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Role of p38 MAPK and RNA-dependent Protein Kinase (PKR) in Hepatitis C Virus Core-dependent Nuclear Delocalization of Cyclin B1*

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Some hepatitis C virus (HCV) proteins, including core protein, deregulate the cell cycle of infected cells, thereby playing an important role in the viral pathogenesis of HCC. Thus far, there are only few studies that have deeply investigated in depth the effects of the HCV core protein expression on the progression through the G_1/S and G_2/M phases of the cell cycle. To shed light on the molecular mechanisms by which the HCV core protein modulates cell proliferation, we have examined its effects on cell cycle in hepatocarcinoma cells. We show here that HCV core protein perturbs progression through both the G_1/S and the G_2/M phases, by modulating the expression and the activity of several cell cycle regulatory proteins. In particular, our data provided evidence that core-dependent deregulation of the G_1/S phase and its related cyclin-CDK complexes depends upon the ERK1/2 pathway. On the other hand, the viral protein also increases the activity of the cyclin B1-CDK1 complex via the p38 MAPK and JNK pathways. Moreover, we show that HCV core protein promotes nuclear import of cyclin B1, which is affected by the inhibition of both the p38 and the RNA-dependent protein kinase (PKR) activities. The important role of p38 MAPK in regulating G_2/M phase transition has been previously documented. It is becoming clear that PKR has an important role in regulating both the G_1/S and the G_2/M phase, in which it induces M phase arrest. Based on our model, we now show, for the first time, that HCV core expression leads to deregulation of the mitotic checkpoint via a p38/PKR-dependent pathway.

The hepatitis C virus (HCV)[3] infection is a rapidly increasing public health problem, with millions of chronically infected patients to date. It is well known that patients with chronic disease have an increased risk of developing hepatocellular carcinoma (HCC) (1). Despite the research already done in this field, HCV-related mechanisms inducing cell transformation are still incompletely understood. Clinical observations indicate that the increased proliferation rate of hepatocytes is a major risk factor for the development of HCC (2). Several *in vivo* studies on hepatic biopsies reported an imbalance between the G_1 and the S phases of the cell cycle in liver cells obtained from chronic hepatitis C patients (3). Moreover, some studies revealed a G_2/M dysfunction in hepatocarcinoma-derived cells (4). A large body of evidence has pointed to the possibility that HCV pathogenesis is due to virus-mediated disruption of several signal transduction pathways that normally regulate cell proliferation (5–7). However, the lack of appropriate cell culture systems and of suitable animal models that mimic viral propagation in humans has hampered the full understanding of HCV-dependent pathogenic mechanisms. The analysis of HCV gene product(s), responsible for the impairment of cell cycle regulation, has already suggested a main role of HCV core protein in regulating hepatocyte proliferation, making cells susceptible to cellular transformation (8–10). In fact, the ability of HCV core protein to accelerate the entry into S phase (8) and to up-regulate cyclin D1 and E expression has been reported *in vitro* (9).

Cell cycle progression is driven by the sequential activation of cyclin-CDK complexes (cyclin-dependent kinases, CDKs), which are in turn regulated by positive (cyclins) and negative (CDK inhibitory proteins) effectors (11, 12). Several cyclin complexes regulate transition through diverse phases of the cell cycle: cyclin D1, which is expressed in the early G_1 phase and forms complexes with CDK4 or CDK6; cyclin E, which controls the G_1/S transition and associates with CDK2; and the cyclin A-CDK2 complex, which instead predominantly directs DNA synthesis, whereas the cyclin B1-CDK1 complex activity regulates the entry and the exit from mitosis (13). The activities of CDKs are regulated by two classes of CDK inhibitory proteins: the INK4 proteins, which interact with CDK4 and CDK6 and act by preventing the formation of the cyclin-CDK dimers (14), and the other class, which includes $p21^{waf1/cip1}$, $p27^{Kip1}$, and p57 of CDK inhibitors (15).

The regulation of cell cycle depends upon several factors such as growth factors, hormones, and cytokines. These induce the expression of cell cycle proteins, in turn leading to the activation of cascade of intracellular signaling molecules, including mitogen-activated protein kinases (MAPKs). MAPKs are a family of serine/threonine kinases involved in the regulation of a wide range of cellular responses such as cell proliferation (16). Based on structural differences, they are divided into three multimer subfamilies: extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK. It is well known that the activation of ERK1/2 as well as p38 and JNK pathways are involved in regulating cell proliferation and cell cycle progression in several cell lines, including liver-derived cells (17–19). The up-regulation of cyclin D1 expression and retinoblastoma protein phosphoryla-

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[3] The abbreviations used are: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; PKR, RNA-dependent protein kinase; FBS, fetal bovine serum; siRNA, small interfering RNA; TRITC, tetramethylrhodamine isothiocyanate; DAPI, 4′,6-diamidino-2-phenylindole; Rb, retinoblastoma; CDK, cyclin-dependent kinase; HA, hemagglutinin.

tion is one of the mechanisms by which MAPKs proteins induce hepatocyte proliferation (20, 21); however, their possible effects on all other cell cycle-related proteins are still under investigation. In an inducible expression system, the HCV core protein was shown to increase the activation of MAPK molecules (22). However, how and which of these MAPK enzymes is involved in HCV core-mediated deregulation of G_1/S and/or G_2/M progression is unknown. Interestingly enough, the protein kinase PKR has been implicated in the deregulation of mitosis driven by HCV core protein (23). The best known activity of PKR is its relationship with the extent of viral infection and its association with double-stranded RNA activation, a property that has led to its designation as an "interferon-inducible RNA-dependent protein kinase." By contrast, the molecular mechanisms by which PKR regulates cell cycle progression remain uncharacterized thus far.

In this work, we have analyzed the changes in the pattern of cell cycle regulators in stable HCC cell lines expressing HCV core protein. Previously reported observations about HCV core protein effects on the cell cycle are incomplete and controversial (24, 25). We show here that in our model, the presence of the viral protein undoubtedly not only impairs the G_1/S transition via ERK1/ERK2 but also increases the cyclin B1-CDK1 activity, causing a prolonged nuclear translocation of the mitotic complex in a p38 MAPK- and PKR-dependent manner.

EXPERIMENTAL PROCEDURES

Cell Culture—The HepG2 cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Polyclonal cell lines were obtained by pooling individual clones of transfected and Geneticin-selected cells stable expressing the wild type genotype 1aWT core protein (23). Cells were grown in 10-cm dishes to ~90% confluence before being placed in 0.5% serum medium for 48 h. Serum-deprived cells were replated in complete medium containing 10% FBS and then cultured for different time points, as indicated in the figure legends.

RNA Interference—The sequence of small interfering RNA (siRNA), selected for the sequence of HCV core cDNA (5′-GAUCGUUGGUGGAGUUUACUU-3′), purchased from MWG-Biotech, was transfected using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's protocol.

Synthesis of DNA—For the DNA synthesis assay, cells were incubated with 10 μCi of [^{3}H]thymidine (PerkinElmer Life Sciences, 82.4 Ci/mM) for 2 h, and then DNAs were precipitated in 5% trichloroacetic acid for 30 min at 4 °C, and finally, cells were lysed with 0.3 M NaOH for 1 h at the same temperature. Extracts were collected, and the measurement of thymidine incorporation by liquid scintillation spectrometry was performed. Data points were obtained in duplicate, and experiments were repeated at least three times.

MAPK Inhibitor Treatment—Cells were pretreated with 10 μM SB203580 (Sigma), 30 μM PD98059 (Sigma), and 10 μM SP600125 (Sigma) for 30 min and restimulated with FBS at different times (0, 1, and 18 h). Then, cells were prepared for thymidine incorporation, for determining of CDK complex activity, and finally, for immunofluorescent staining.

Western Blot Analysis and Antibodies—Cells were collected and lysed with radioimmune precipitation assay buffer containing 50 mM Tris (pH 7.5), 100 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin and phosphatase inhibitors. Protein concentrations were measured using a colorimetric assay (Bio-Rad). Equal amounts of proteins were electrophoresed on SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore), treated with specific primary antibodies, and incubated overnight at 4 °C. Then, filters were washed four times with phosphate-buffered saline-Tween 20 and newly incubated with peroxidase-coupled secondary antibodies for 1 h at room temperature. After incubation, the sheets were visualized by ECL kits (Amersham Biosciences). Antibodies directed against cell cycle proteins, p38, phospho-p38, and JNK were purchased from Santa Cruz Biotechnology, Inc. Anti-ERK1/2, anti-activated-ERK1/2, and anti-activated-JNK were purchased from Sigma.

Determination of Cdk Complex Activity—All cell lysates were prepared using a buffer containing 50 mM Tris, pH 7.4, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 1% Triton, 0.1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, and 1 mM sodium orthovanadate. 500 μg of cell extract were incubated overnight at 4 °C with 6 μg of anti-cyclin D1, E, A, B1, CDK4, CDK2, or CDK1 antibodies (Santa Cruz Biotechnology) and protein A-Agarose (Roche Applied Science) beads (26). The samples were resuspended in kinase reaction buffer supplemented with 20 μM ATP, 3 μg of histone H1 (Roche Applied Science), or 5 μg of glutathione *S*-transferase-Rb (Santa Cruz Biotechnology) and 5–10 μCi of [γ-^{32}P]ATP (Amersham Biosciences 3,000 Ci/mM). After 25 min of incubation at 30 °C, the reaction was terminated by adding 10 μl of Sample buffer, and the samples were resolved on 10% SDS-PAGE, blotted on polyvinylidene difluoride membrane, and analyzed by autoradiography.

Immunofluorescence Staining—Cells were plated onto coverslips, fixed with 3% paraformaldehyde, 2% sucrose in phosphate-buffered saline for 20 min, and permeabilized with ice-cold methanol for 3 min. After the blocking reaction with 5% bovine serum albumin for 20 min at 25 °C, the cells were incubated with different primary antibodies: mouse monoclonal cyclin B1 antibody, rabbit polyclonal CDK1, and PKR antibodies (Santa Cruz Biotechnology). Bound primary antibodies were revealed due to the incubation period of 45 min at room temperature with two secondary antibodies (TRITC-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit, Sigma). Finally, the slides were mounted in Vectashield (Vector Laboratories) in the presence of 0.2 μg/ml 4,6-diamino-2-phenylindole (DAPI) for 1 min, washed three times, and examined with a fluorescence microscope (Nikon).

Statistical Analysis—Phosphorylated proteins were quantified by Phoretix 1D software. The values of the figures are means ± S.D. Data of cyclin-CDK complex activities were analyzed by Student's *t* test. A p value less than 0.05 was considered statistically significant.

RESULTS

HCV Core Protein Up-regulates the Expression and the Activity of All Cyclin-CDK Complexes—To gather further insight into the growth properties of core-expressing cells, we have generated HepG2 polyclonal cell lines. These cells were used to characterize the expression and the activity of genes involved in regulating cell cycle progression. HepG2 polyclonal cells were synchronized by starvation for 48 h with 0.5% FBS, and a time course of DNA synthesis (0, 6, 12, 18, 24, and 30 h) was performed. Cells expressing HCV core protein displayed a higher peak of DNA synthesis, between 12 and 18 h, when compared with the control (Fig. 1*a*). As can be seen, the extent of expression of HCV core protein, measured at 0, 18, and 30 h after serum starvation, was constant all along the study (Fig. 1*b*) in our experimental conditions.

Rb or histone H1 kinase assays were next performed to directly analyze the activity of cyclin-CDK complexes involved in either G_1/S or G_2/M transitions at 0, 12, 18 ,and 24 h. Cell extracts were obtained from synchronized polyclonal HCV core and control cells. In our model,

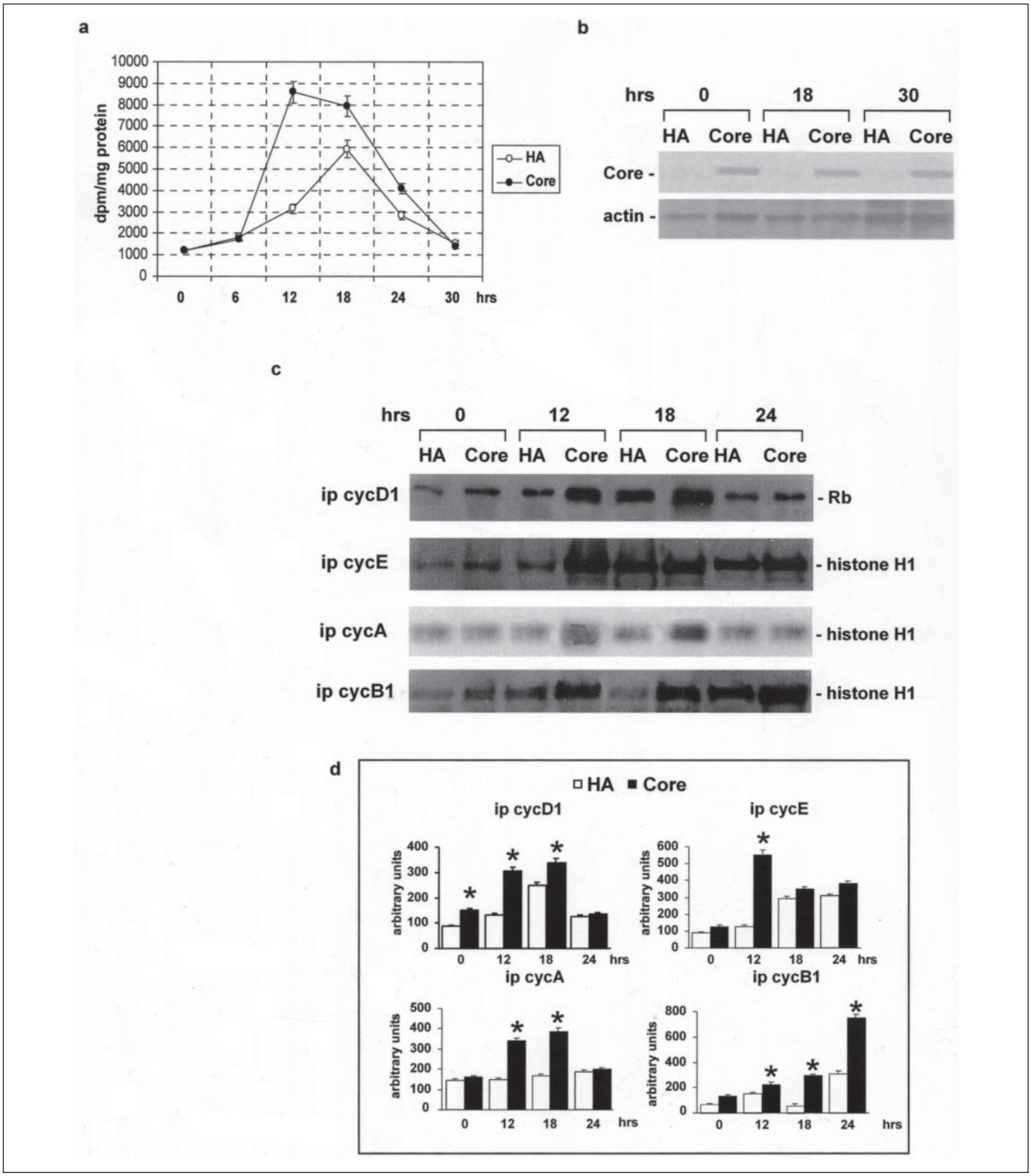

FIGURE 1. **The HCV core protein increases DNA synthesis and modifies the activity of all cell cycle-related proteins in HepG2 polyclonal cells.** *a*, HepG2 polyclonal cell lines, control (hemagglutinin (*HA*)), and core-expressing (*Core*) were synchronized and subjected to DNA synthesis analysis at the indicated time points (0, 6, 12, 18, 24, and 30 h), as described under "Experimental Procedures." The data expressed as dpm/mg of protein are the mean value of three different experiments, each performed in duplicate. S.E. does not exceed 10%. *b*, Western blotting of the HCV core protein at 0, 18, and 30 h. *c*, the cell extracts were used to perform kinase assay at 0, 12, 18, and 24 h. The cyclin D1-CDK4 complex activity was evaluated by the Rb phosphorylation, whereas the activities of the cyclin E/A-CDK2 and cyclin B1-CDK1 complexes were evaluated by the histone H1 phosphorylation. The immunoblots are representative of three different experiments. *ip*, immunoprecipitation. *d*, histograms represent densitometric analysis by Phoretix 1D software. The data, expressed as arbitrary units (means ± S.D.), were obtained from three different experiments. *, $p < 0.001$; **, $p < 0.01$ *versus* control HA; Student's *t* test was performed.

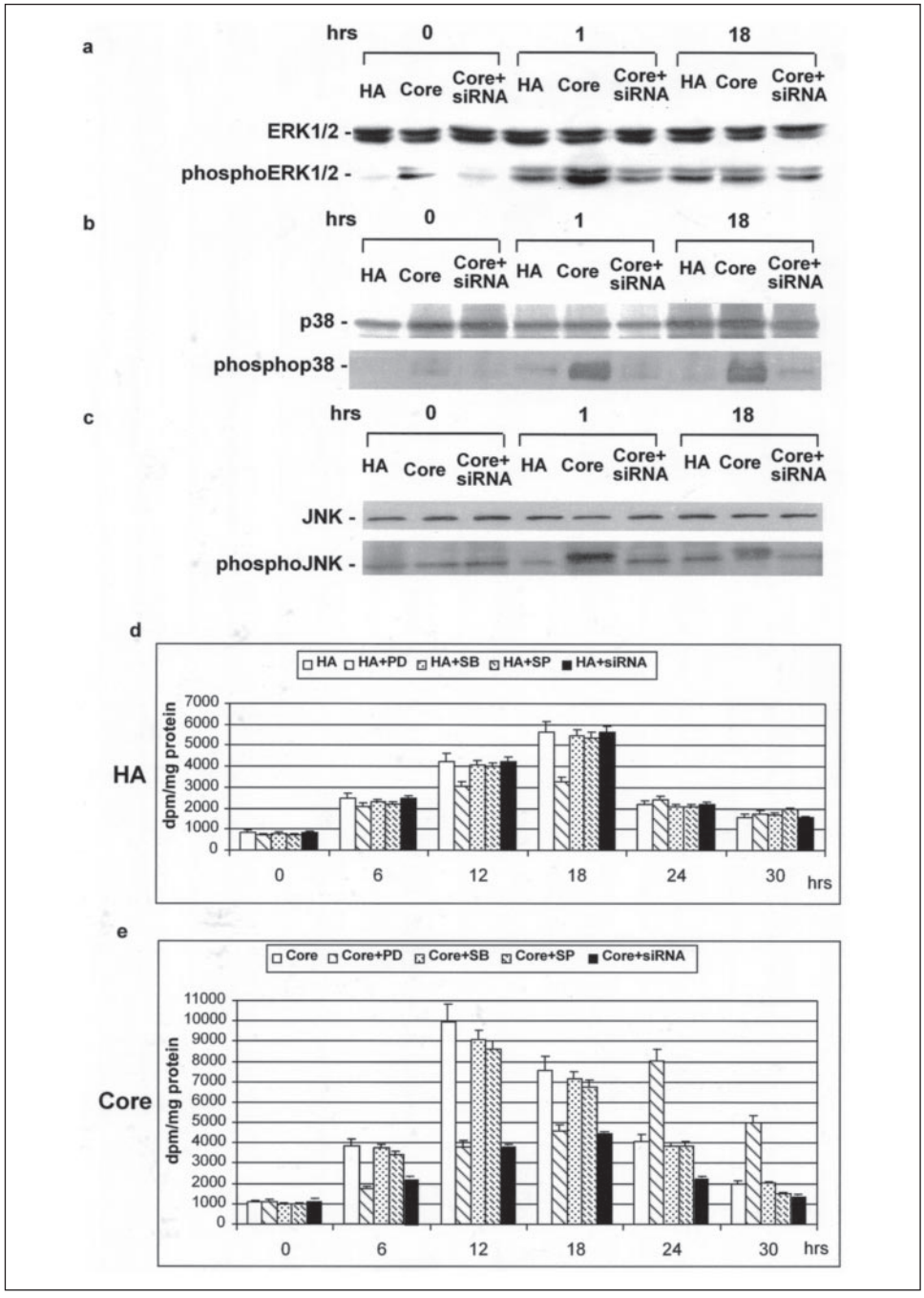

FIGURE 2. **The effects of HCV core protein on DNA synthesis require ERK1/2 activity.** *a*, the expression levels of ERKs (*upper panel*) and the activated forms of ERKs at 0, 1, and 18 h (*lower panel*) were assayed by immunoblotting analysis of total cell extracts from HCV core-positive cells transfected or not with siRNA core (5 μM). *b*, the expression levels of p38 MAPK (*upper panel*) and activated forms of p38 MAPK (*lower panel*), at different time points (0, 1, and 18 h), were assayed by immunoblotting analysis of total cell extracts from the HCV core-expressing (*Core*) cells transfected or not with siRNA core (5 μM). *c*, the expression levels of JNK (*upper panel*) and activated forms of JNK (*lower panel*), at different time points (0, 1, 18 h), were assayed by immunoblotting analysis of total cell extracts from HCV core-positive cells transfected or not with siRNA core (5 μM). *d* and *e*, the histograms represent the thymidine incorporation after PD98059 (*PD*, (0 μM), SB203580 (*SB*, 10 μM), SP600125 (*SP*, 10 μM) pretreatment, and siRNA core (5 μM) transfection of the control and the HCV core-expressing polyclonal cells. The values are mean ± S.E. (<10%) of three independent experiments.

when HCV core protein was expressed, the activities of all cyclin-CDK complexes were increased but especially that of the cyclin B1-CDK1 complex, which peaked at 24 h (Fig. 1, *c* and *d*). Fig. 1*d* represents the data, expressed in -fold, and shows the data obtained by densitometric analysis of cyclin-CDK complexes in the presence of HCV core protein, expressed as -fold of activation. All this information, taken together, demonstrates that HCV core protein induces modifications of the activity of cyclin-CDK complexes and indicates that hepatoma cells, chronically expressing the HCV core protein, display a higher activity of all cyclin-CDK complexes.

The ERK1/ERK2 Activity Correlates with HCV Core Protein Effects on Cell Growth—The three best characterized mammalian proteins are MAPKs, ERK1/2, p38 MAPK, and JNK, all of which are known to play an important role in coordinating a variety of cellular processes including growth, differentiation, and in some cases, apoptosis. We then assessed whether the stable expression of HCV core protein is able to activate MAPK intracellular signaling pathways and whether this phenomenon correlates with the increased rate of DNA synthesis. Western blot analysis of activated forms of MAPKs demonstrated that their activity was increased, whereas their expression levels remained unchanged in HCV core-expressing cells (Fig. 2, *a–c*). To examine whether and which of the three different MAPK-dependent pathways is essential in promoting the HCV core-dependent pro-proliferative effects, we incubated the starved HCV core-expressing polyclones for 30 min with inhibitors of ERK1/2 (30 μM PD98059), of p38 MAPK (10 μM SB203580), or of JNK (10 μM SP600125). Furthermore, to establish that the effects on MAPKs were, indeed, HCV core protein-dependent, we transfected our polyclones with small inhibitor RNA specific for this protein (Fig. 2, *a–c*).

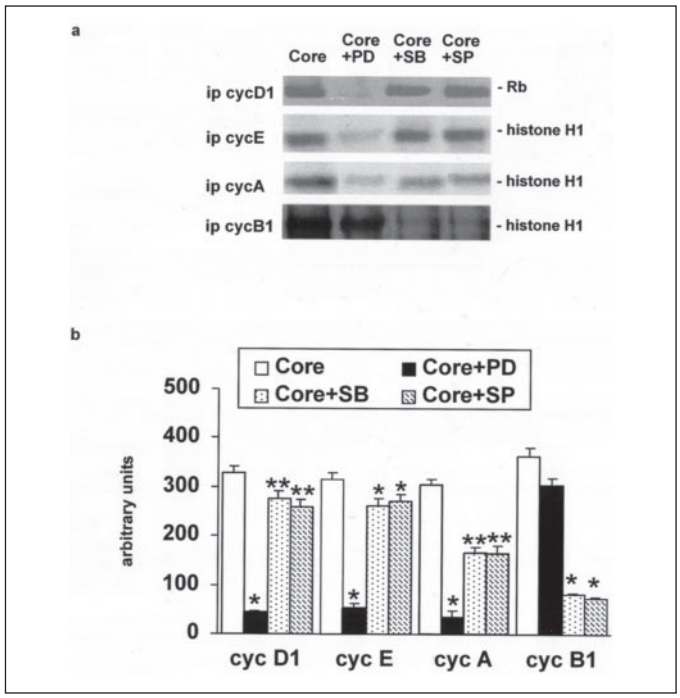

FIGURE 3. **The HCV core protein modulates the G_1/S and G_2/M transition via ERKs or p38/JNK pathways, respectively.** *a*, the cell extracts of the core-expressing (*Core*) HepG2 polyclones (in the absence or in the presence of pretreatment with inhibitors of MAPKs; see "Experimental Procedures") were used to perform the kinase assays at 18 h. The cyclin (*cyc*) D1-CDK4 complex activity was evaluated by the Rb phosphorylation, whereas the activities of the cyclin E/A-CDK2 and the cyclin B1-CDK1 complexes were evaluated by histone H1 phosphorylation. The immunoblots are representative of three different experiments. *ip*, immunoprecipitation; *PD*, PD98059; *SB*, SB203580; *SP*, SP600125. *b*, the histograms represent densitometric analysis by Phoretix 1D software. The data, expressed as arbitrary units (means ± S.D.), were obtained from three different experiments. *, $p < 0.001$; **, $p < 0.01$ *versus* control core; Student's *t* test was performed.

MAPK inhibitor-pretreated cells and cells transfected with HCV core-specific small inhibitor RNA were restimulated with 10% FBS, and the incorporation of methyl-[^{3}H]thymidine incorporation was then measured at different time points (0, 6, 12, 18, 24, and 30 h). As shown in Fig. 2, *d* and *e*, the pretreatment with the ERK1/ERK2 inhibitor caused an evident delay (from 12 to 24 h) in the rate of DNA synthesis rate, whereas no significant effect on HCV core-expressing cells was noted in the presence of p38 MAPK and JNK inhibitors. All of the inhibitory effects were lost after 18 h, probably due to a time-dependent decline in the activity of the MAPK inhibitors (Fig. 2, *d* and *e*). Finally, Fig. 2, *d* and *e*, show that the pro-proliferative effects were, indeed, due to the presence of the core protein. Our findings indicate that the effects of HCV core protein on DNA synthesis depend essentially on ERK1/ERK2.

HCV Core Modulates G_1/S and G_2/M Transition via ERK1/ERK2 or p38 MAPK/JNK, Respectively—To better understand the functional role of each member of the MAPK family in HCV core-dependent cell cycle alterations, we investigated the impact of specific MAPK inhibition on the activity of cyclin-CDK complexes involved in the control of G_1/S and G_2/M transition. To this end, we performed Rb and histone H1 kinase assays in our HCV core-positive cells starved and pretreated with 30 μM PD98059, 10 μM SB203580, or 10 μM SP600125. The activity of cyclin-CDK complexes was examined 18 h after the serum replacement. As demonstrated by data shown in Fig. 3, the pretreatment of polyclones with PD98059 induced a significant down-regulation of the activity of the cyclin D1-CDK4 and cyclin E/A-CDK2 complexes, which essentially regulate G_1/S transition, whereas the activity of the mitotic complex cyclin B1-CDK1 was uninfluenced. Interestingly enough, the pretreatment with SB203580 and SP600125 does not influence the activity of the cyclin D1-CDK4 and cyclin E-CDK2 complexes but provokes a weak decrease of cyclin A-CDK2 and a significant down-regulation in the cyclin B1-CDK1 complex activities (Fig. 3, *a* and *b*). Our results confirm that Raf-1/MAPKs pathways could be necessary for HCV core protein induction of cell growth and highlight the fact that p38 MAPK and JNK pathways are relevant for HCV core-dependent effect on G_2/M transition.

HCV Core-dependent Cyclin B1-CDK1 Complex Delocalization Is Mediated by p38 MAPK—We further investigated whether HCV core could also affect the intracellular localization of the cyclin B1-CDK1 complex. As shown in Fig. 4*a*, in the HCV core-expressing cells, 1 h after the serum replacement, the cyclin B1 expression was up-regulated and the protein was localized in the perinuclear region, whereas CDK1 was up-regulated and delocalized in the nucleus. After 18 h, a time at which cells accumulate in G_2/M phase (data not shown; 15–20% of the HCV core-positive cells accumulated in G_2/M phase at 18 h), HCV core induced a significant nuclear import of cyclin B1 as well. On the other hand, in the control cells, at the same time point (18 h), cyclin B1 and CDK1 were weakly visible in the perinuclear region.

To understand whether MAPKs were involved in cyclin B1-CDK1 complex delocalization, we performed an immunofluorescent assay after pretreatment of cells with all the three MAPK inhibitors. As shown in Fig. 4*b*, only the p38 inhibitor impairs nuclear import of cyclin B1 and CDK1.

Finally, we asked whether this phenomenon could be related to a direct interaction between cyclin B1 and p38 MAPK in HCV core-positive HepG2 cells. With this aim, we performed an immunoprecipitation assay with an anti-cyclin B1 rabbit polyclonal antibody, and the resulting immunoprecipitates were then blotted with an anti-p38 MAPK antibody. As shown in Fig. 4*c*, cyclin B1 was able to coimmunoprecipitate with p38. Finally, Fig. 4*d* shows the perinuclear co-localization of p38 and cyclin B1, demonstrated by confocal microscopy after immunofluorescent double labeling assay. Thus, our data suggest that HCV core protein could influence the correct exit from mitosis, inducing a significant and prolonged nuclear import of cyclin B1-CDK1 complex, which is directly p38 MAPK-related.

P38 and PKR Cooperate to Induce Nuclear Import of cyclin B1-CDK1 Complex in HCV Core-positive Cells—It has been recently demonstrated that PKR interacts with and activates a member of the MAPKs family, p38 MAPK (27). In addition, we have already highlighted the role of PKR in determining a G_2/M accumulation in cells stably expressing HCV core protein (23). Thus, we studied the potential role of PKR in HCV core-related nuclear import of cyclin B1 and the relationship of this kinase with p38 MAPK. With this aim, we have transfected a dominant-negative plasmid (pcDNA-K296R) of the catalytic domain of PKR (23) into our HCV core-positive cells, and the localization of cyclin B1 and CDK1 was examined by immunofluorescence. As demonstrated in Fig. 5, the lack of the activity of PKR hampers the nuclear translocation of cyclin B1 and CDK1. These results clearly indicate that PKR plays an important role in regulating the cellular distribution of the cyclin B1-CDK1 complex in the presence of HCV core protein. Considering that both PKR and p38 MAPK seem to be implicated in HCV core-related nuclear localization of cyclin B1, two additional sets of experiments were performed. First, we investigated to see whether a relationship between p38 MAPK and PKR does exist. As shown in Fig. 6*a*, PKR strongly interacts with p38 MAPK in HCV core-expressing cells. Second, we asked whether cyclin B1 interacts with PKR, as well as with p38 MAPK (Fig. 4*c*). The analysis of immunoprecipitates obtained with an anti-cyclin B1 antibody clearly demonstrates the existence of a direct interaction between cyclin B1, PKR, and p38 MAPK in HCV core-positive cells (Fig. 6*b*). Interestingly enough, the activity of p38 MAPK

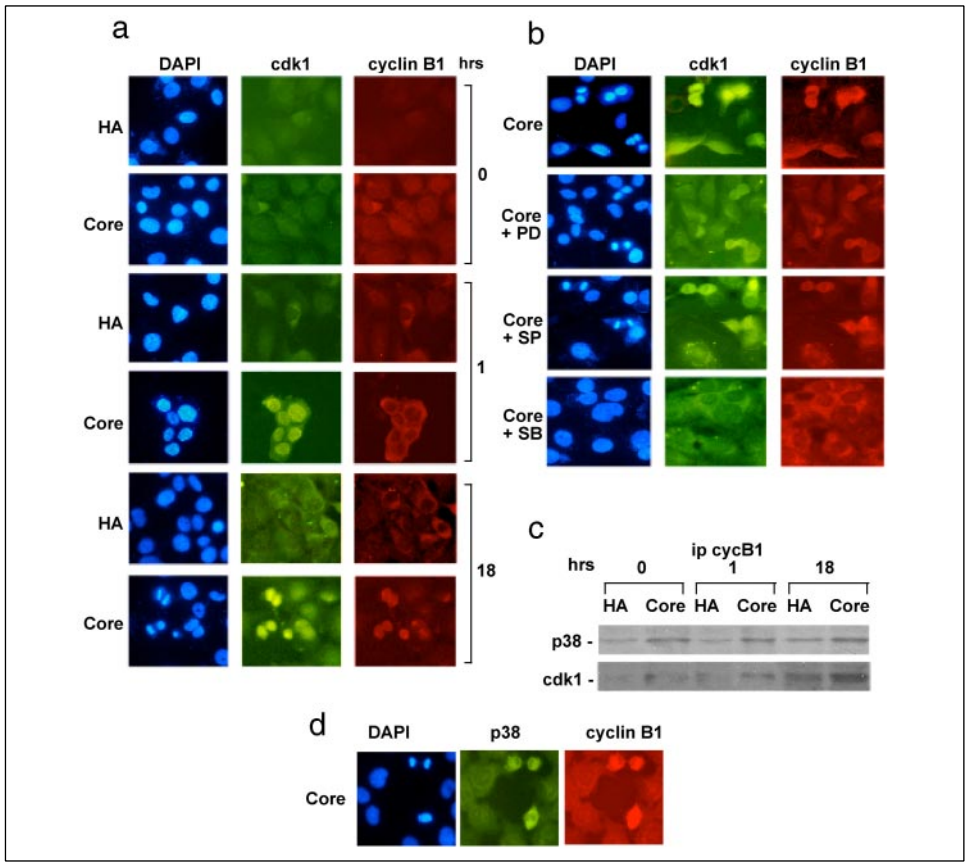

FIGURE 4. **HCV core-dependent nuclear localization of the cyclin B1 and CDK1 is mediated by p38 MAPK.** *a*, the immunofluorescent staining of the cyclin B1 and CDK1 in the control (*HA*) and core-expressing (*Core*) HepG2 polyclones after 0, 1, and 18 h after serum replacement. *Red* and *green* identify the intracellular localization of the cyclin B1 and CDK1, respectively. Simultaneously, the detection of the nuclei was obtained by counterstaining cells with DAPI. *b*, the immunofluorescent staining of the cyclin B1 and CDK1 in core-expressing HepG2 polyclones, pretreated or not with the MAPKs inhibitors, after 18 h from the serum replacement. *Red* identifies the intracellular localization of the cyclin B1. Simultaneously, the detection of the nuclei was obtained by counterstaining cells with DAPI. *PD*, PD98059; *SB*, SB203580; *SP*, SP600125. *c*, the control (*HA*) and core-expressing extracts, after 0, 1, and 18 h after the serum replacement, were immunoprecipitated (*ip*) with an anti-cyclin B1 rabbit polyclonal antibody and revealed with an anti-p38 MAPK antibody. Representative profiles of three independent experiments are reported. *d*, the immunofluorescent staining of the cyclin B1 and p38 MAPK in core-expressing HepG2 polyclones, after 18 h from the serum replacement.

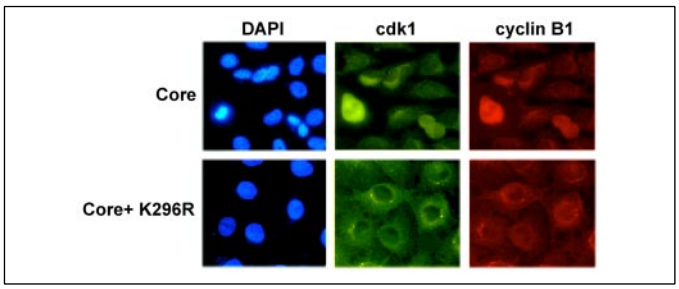

FIGURE 5. **Involvement of PKR in HCV core-dependent nuclear import of cyclin B1-CDK1.** The immunofluorescent staining of the cyclin B1 and CDK1 in the core-expressing (*Core*) HepG2 polyclones, control and transfected cells with the pcDNA-K296R plasmid, at 18 h after the serum replacement, is shown. *Red* and *green* identify the intracellular localization of the cyclin B1 and CDK1, respectively. Simultaneously, the detection of the nuclei was obtained by counterstaining cells with DAPI.

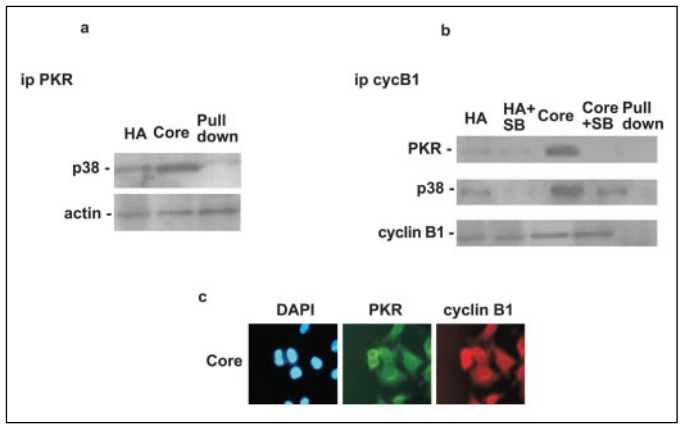

FIGURE 6. **p38 MAPK, PKR, and cyclin B1 interaction in HCV core polyclones.** *a*, the extracts of the control (*HA*) and core-expressing (*Core*) cells, after 18 h from the serum replacement, were immunoprecipitated (*ip*) with an anti-PKR rabbit polyclonal antibody and revealed with an anti-p38 MAPK antibody. α-Actin of the supernatants in the *lower panel* was used as control. The controls for nonspecific pull-down of the proteins are represented. *b*, the extracts of the core-expressing cells, pretreated or not with the p38 MAPK inhibitor after 18 h from the serum replacement, were immunoprecipitated with an anti-cyclin B1 rabbit polyclonal antibody and revealed with anti-PKR and p38 MAPK antibodies. The labeling with anti-cyclin B1 antibody in the *lower panel* was used as positive control. The immunoblots are representative of three different experiments. The controls for nonspecific pull-down of the proteins are represented. *SB*, SB203580. *c*, the immunofluorescent staining of the cyclin B1 and PKR in the core-expressing HepG2 polyclones, after 18 h from the serum replacement.

seems to be necessary for this interaction, which disappeared when our polyclones were pretreated with an inhibitor of p38 activity (shown in Fig. 6*b*). The co-localization between cyclin B1 and PKR was demonstrated by confocal microscopy analysis (Fig. 6*c*).

DISCUSSION

Among HCV carcinogenic proteins, the HCV core protein modulates gene transcription, proliferation, and cellular apoptosis (28). Moreover, it has also been shown to induce hepatic adenomas in transgenic mice. These adenomas typically progress morphologically and biochemically to full malignant HCC (29). In this report, we evaluated the relationships between stable HCV core protein expression and its impact on the different cyclin-CDK complex activities involved in the control of cell growth. We show that HCV core protein expression deeply influences cell proliferation mainly by modulating MAPK pathways: ERK1/2, p38 MAPK, and JNK. First, we demonstrated that HCV core protein increases the number of cells that are engaged in DNA synthesis. In particular, the viral protein causes an increased activity of the cyclin D1-CDK4 and cyclin E-CDK2 complexes involved in the early S and G_1/S transition phase. Moreover, it can also up-regulate the cyclin A-CDK2/CDK1 complex activity, which regulates both the late S and the G_2/M phases. Finally, the cyclin B1-CDK1 complex activity was

clearly increased in core-expressing HepG2 cells and, of most significance, the mitotic complex delocalized into the nucleus in presence of the viral protein. This leads to a deregulation of the mitotic phase; in fact, 15–20% of cells accumulate in G_2/M phase (data not shown).

To be protected from a variety of different types of stress, eukaryotic cells have developed a system of checkpoints that delay progression to the next phase of the cell cycle. A critical evolutionary conserved checkpoint exists at the boundary of the G_2 phase of the cell cycle and mitosis. Chromosome instability is known as the most common form of genetic instability in human malignancies, which can be caused by aberrant chromosomal segregation during mitosis. Cells with persistent chromosomal instability may undergo cell death, but some of them could survive and become malignant clones bearing defective genomic information. These cells can be characterized by gains or losses of whole chromosomes along with eccentric cytologic alterations such as nuclear pleomorphism and multi-/giant-nucleated cells (30). The formation of multinucleated cells was also found to occur in human T-cell lymphotropic virus type I Tax-expressing cells (31) as well as in Epstein-Barr virus EBNA3C-expressing cells (32). It has been suggested that these cells originate from viral interference with the mitotic checkpoint. Interestingly enough, E6 and E7 oncoproteins from the high risk type of human papillomavirus actively induce numerical and structural centrosome abnormalities, increasing the propensity for chromosome missegregation (33). Centrosome abnormalities, such as excess number or variability in shape and size, have been found in human cancer of multiple origins, including HCC (34, 35). All these observations suggest that chromosomal aberrations resulting from aberrant progression of mitosis might be an important step during multistage carcinogenesis.

In our model, the deregulation of mitosis is linked to the phosphorylation state of PKR (23) that, in turn, seems to be related to HCV core-dependent up-regulation of p38 MAPK activity. The presence of activated PKR in association with pathology is particularly intriguing since its activation is typically associated with HCV pathology (36). Originally described in the early cellular response to viral infection, PKR activation is now widely recognized as a component of stress-activated pathways that mobilize somatic cell death programs (37). PKR is an interferon-induced serine-threonine protein kinase that is best known for its activation, by auto- or trans-phosphorylation, upon binding to double-stranded RNA. PKR is also activated by a number of other signals that induce cell stress, including tumor necrosis factor, heat shock, and trophic factor withdrawal via molecular mechanisms that are not yet clear. Once activated, PKR is known to phosphorylate the α-subunit of eukaryotic initiation factor 2 that leads to an inhibition of protein synthesis and eventually to cell death. PKR also mediates cell death programs triggered by upstream activation of the JNK and p38 MAPK pathways (27), both of which have been implicated in the development of HCC in HCV-positive patients. Since PKR has been shown to promote and participate in cell-cycle arrest at G_0/G_1 and G_2/M (38, 39), it can be argued that a relationship between p38 signal transduction, PKR activation, and cell cycle deregulation in HCC does exist. Accordingly, our findings clearly indicated that p38 MAPK, PKR, and cyclin B1 physically interact, and this interaction seems to be essential in determining the effect of HCV core protein in mitosis.

In conclusion, we have demonstrated that HCV core protein expression deeply influences cell cycle progression, causing changes during both the S and the M phases. We have also discovered that the p38 MAPK/PKR-dependent pathway works as a downstream effector of HCV core protein and as a key mediator of its effects on mitosis. Although the role of the mitotic checkpoint and its deregulation during carcinogenesis in humans is only starting to emerge, the data presented here emphasize its importance in liver cancer development.

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Role of p38 MAPK and RNA-dependent Protein Kinase (PKR) in Hepatitis C Virus Core-dependent Nuclear Delocalization of Cyclin B1

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