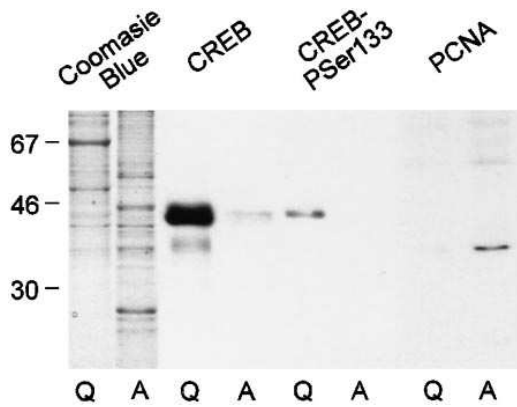


Figure 2. CREB and CREB-PSer133 are expressed in quiescent stellate cells. Protein immunoblots were performed with nuclear extracts (10 μ g) from quiescent (Q) and activated (A) stellate cells as described in Methods, using antibodies against CREB, CREB-PSer133, or PCNA as indicated. Coomassie blue staining of nuclear extracts shows the protein pattern. Molecular markers (kD) are shown.

cells, but not in nuclear extract from quiescent cells (Fig. 2), indicating that the decrease in CREB/CREB-PSer133 in activated cells was not due to a spurious effect of nuclear extraction. Moreover, the results shown in Fig. 2 are representative of four independent nuclear extracts from quiescent and activated stellate cells.

In subsequent experiments, we analyzed whether phosphorylation of CREB on Ser 133 is critical for the inhibition of stellate cell proliferation. Activated cells were transfected with vectors expressing the protein of interest (21, 22), and cultured on collagen type I to maintain their activated phenotype (11). Most of the cells expressing the control β -galactosidase alone entered S-phase (> 80%), as indicated by the expression of PCNA (cyclin; polymerase δ accessory protein) (29) (Fig. 3 A). Cells underwent organic fixation which permits the detection of PCNA in the nucleus during S-phase (30). Of interest, coex-

pression of CREB and a constitutively active PKA (25, 31) together with β -galactosidase inhibited the proliferation of these activated cells. In contrast, expression of β -galactosidase with CREB alone, PKA alone, or CREB-Ala 133 (which lacks the Ser 133 phosphoacceptor [25, 26]) with PKA did not affect stellate cell proliferation (Fig. 3 A). These results suggest that PKA-induced phosphorylation of CREB on Ser 133 is necessary for the inhibition of stellate cell proliferation, since unlike wt CREB, CREB-Ala 133 was refractory to the effects of PKA on cell entry into S-phase. Neither the expression of CREB nor the expression of a constitutively active PKA was sufficient to prevent cells to enter S-phase. In Fig. 4, representative examples of the dissociation between CREB/PKA expression and stellate cell cycle S-phase, assessed by PCNA (bright green-yellow), are shown. The transfected cells were identified by the expression of cytoplasmic β -galactosidase (in red). Nuclear expression of PCNA was high in control activated cells but it was markedly inhibited in stellate cells expressing both CREB and PKA. However, the nuclear expression of PCNA in cells transfected with CREB-Ala133 and PKA or CREB and an inactive PKA mutant (25) was comparable with control cells (Fig. 4). Because CAMK-II is activated by Ca^{2+} fluxes and under some experimental conditions is capable of inducing phosphorylation of CREB on Ser 133 (17), we also analyzed the effects of this pathway on the stellate cell cycle. Coexpression of CREB and a constitutively active CAMK-II (32) inhibited proliferation of activated cells (Fig. 3 B). In contrast, expression of CAMK-II alone, CREB-Ala133 with CAMK-II, or CREB with an inactive CAMK-II mutant (32) did not alter the stellate cell cycle. Taken together, these results indicate that phosphorylation of CREB on Ser 133 by cAMP/PKA or Ca^{2+} /CAMK-II pathways arrests the stellate cell cycle.

To analyze further the role of CREB-PSer133 on the stellate cell cycle, we performed immunofluorescence for CREB-PSer133 in activated cells using specific antibodies that do not cross-react with nonphosphorylated CREB at Ser 133 (28). As depicted in Fig. 5, when proliferating, activated stellate cells were treated with 8Br-cAMP, IBMX, forskolin (not shown),

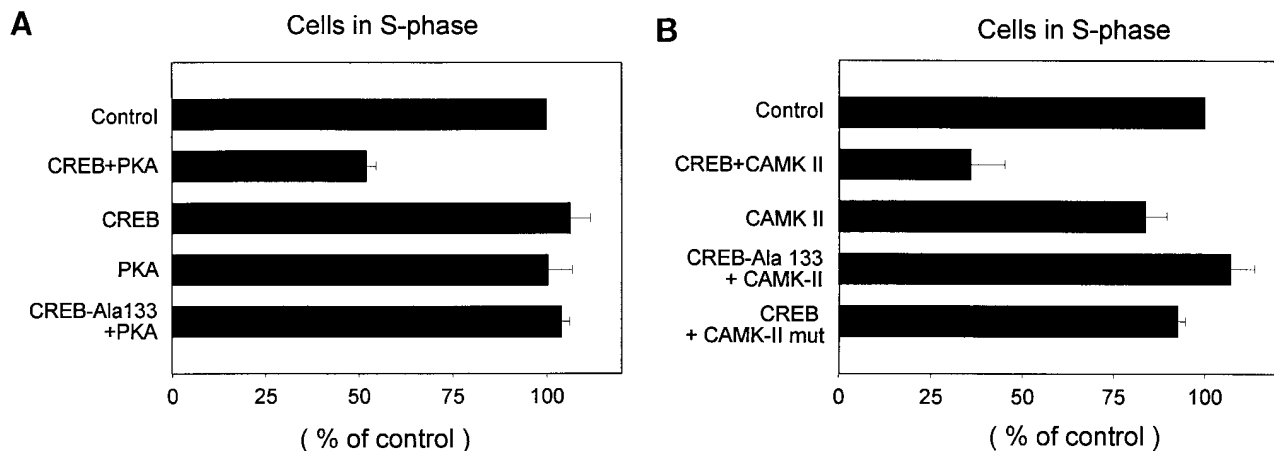


Figure 3. CREB-PSer133 inhibits stellate cell entry into S-phase. Activated stellate cells were transfected with vectors expressing β -galactosidase alone (control) or together with vectors expressing CREB, CREB-Ala133, PKA (C-subunit plasmid MtC) (A), CREB, CREB-Ala 133, CAMK-II, or CAMK-II inactive mutant (M42) (B) as indicated. Cells were immunostained for β -galactosidase and PCNA 48 h after transfection. Values represent the percentages of cells expressing the transfected DNA that were also in S-phase, setting the percentage for control cells at 100%; $P < 0.05$ for CREB + PKA and CREB + CAMK-II compared with controls.

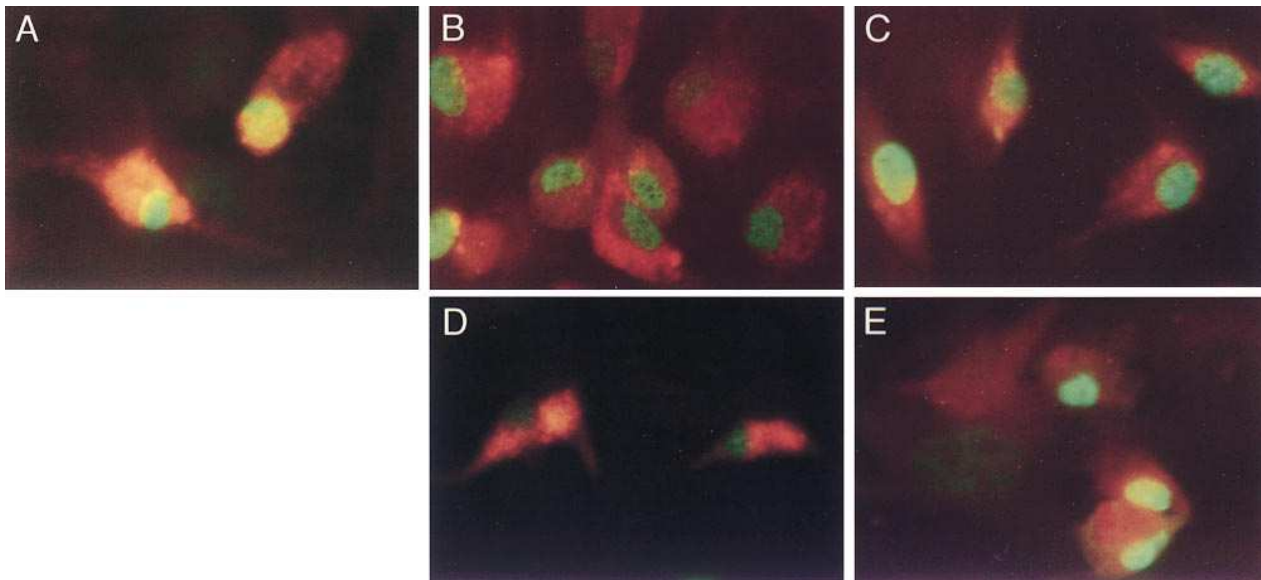


Figure 4. CREB phosphorylation on Ser133 is associated with arrest of the hepatic stellate cell cycle. Activated stellate cells were transfected as described in Fig. 3 with vectors expressing CREB alone (A); CREB + PKA (B); CREB + PKA inactive mutant (pCaK72M) (C); CREB + CAMK-II (D); and CREB + CAMK-II mutant (E). Cells were fixed 48 h after transfection and dual immunofluorescence was performed for β -galactosidase (red) and PCNA (bright green-yellow). Cells expressing CREB + PKA or CREB + CAMK-II did not enter S-phase.

or A-23187 they expressed nuclear CREB-PSer133, whereas untreated activated stellate cells did not.

Next, we studied whether CREB/CREB-PSer133 contribute to the inhibition of the cell cycle in quiescent stellate cells. In these cells, expression of the *trans*-dominant negative CREB-Ala133 induced a greater than fivefold entry into S-phase of quiescent stellate cells, compared with control cells expressing either β -galactosidase alone or with wt CREB (Fig. 6). The mutant CREB-Ala133 lacks the Ser 133 phosphoacceptor but competes with wt CREB for cognate DNA binding sites, and behaves as an antagonist (25). Thus, expression of a CREB mutant that cannot be phosphorylated on Ser 133 is sufficient to stimulate stellate cell proliferation.

Because oxidative stress and malondialdehyde (MDA), a product of lipid peroxidation, are strong inducers of stellate cell proliferation (11), we tested whether these effects of MDA could be neutralized by CREB-PSer133. We found that day 6 stellate cells cultured on a collagen type I matrix and expressing wt CREB and active PKA proliferated as much as control

cells when treated with MDA (Fig. 7). Conversely, butylated hydroxytoluene, an antioxidant that prevents the entry of stellate cells into S-phase induced by collagen type I matrix or TGF α (11), also blocked the proliferation of stellate cells cultured on EHS matrix, when stimulated by the expression of the mutant CREB-Ala133. These results suggest that oxidative stress affects the cascade leading to stellate cell proliferation at a site distal to the effects of CREB-PSer133.

Discussion

Although overproduction of collagen type I by activated hepatic stellate cells is a critical step in the development of liver cirrhosis (4, 5, 7, 8), the mechanisms responsible for the proliferation and activation of hepatic stellate cells remain unclear (3). In this study, we have characterized some of the signal transduction pathways that are involved in the modulation of the stellate cell cycle. Induction of either the cAMP/PKA or Ca²⁺/CAMK-II pathway (16–18) inhibits proliferation of acti-

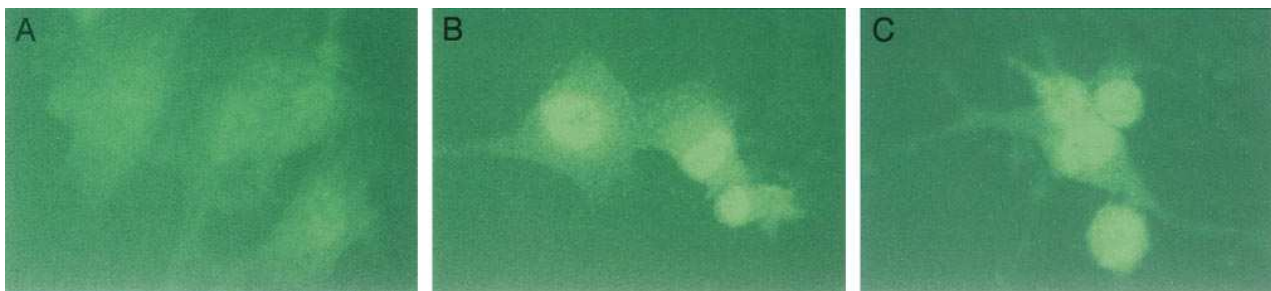


Figure 5. CREB-PSer133 expression is induced in hepatic stellate cells by cAMP or Ca²⁺ fluxes. CREB-PSer133 was detected using specific antibodies and fluorescein-labeled second antibodies. CREB-PSer133 immunofluorescence is shown for activated stellate cells control (A); 500 μ M IBMX (B); and 1 μ M A-23187 (C) as described in Fig. 1. Cells treated with IBMX or A-23187 expressed nuclear CREB-PSer133.

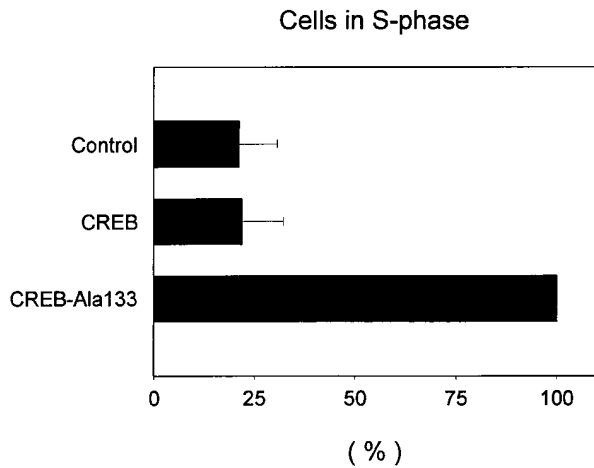


Figure 6. Expression of the *trans*-dominant negative CREB-Ala133 induces stellate cell proliferation. Quiescent primary stellate cells were transfected with vectors expressing β -galactosidase alone, or with vectors expressing CREB or CREB-Ala133 for 120 h. Transfected cells were immunostained for β -galactosidase, and S-phase was determined by the expression of PCNA. Values represent the percentages of cells expressing the transfected DNA that were also in S-phase, setting the percentage for CREB-Ala133 cells at 100%. $P < 0.05$ for CREB-Ala133 compared with controls.

vated stellate cells. Moreover, we have identified a molecular mechanism leading to entry of stellate cells into S-phase or arrest before the G_1/S boundary.

Our results suggest an important role of CREB Ser133 phosphorylation on the stellate cell cycle. The stellate cell cycle arrest induced by CREB-PSer133 results from the interaction between wt CREB and either active PKA or active CAMK-II, as strongly suggested by the ineffectiveness of: (a)

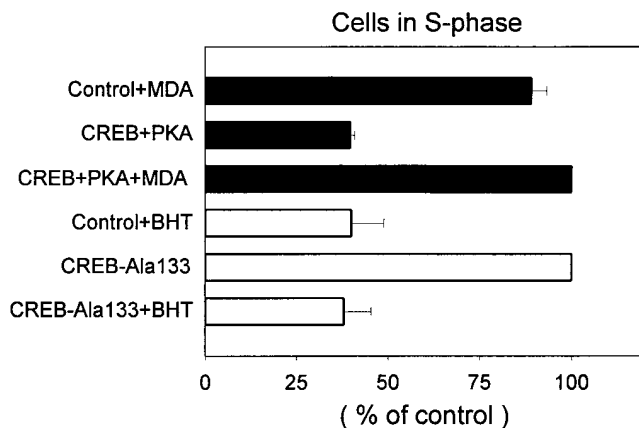


Figure 7. Oxidative stress affects the stellate cell cycle independently of CREB-PSer133. Stellate cells were cultured on collagen type I matrix (closed bars) or EHS (open bars), respectively. Cells were transfected with expression vectors and treated for 120 h with MDA (200 μ M), or for 48 h with butylated hydroxytoluene (BHT) (50 μ M) as indicated. Cells were stained as described in Fig. 4. Values represent the percentages of cells expressing the transfected DNA that were also in S-phase setting the percentage for MDA (closed bars) and CREB-Ala133 (open bars) at a 100%. $P < 0.05$ for CREB + PKA compared with CREB + PKA + MDA; and CREB-Ala133 compared with CREB-Ala133 + BHT.

CREB-Ala133 in the presence of constitutively active PKA or CAMK-II; (b) active PKA or active CAMK-II; and (c) wt CREB in the presence of mutant PKA or mutant CAMK-II. Also, we determined that quiescent cells can be induced to enter S-phase when they expressed the *trans*-dominant negative CREB-Ala133. In agreement with these results, we found that stellate cell cycle can be arrested by elevation of cAMP with 8Br-cAMP or IBMX or by inducing Ca^{2+} fluxes with the Ca^{2+} ionophore A-23187.

In contrast, quiescent stellate cell entry into S-phase is markedly induced by interfering with the activity of CREB/CREB-PSer133, with the *trans*-dominant negative CREB-Ala133. As for many other cellular functions (33), intracellular Ca^{2+} as well as cAMP seems to modulate the stellate cell cycle. In addition, activation of the cAMP/PKA pathway by M-phase-promoting factor is required for the transition from mitosis to interphase (34). Interestingly, either increased cAMP (35) or Ca^{2+} mobilization (24) inhibits collagen production by fibroblasts. A similar inhibitory effect of cAMP or Ca^{2+} fluxes on collagen production by stellate cells is expected, since quiescent stellate cells have a low expression of collagen type I gene compared with their activated, proliferating counterparts (4, 5, 19).

Because oxidative stress and reactive aldehydes induce the proliferation of quiescent stellate cells (11), and stimulate collagen transcription (36, 37), and antioxidants suppress the proliferation of activated stellate cells (11) as well as collagen gene expression (38), we analyzed whether oxidative stress affects the cell cycle at a site proximal or distal to the effects of CREB-PSer133. The antioxidant butylated hydroxytoluene (38) prevented the stimulation of quiescent stellate cell entry into S-phase by the mutant CREB-Ala133 (an antagonist of CREB). Moreover, exposure of proliferating, activated stellate cells to MDA, an aldehyde product of lipid peroxidation (37), prevented the cell cycle arrest by wt CREB and active PKA. Collectively, these results suggest that oxidative stress modulates the stellate cell cycle cascade at a site distal to the effects of CREB-PSer133. In this context, we have also demonstrated that quiescent stellate cells express nuclear CREB and CREB-PSer133, whereas nuclear expression of CREB-PSer133 in activated stellate cells is stimulated by the elevation of cAMP or by Ca^{2+} mobilization. Given the results of this study, we expect that stellate cell proliferation will be facilitated by a targeted disruption of the CREB gene (39). In contrast to CREB-PSer133, CREB phosphorylation on Ser 119 seems to be required for T cell activation and cell-cycle progression (40).

Although little is known about the mechanisms by which CREB/CREB-PSer133 affects the cell cycle, CREB/CREB-PSer133 function can be modulated by CREB binding protein (CBP) (41, 42). Recent studies indicate that CBP binding affinity can be regulated by site-specific phosphorylations mediated by PKA (16) and p90^{rsk} (43). The precise molecular interaction of CREB/CREB-PSer133 with CBP and other transcription factors is poorly understood. However, Kamei et al. (44) have proposed that CBP could act as a key integrator of cellular functions by selectively interacting with different transcription factors. For example, CBP is known to bind preferentially to the oncoproteins *c-myc* and *c-jun*, or to the cell-cycle arresting factors RARs (retinoic acid receptors) and CREB, according to the biological conditions (41–45). Taken together, these results suggest the possibility that CREB/CREB-PSer133 could compete with *c-myc* for interaction with CBP (45), thereby af-

fecting the role of *c-myb* in the activation of stellate cells (11). Likewise, the important role of retinoids on quiescent stellate cell phenotype (7, 9) could be related to their activation of RARs and the potential facilitation of the interaction between CBP and CREB-P_{Ser133} (41, 42). Alternatively or in addition, the role of cAMP may reflect its modulation of the retinoic acid-dependent RAR's transcription, given the presence of CREB binding sites on the RAR- β_2 promoter (46).

In this study, we have identified a novel function of CREB-P_{Ser133} as a regulator of the cell cycle in highly differentiated cells. A similar inhibitory role on the cell cycle has also been found for other transcription factors such as MyoD, C/EBP α , and LAP (21, 47, 48), known to induce differentiated phenotypes in skeletal muscle, adipocytes, and hepatocytes (49). However, in poorly differentiated cell lines, such as erythroleukemia K562 and pheochromocytoma PC-12, growth factors activate the RSK2 kinase which induces phosphorylation of CREB on Ser 133 (50), indicating that under certain growth conditions, CREB-P_{Ser133} is not sufficient to block cell cycle progression, as suggested by our results with oxidative stress (Fig. 7). In summary, our study provides insights into the molecular mechanisms modulating hepatic stellate cell cycle, as well as a rationale for potential therapeutic approaches for hepatic fibrosis.

Acknowledgments

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References

- Martinez-Hernandez, A. 1985. The hepatic extracellular matrix. II. Electron immunohistochemical studies in rats with CCl₄-induced cirrhosis. *Lab. Invest.* 53:166-186.
- Clément, B., J.A. Grimaud, J.P. Campion, Y. Deugnier, and A. Guilouzo. 1986. Cell types involved in collagen and fibronectin production in normal and fibrotic human liver. *Hepatology.* 6:225-234.
- Chojkier, M. 1993. Pathogenesis of hepatic fibrosis. In *Extracellular Matrix*. M. Zern and L. Reid, editors. Marcel Dekker Inc., New York. 541-557.
- Friedman, S., F. Roll, J. Boyles, and D. Bissell. 1985. Hepatic stellate cells: the principal collagen-producing cells of the liver. *Proc. Natl. Acad. Sci. USA.* 82:8681-8685.
- Maher, J.J., and R.F. McGuire. 1990. Extracellular matrix gene expression increases preferentially in rat stellate cells and sinusoidal endothelial cells during hepatic fibrosis in vivo. *J. Clin. Invest.* 86:1641-1648.
- Brenner, D.A., J.M. Alcorn, H.L. Leffert, S.P. Feitelberg, and M. Chojkier. 1990. Expression of collagen genes in the liver. *Mol. Biol. Med.* 7:105-115.
- Davis, B.H., B.M. Pratt, and J.A. Madri. 1987. Retinol and extracellular collagen matrices modulate hepatic Ito cell collagen phenotype and cellular retinol binding protein levels. *J. Biol. Chem.* 262:10280-10286.
- Friedman, S.L., F.J. Roll, J. Boyles, D.M. Aronson, and D.M. Bissell. 1989. Maintenance of differentiated phenotype of cultured rat hepatic stellate cells by basement membrane matrix. *J. Biol. Chem.* 264:10756-10762.
- Geerts, A., P. De Bleser, M.L. Hautekeete, T. Niki, and E. Wisse. 1994. Fat-storing (Ito) cell biology. In *The Liver: Biology and Pathobiology*. I.M. Arias, J.L. Boyer, N. Fausto, W.B. Jakoby, D.A. Schachter, and D.A. Shafritz, editors. Raven Press, Ltd., New York. 819-838.

- Pinzani, M., L. Gesulado, G. Sabbah, and H. Abboud. 1989. Effects of platelet derived growth factor and other polypeptide mitogens on DNA synthesis and growth of cultured rat liver fat storing cells. *J. Clin. Invest.* 84:1786-1793.
- Lee, K.S., M. Buck, K. Houglum, and M. Chojkier. 1995. Activation of hepatic stellate cells by TGF α and collagen type I is mediated by oxidative stress through *c-myb* expression. *J. Clin. Invest.* 96:2461-2468.
- Mallat, A., L. Fouassier, A.-M. Préaux, C. Serradeil-Le Gal, D. Rautaste, J. Rosenbaum, D. Dhumeaux, C. Jouneaux, P. Mavier, and S. Lotersztajn. 1995. Growth inhibitory properties of endothelin-1 in human hepatic myofibroblastic Ito cells. *J. Clin. Invest.* 96:42-49.
- Gamet, L., J.-C. Murat, A. Remaury, C. Remesy, P. Valet, H. Paris, and C. Denis-Pouxviel. 1992. Vasoactive intestinal peptide and forskolin regulate proliferation of the HT29 human colon adenocarcinoma cell line. *J. Cell. Physiol.* 150:501-509.
- Verheijen, M.H.G., and L.H.K. Defize. 1995. Parathyroid hormone inhibits mitogen-activated protein kinase activation in osteosarcoma cells via a protein kinase A-dependent pathway. *Endocrinology.* 136:3331-3337.
- Yokozaki, H., G. Tortora, S. Pepe, E. Maronde, H.-G. Genieser, B. Jastorff, and Y.S. Cho-Chung. 1992. Unhydrolyzable analogues of adenosine 3':5'-monophosphate demonstrating growth inhibition and differentiation in human cancer cells. *Cancer Res.* 52:2504-2508.
- Brindle, P., T. Nakajima, and M. Montminy. 1995. Multiple protein kinase A-regulated events are required for transcriptional induction by cAMP. *Proc. Natl. Acad. Sci. USA.* 92:10521-10525.
- Sheng, M., M.A. Thompson, and M.E. Greenberg. 1991. CREB: a Ca²⁺-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science (Wash. DC).* 252:1427-1430.
- Liu, F., M.A. Thompson, S. Wagner, M.E. Greenberg, and M.R. Green. 1993. Activating transcription factor-1 can mediate Ca²⁺- and cAMP-inducible transcriptional activation. *J. Biol. Chem.* 268:6714-6720.
- Houglum, K., M. Buck, J. Alcorn, S. Contreras, P. Bornstein, and M. Chojkier. 1995. Two different *cis*-acting regulatory regions direct cell-specific transcription of the collagen $\alpha_1(I)$ gene in hepatic stellate cells and in skin and tendon fibroblasts. *J. Clin. Invest.* 96:2269-2276.
- Bedossa, P., K. Houglum, C. Trautwein, A. Holstege, and M. Chojkier. 1994. Stimulation of collagen $\alpha_1(I)$ gene expression is associated with lipid peroxidation in hepatocellular injury. A link to tissue fibrosis? *Hepatology.* 19:1262-1271.
- Buck, M., H. Turler, and M. Chojkier. 1994. LAP, a tissue-specific transcriptional activator, is an inhibitor of hepatoma cell proliferation. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:851-860.
- Trautwein, C., C. Caelles, P. van der Geer, T. Hunter, M. Karin, and M. Chojkier. 1993. Transactivation by NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature (Lond.)* 364:544-547.
- Buck, M., and M. Chojkier. 1996. Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:1753-1765.
- Flaherty, M., and M. Chojkier. 1986. Selective inhibition of collagen synthesis by the Ca²⁺ ionophore A23187 in cultured human fibroblasts. *J. Biol. Chem.* 261:12060-12065.
- Gonzalez, G., and M.R. Montminy. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell.* 59:675-680.
- Yamamoto, K.K., G.A. Gonzalez, W.H. Biggs III, and M.R. Montminy. 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature (Lond.)* 334:494-498.
- Descombes, P., M. Chojkier, S. Lichtsteiner, E. Falvey, and U. Schibler. 1990. LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* 4:1541-1551.
- Ginty, D.D., J.M. Kornhauser, M.A. Thompson, H. Bading, K.E. Mayo, J.S. Takahashi, and M.E. Greenberg. 1993. Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock. *Science (Wash. DC).* 260:238-242.
- Bravo, R., R. Frank, P.A. Blundell, and H. MacDonald-Bravo. 1987. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. *Nature (Lond.)* 326:515-517.
- Bravo, R., and H. MacDonald-Bravo. 1987. Existence of two populations of cyclin/proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. *J. Cell Biol.* 105:1549-1554.
- Mellon, P.L., C.H. Clegg, L.A. Correll, and G.S. McKnight. 1989. Regulation of transcription by cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA.* 86:4887-4891.
- Wegner, M., Z. Cao, and M.G. Rosenfeld. 1992. Calcium-regulated phosphorylation within the leucine zipper of C/EBP β . *Science (Wash. DC).* 256:370-373.
- Rasmussen, H., and P.Q. Barrett. 1984. Calcium messenger system: an integrated view. *Physiol. Rev.* 64:938-984.
- Grieco, D., A. Porcellini, E.V. Avvedimento, and M.E. Gottesman. 1996. Requirement for cAMP-PKA pathway activation by M phase-promoting factor in the transition from mitosis to interphase. *Science (Wash. DC).* 271:1718-1723.

35. Berg, R.A., J. Moss, B.J. Baum, and R.G. Crystal. 1981. Regulation of collagen production by the β -adrenergic system. *J. Clin. Invest.* 67:1457-1462.
36. Brenner, D.A., and M. Chojkier. 1987. Acetaldehyde increases collagen gene transcription in cultured human fibroblasts. *J. Biol. Chem.* 262:17690-17695.
37. Chojkier, M., K. Houglum, J. Solis-Herruzo, and D.A. Brenner. 1989. Stimulation of collagen gene expression by ascorbic acid in cultured human fibroblasts. *J. Biol. Chem.* 264:16957-16962.
38. Houglum, K., D.A. Brenner, and M. Chojkier. 1991. Inhibition of collagen gene expression by D- α -tocopherol in cultured human fibroblasts. *J. Clin. Invest.* 87:2230-2235.
39. Maldonado, R., J.A. Blendy, E. Tzavara, P. Grass, B.P. Roques, J. Hannon, and G. Schütz. 1996. Reduction of morphine abstinence in mice with a mutation in the gene encoding CREB. *Science (Wash. DC)*. 273:657-659.
40. Barton, K., N. Muthusamy, M. Chanyangam, C. Fischer, C. Clendenin, and J.M. Leiden. 1996. Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB. *Nature (Lond.)*. 379:81-85.
41. Arias, J., A.S. Alberts, P. Brindle, F.X. Claret, T. Smeal, M. Karin, J. Ferramisco, and M. Montminy. 1994. Activation of cAMP and mitogen responsive genes relies on a common nuclear factor. *Nature (Lond.)*. 370:226-229.
42. Kwok, R.P.S., J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bächinger, R.G. Brennan, S.G.E. Roberts, M.R. Green, and R.H. Goodman. 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature (Lond.)*. 370:223-226.
43. Nakajima, T., A. Fukamizu, J. Takahashi, F.H. Gage, T. Fisher, J. Blenis, and M.R. Montminy. 1996. The signal-dependent coactivator CBP is a nuclear target for pp90^{RSK}. *Cell*. 86:465-474.
44. Kamei, Y., L. Xu, T. Heinzl, J. Torchia, R. Kurokawa, B. Gloss, S.-C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass, and M.G. Rosenfeld. 1996. A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*. 85:403-414.
45. Oelgeschläger, M., R. Janknecht, J. Krieg, S. Schreek, and B. Lüscher. 1996. Interaction of the co-activator CBP with Myb proteins: effects on Myb-specific transactivation and on the cooperativity with NF-M. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:2771-2780.
46. Krutz, F.A.E., G. Folkers, C.E. van den Brink, and P.T. van der Saag. 1992. A cyclic AMP response element is involved in retinoic acid-dependent RAR β_2 promoter activation. *Nucl. Acids Res.* 20:6393-6399.
47. Sorrentino, V., R. Pepperkok, R.L. Davis, W. Ansorge, and L. Philipson. 1990. Cell proliferation inhibited by MyoD1 independently of myogenic differentiation. *Nature (Lond.)*. 345:813-815.
48. Umek, R.M., A.D. Friedman, and S.L. McKnight. 1991. CCAAT-enhancer binding protein: a component of a differentiation switch. *Science (Wash. DC)*. 251:288-291.
49. Chojkier, M. 1995. Regulation of liver-specific gene expression. In *Progress in Liver Diseases*. J.L. Boyer and R.K. Ockner, editors. W.B. Saunders Co., Philadelphia, PA. 37-61.
50. Xing, J., D.D. Ginty, and M.E. Greenberg. 1996. Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. *Science (Wash. DC)*. 273:959-963.