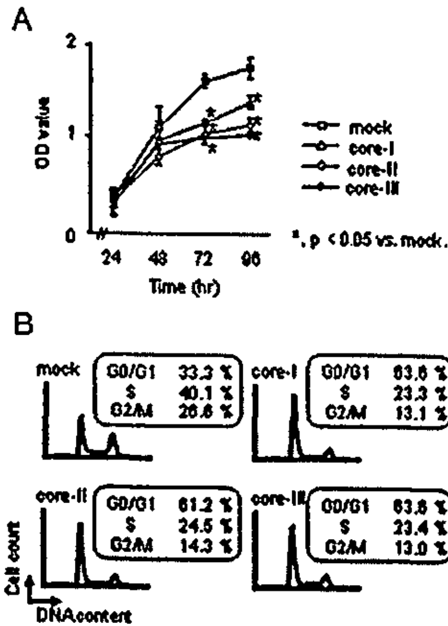

Fig. 1. Cellular proliferation and cell cycle profile in CL2 mock and core cells. *A*, CL2 mock and core cells were seeded on a 96-well culture plate, and viable cells were assessed by the water-soluble tetrazolium colorimetric assay. *B*, CL2 mock and core cells were incubated with propidium iodine and subjected to flow cytometry to examine the cell cycle profile.

translation was conducted using the TNT T3 coupled rabbit reticulocyte lysate system (Promega).

Synthesis for GST Fusion Proteins and Binding Assay—To construct various GST fusion proteins, the *Escherichia coli* BL21 (Stratagene) was transformed with p5GEX/hCDK2, p5GEX/hCDK7, p5GEX/hcycH, p5GEX/hpRB(379–793), p5GEX/core(1–122), pGCTD, and the empty plasmid p5GEX-1. The bacteria were grown in the medium containing 100 μg/ml of ampicillin sodium, and the proteins were expressed by the addition of isopropyl-1-thio-β-D-galactopyranoside. After sonication of the bacterial pellet, the proteins were purified with glutathione-Sepharose 4B beads (Amersham Biosciences). For the binding assay, ~5 μg of the various GST fusion protein or GST protein (negative control) was bound to the beads and incubated with 150 μg of the cellular lysate or 10 μl of *in vitro* translated protein at 4 °C for 2 h. After the extensive washing, the bound protein was used for Western blotting.

Kinase Assay—For the kinase assay, 250–500 μg of the total cellular protein was immunoprecipitated with CDK4, CDK2, and CDK7 antibodies as above. After extensive washing, the precipitate was subjected to the kinase assay in the presence of 12.5 mM MOPS, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM sodium vanadate, 5 mM dithiothreitol, 100 μM ATP, and 10 mCi of [γ-^{32}P]ATP in a total volume of 30 μl. Also added as a substrate was 2 μg of the GST-pRB fusion protein for the CDK4 kinase assay, the histone H1 (Calbiochem) for the CDK2 kinase assay, the GST-CDK2 fusion protein for the CAK assay, or the GST-CTD fusion protein for the CTD kinase assay. The reaction was carried out at 30 °C for 30 min. After the elution, the supernatant was fractionated by SDS-PAGE, and the gel was dried and autoradiographed. As for the CAK assay, the kinase reaction was carried out without [γ-^{32}P]ATP, and the phosphorylated product was detected by Western blotting using a pT^{160}-CDK2 antibody. All kinase assays were carried out with scaling up by 1.3–1.5-fold. Before the kinase reaction, the sample was divided into two tubes, and the remaining product was immunoblotted with the same antibody as that used for the IP reaction as a loading control (data not shown).

Statistical Analysis—Statistical analysis was performed using Student's nonpaired *t* test as appropriate. *p* values less than 0.05 were considered to be statistically significant.

RESULTS

HCV Core Protein Impairs G_1/S Transition in the Cell Cycle through Suppression of CDK4 and CDK2 Activities—Fig. 1*A* shows the cellular growth curve of CL2 mock and core cells. Cell growth was significantly suppressed in the CL2 core cells, compared with the mock cells. Their cell cycle profiles were then assessed by flow cytometry (Fig. 1*B*). In the mock cells, ~30% of the cells were in the G_0/G_1 fraction, whereas in the CL2 core cells, more than 60% of cells were in this phase. The cells representing S and G_2/M phases were decreased by expression of the HCV core. The effect of the HCV core on the E2F-mediated transcription activity was next studied (Fig. 2*A*). In the cotransfection experiment using the CL2 cells, cotransfection of the HCV core-expressing plasmid pc/3EFΔNCTH significantly reduced the E2F-mediated transcription activity, compared with that of the negative control plasmid, pc/3EFpro. Significant reduction in E2F-mediated transcription by HCV core was also seen in the experiment using the CL2 mock and core cells. When the phosphorylation status of pRB was compared between CL2 mock and core cells (Fig. 2*B*), expression of HCV core caused a decrease of the hyperphosphorylated pRB

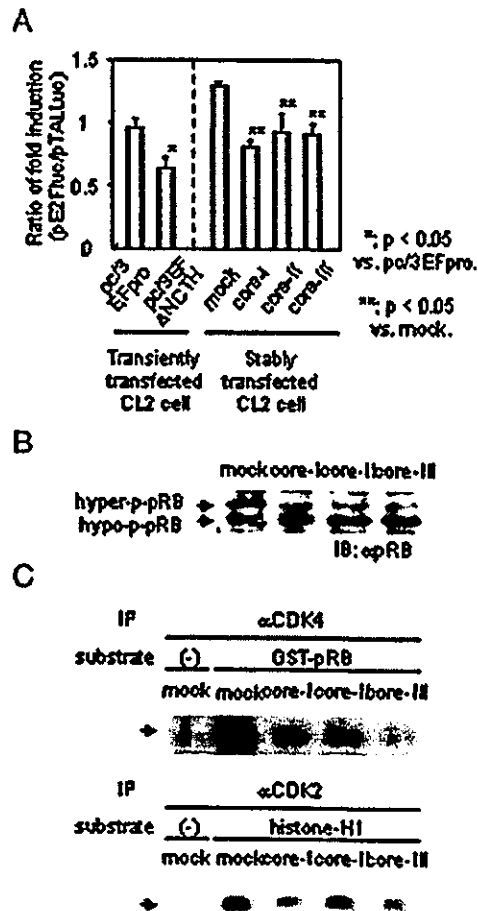

Fig. 2. E2F-mediated transcription, pRB phosphorylation, and CDK4 and CDK2 kinase activities in CL2 mock and core cells. *A*, CL2 cells were cotransfected of the reporter plasmid (pE2Fluc or pTALluc) with the effector plasmid (pc/3EFpro or p/3EFΔNCTH) (*left panel*). For the assay using CL2 mock and core cells, the cells were transfected with the reporter plasmid (pE2Fluc and pTALluc) (*right panel*). The E2F-mediated transcription activity was then assessed by the luciferase assay. The ratio of the fold activity in transfection with the pE2Fluc to that in transfection with pTALluc (pE2Fluc/pTALluc) was regarded as the E2F-mediated transcription activity. *B*, phosphorylation status of pRB was examined in CL2 mock and core cells by Western blotting. *C*, cellular proteins from CL2 mock and core cells were precipitated with a CDK4 or CDK2 antibody, and the precipitate was used for the kinase reaction with the GST-pRB fusion protein (for the CDK4 kinase assay, *upper panel*) or the histone H1 (for the CDK2 kinase assay, *lower panel*) as a substrate. *IB*, immunoblot.

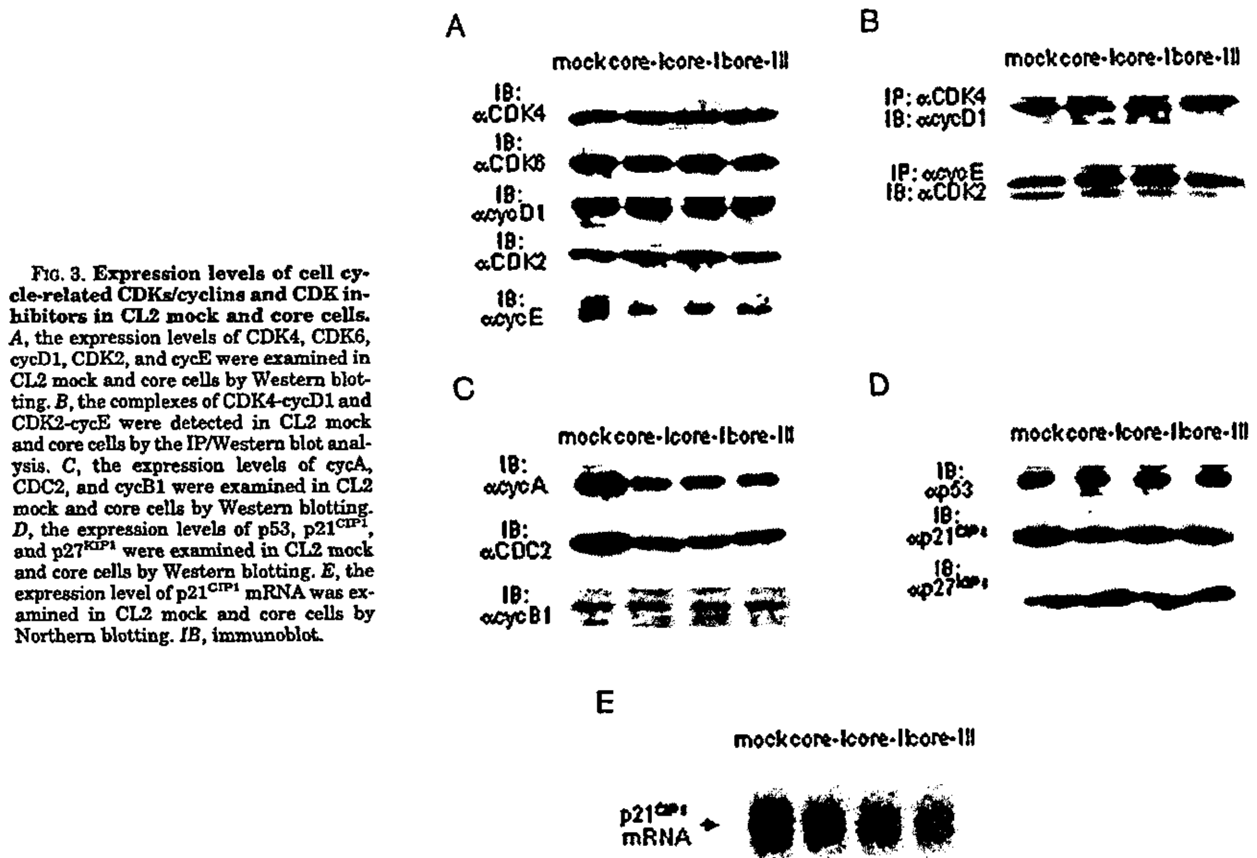

FIG. 3. Expression levels of cell cycle-related CDKs/cyclins and CDK inhibitors in CL2 mock and core cells. *A*, the expression levels of CDK4, CDK6, cycD1, CDK2, and cycE were examined in CL2 mock and core cells by Western blotting. *B*, the complexes of CDK4-cycD1 and CDK2-cycE were detected in CL2 mock and core cells by the IP/Western blot analysis. *C*, the expression levels of cycA, CDC2, and cycB1 were examined in CL2 mock and core cells by Western blotting. *D*, the expression levels of p53, $p21^{CIP1}$, and $p27^{KIP1}$ were examined in CL2 mock and core cells by Western blotting. *E*, the expression level of $p21^{CIP1}$ mRNA was examined in CL2 mock and core cells by Northern blotting. *IB*, immunoblot.

and an increase of the hypo-phosphorylated pRB. As for CDK4 and CDK2 kinase activities (Fig. 2*C*), both kinase activities were substantially lower in the CL2 core cells than in the mock cells. Thus, persistent expression of the HCV core impaired the G_1/S transition in the cell cycle, which may be due to the decreased CDK4 and CDK2 activities and the subsequently occurring inhibition of pRB phosphorylation and E2F-mediated transcription.

HCV Core Protein Does Not Influence the Expression Levels of G_1 Phase-related CDKs, Cyclins, and CDK Inhibitors—The expression levels of various CDKs and cyclins were studied in CL2 mock and core cells. When the G_1 phase-related CDKs and cyclins were examined (Fig. 3*A*), the levels of CDK4, CDK6, cycD1, and CDK2 did not differ between CL2 mock and core cells. Also, the expression level of cycE was not substantially affected by expression of the HCV core. The levels of both CDK4-cycD1 and CDK2-cycE complexes, as examined by the IP/Western blot analysis, were not decreased by HCV core (Fig. 3*B*). As for the expression levels of cycA, CDC2, and cycB1 (Fig. 3*C*), cycA and CDC2 were expressed more weakly in the CL2 core cells than in the mock cells. The cycB1 level did not differ between CL2 mock and core cells. When the expression levels of tumor suppressor p53 and its transcriptional target $p21^{CIP1}$ (29) were examined in CL2 mock and core cells, the p53 expression did not differ between them (Fig. 3*D*, *top panel*). The CL2 core cells showed slightly lower expression levels of $p21^{CIP1}$ mRNA than the mock cells (Fig. 3*E*), although its protein level was not much affected by HCV core (Fig. 3*D*, *middle panel*). Also, the $p27^{KIP1}$ expression was not different between CL2 mock and core cells (Fig. 3*D*, *bottom panel*). When the expression levels of proteins belonging to an INK4 family were determined by Western blot analyses, $p16^{INK4A}$, $p15^{INK4B}$, and $p18^{INK4C}$ were faintly detected in CL2 mock and core cells with no substantial differences between them. The $p19^{INK4D}$ expression did not differ between CL2 mock and core cells (data not shown). According to these results, HCV core suppressed the expression levels of cycA and CDC2, which play a role in the S and G_2/M phases, but did not influence the expression levels of G_1 phase-related CDKs, cyclins, and CDK inhibitors. The reduced expression of cycA and CDC2 may occur as a secondary effect of the decreased E2F-mediated transcription, because these two genes are known to be regulated by E2F (46, 47).

HCV Core Expression Results in Suppression of CAK Assembly and Activity—We further investigated the level of activating phosphorylation in CDK2 and the degrees of CAK expression, assembly, and activity in CL2 mock and core cells. The level of pT^{160}-CDK2 was significantly lower in the CL2 core cells than in the mock cells (Fig. 4*A*). For the expression levels of CAK components, CL2 core cells expressed the same levels of CDK7, cycH, and MAT1 as the mock cells. (Fig. 4*B*). For the complex formation of CAK examined by the IP/Western blot analysis, the level of the CDK7-cycH complex did not differ between CL2 mock and core cells (Fig. 4*C*, *top panel*). When the ternary form of CAK (CDK7-cycH-MAT1 complex) was investigated by means of the IP reaction using the mixture of CDK7 and cycH antibodies followed by the immunoblot using a MAT1 antibody, CL2 core cells displayed a lower level of ternary CAK complex formation than the mock cells (Fig. 4*C*, *bottom panel*). A similar result was also observed in the experiment with the IP reaction using a CDK7 or cycH antibody alone followed by the immunoblot with a MAT1 antibody (data not shown). The kinase assays using the GST-CDK2 substrate (CAK assay) and

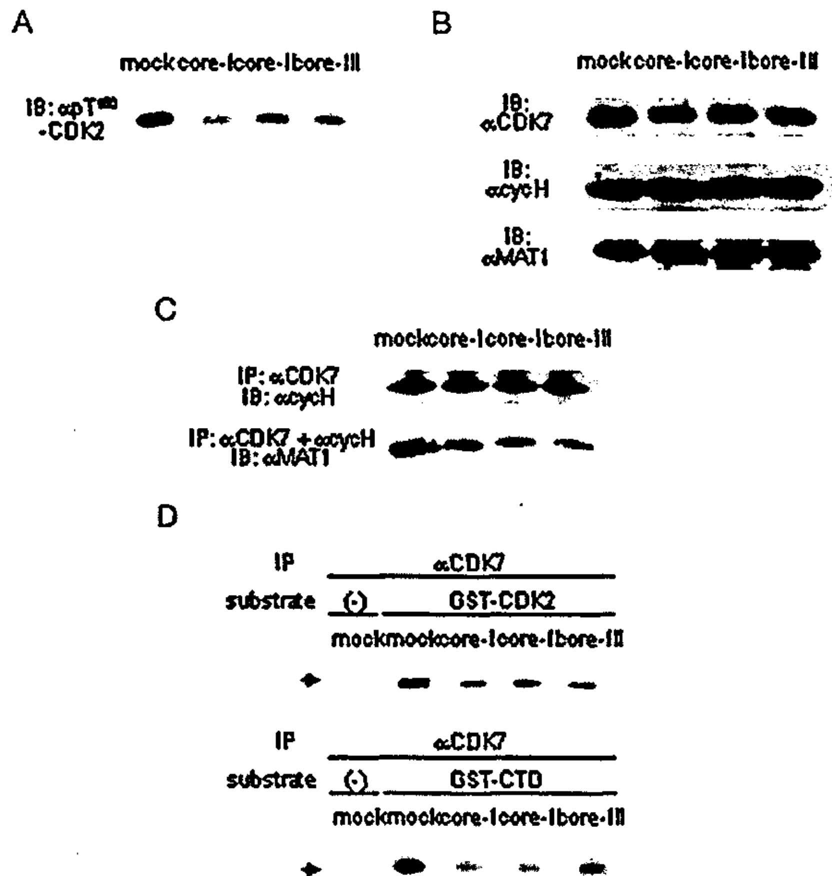

FIG. 4. Expression, assembly, and activity of CAK in CL2 mock and core cells. *A*, cellular proteins from CL2 mock and core cells were blotted with an antibody to pT[160]-CDK2. *B*, the expression levels of CDK7, cycH, and MAT1 were examined in CL2 mock and core cells by Western blotting. *C*, the CDK7-cycH complex and the CDK7-cycH-MAT1 (ternary CAK) complex were detected in CL2 mock and core cells by the IP/Western blot analysis. *D*, cellular proteins from CL2 mock and core cells were precipitated with a CDK7 antibody, and the precipitate was used for the kinase reaction with the GST-CDK2 fusion protein (for the CAK assay, *upper panel*) or the GST-CTD fusion protein (for the CTD kinase assay, *lower panel*) as a substrate. *IB*, immunoblot.

the GST-CTD substrate (CTD kinase assay) were further carried out (Fig. 4*D*). Both CAK and CTD kinase activities were suppressed in the CL2 core cells, compared with the mock cells. These findings indicate that persistent expression of HCV core may inhibit the ternary CAK complex formation, resulting in suppression of CAK activity. We also examined CDC25A expression by Western blotting and the tyrosine-phosphorylated CDK2 level using the IP reaction with a CDK2 antibody, followed by immunoblotting with a phosphorylated tyrosine antibody pY20 in CL2 mock and core cells. The expression level of CDC25A was not different between these cells. The tyrosine-phosphorylated CDK2 was below the detection limit in both CL2 mock and core cells (data not shown). According to these results, HCV core protein may not affect dephosphorylation of CDKs, although the CDC25A activity was not directly assessed in this study.

HCV Core Protein Directly Dissociates MAT1 from CAK Complex and Suppresses CAK Activity—As the next step, the direct effect of HCV core on CAK assembly and activity was investigated *in vitro*. Total cellular proteins from murine CL2 and human HepG2 cells were immunoprecipitated with a CDK7 antibody, followed by the binding reaction of the *in vitro* synthesized HCV core protein (aa 1–191) with the IP product. The *in vitro* translation product from the empty plasmid pCMVtag2B was also used as a negative control. The eluted product was then subjected to immunoblots using CDK7, cycH, and MAT1 antibodies and the CAK assay. As shown in Fig. 5*A*, the addition of the HCV core did not affect the levels of bound CDK7 and cycH but decreased the bound MAT1 level in both cases using CL2 and HepG2 cellular proteins. The CAK activity was also suppressed by the addition of HCV core (Fig. 5*B*). In these assays, the HCV core was detected very faintly in the eluted product by Western blotting (data not shown), indicating that the inhibitory effect on CAK assembly and activity may not have been due to an excess amount of HCV core. Furthermore, when the *in vitro* translated hepatitis B virus surface protein was used for this assay instead of the HCV core, the CAK activity was not suppressed by the addition of this protein (data not shown). Our findings strongly suggest that HCV core may dissociate MAT1 from the CAK complex through possible direct interaction with the particular CAK component, resulting in the suppression of CAK activity.

HCV Core Protein Interacts with CDK7—The direct binding of HCV core to CAK was further studied. The total cellular protein from the CL2 core-I cells was immunoprecipitated with a CDK7, cycH, or MAT1 antibody, and each precipitate was blotted with an antibody to the HCV core (Fig. 6*A*). In all cases using antibodies to CDK7, cycH, and MAT1 on the IP reaction, the three CAK components were precipitated efficiently. Nevertheless, HCV core was detected in the precipitates using CDK7 and cycH antibodies but not in that using a MAT1 antibody. This indicates that the HCV core may be directed against either CDK7 or cycH and that the interaction of the HCV core with the CDK7-cycH complex may induce dissociation of MAT1, as suggested above. To clarify the direct target molecule of HCV core, the *in vitro* GST fusion protein binding assay was carried out. The truncated HCV core protein (aa 1–122) fused to GST was used for this assay, because the full length of the HCV core fusion protein could not be obtained because of poor solubility. Total cellular proteins from murine CL2 and human HepG2 cells were incubated with the purified truncated HCV core protein, and the bound complex was checked by Western blotting using an antibody to CDK7 or cycH. The bound complex included the detectable level of

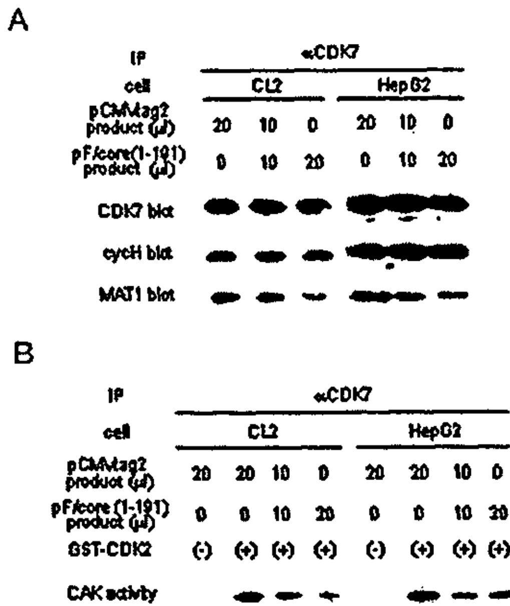

FIG. 5. Direct effect on CAK assembly and activity by HCV core. *A*, cellular proteins from CL2 (*left panel*) and HepG2 (*right panel*) cells were precipitated with a CDK7 antibody. The *in vitro* translation products containing various amounts of HCV core were added to the IP product, followed by the binding reaction. The product was then blotted with antibodies to CDK7, cycH, and MAT1. *B*, cellular proteins from CL2 (*left panel*) and HepG2 (*right panel*) cells were precipitated with a CDK7 antibody, and the precipitate was subjected to the binding reaction with the *in vitro* translation product containing the various amounts of HCV core. Then the sample was used for the CAK assay, as shown in Fig. 4*D*.

CDK7, whereas cycH was not detected in both cases using CL2 and HepG2 cellular proteins (Fig. 6*B*). Furthermore, human CDK7 and cycH fused to GST were synthesized and used for the binding reaction together with the *in vitro* translated full length of the HCV core protein (aa 1–191). HCV core was detected in the bound complex with GST-CDK7 but not in that with GST-cycH (Fig. 6*C*). Taken together, the HCV core protein could bind directly to both murine and human CDK7 proteins, whereas its binding to cycH may be indirect. Also, the region within aa 1–122 of HCV core was important for interaction with CDK7.

HCV Core Protein Does Not Influence Intracellular Localization of CAK—Finally, we investigated whether HCV core would affect the nuclear localization of CAK components. When the expression levels of CDK7, cycH, and MAT1 in the nuclear fraction were examined (Fig. 7*A*), they were not different between CL2 mock and core cells. We also investigated the intracellular localization of HCV core and CDK7 proteins in the CL2 core-I cells by immunostaining. As shown in Fig. 7*B*, the HCV core was expressed mainly in the cytoplasm but was also detected in the nucleus. On the other hand, CDK7 was expressed strongly in the nucleus, although the signal was seen sparsely in the cytoplasm. For the colocalization of both proteins, the HCV core was colocalized with a portion of CDK7 generally in the nucleus. Thus, the HCV core did not influence the nuclear translocation of CAK. The inhibitory effect of the HCV core on CAK may be exerted by its direct interaction with CDK7 in the nucleus.

DISCUSSION

In the present study, we aimed to elucidate the biological properties of the HCV core protein toward cell cycle progression and cell cycle-related molecules in the host cell. The HCV core-expressing stable transfectant, CL2 core, which was established from a murine normal liver-derived cell line (15, 41), was used for this purpose, because cells constitutively expressing the HCV core protein could not be isolated from the human hepatoma-derived cell lines Huh-7 (37), HepG2, and Hep3B (38). These CL2 core cells were shown to possess an only ~2–5-fold higher expression level of HCV core compared with that in the HCV-infected noncancerous liver tissue obtained from a patient with hepatocellular carcinoma who underwent partial hepatectomy (41). The HCV core has been shown to be immunohistochemically detected in a small portion of the hepatocytes of the HCV-infected liver specimen from chronic hepatitis C patients (48), suggesting that, unlike the cultured cells with forced expression of HCV core, the expression level of HCV core may differ among hepatocytes in the HCV-infected liver tissue. According to these, our CL2 core cells were speculated to express the physiological level of HCV core at least in a single cell.

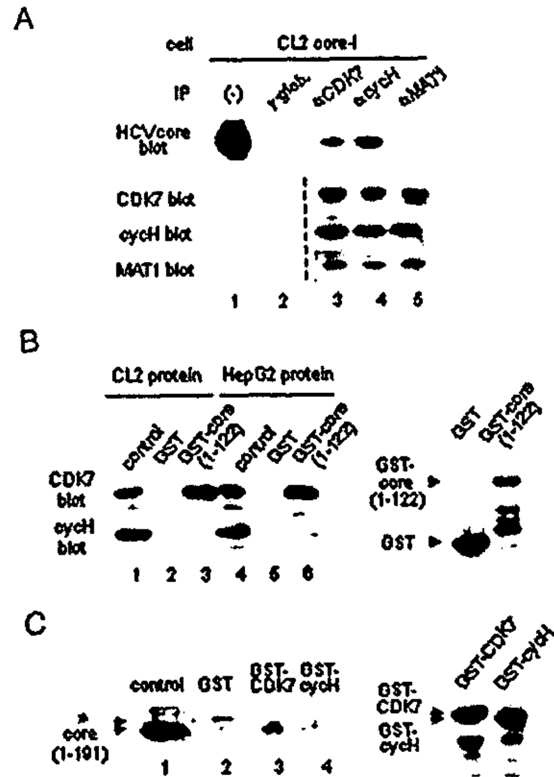

FIG. 6. Binding of HCV core to CAK. *A*, total cellular protein from CL2 core-I cells was precipitated with a rabbit nonspecific γ-globulin (negative control, *lane 2*), and antibodies to CDK7 (*lane 3*), cycH (*lane 4*), and MAT1 (*lane 5*), and the precipitates were blotted with antibodies to HCV core, CDK7, cycH, and MAT1. *Lane 1*, 5% of the cellular protein was used for Western blotting. *B*, *left panel*, GST alone (*lanes 2* and *5*) or the truncated HCV core protein (aa 1–122) fused to GST (*lanes 3* and *6*) was bound to the affinity resin and incubated with cellular proteins from CL2 (*lanes 2* and *3*) and HepG2 (*lanes 5* and *6*) cells. The bound protein complex was blotted with antibodies to CDK7 and cycH. *Lanes 1* and *4*, 10% of the cellular proteins from CL2 (*lane 1*) and HepG2 (*lane 4*) cells were used for Western blotting. *Right panel*, small portions of the products identical to *lanes 2* and *3* of the *left panel* were blotted with an antibody to GST. *C*, *left panel*, GST alone (*lane 2*), human CDK7 fused to GST (*lane 3*), or human cycH fused to GST (*lane 4*) was bound to the affinity resin and incubated with the *in vitro* translated full length of the HCV core protein. The bound protein complex was blotted with an antibody to HCV core. *Lane 1*, 10% of the HCV core protein was used for Western blotting. The *asterisk* shows the band possibly arising because of a weak cross-reaction of the GST protein with the antibody (*lane 2*). *Right panel*, small portions of the products identical to *lanes 3* and *4* of the *left panel* were blotted with an antibody to GST.

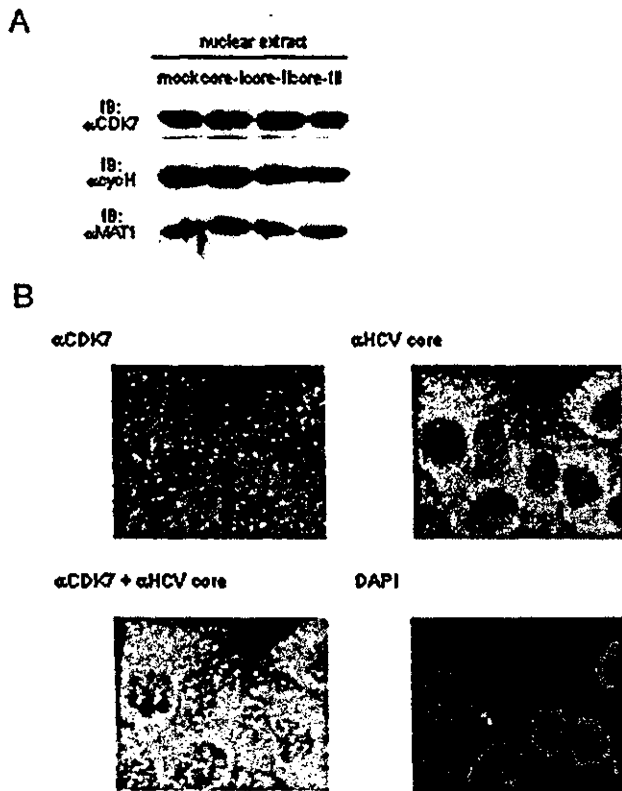

FIG. 7. **Intracellular localization of CAK in CL2 mock and core cells.** *A*, the nuclear fraction was obtained from CL2 mock and core cells, and the CDK7, cycH, and MAT1 proteins were detected by Western blotting. *B*, the CL2 core-I cells were double-stained with the mouse monoclonal HCV core antibody and the rabbit polyclonal CDK7 antibody and analyzed by the confocal microscopic analysis. Images recorded in *green* (HCV core) and *red* (CDK7) channels are shown separately in the *upper panels*, and a composite image is shown in the *left lower panel*. The *right lower panel* shows the nuclear staining with 4',6-diamidino-2'-phenylindole-dihydrochrolide (*DAPI*). *IB*, immunoblot.

When the cell growth curve and the cell cycle profile were compared between CL2 mock and core cells, expression of HCV core impaired cell growth and the G_1/S transition in the cell cycle. It was also shown that persistent expression of HCV core suppressed CDK4 and CDK2 activities and subsequently inhibited the pRB phosphorylation and E2F-mediated transcription. Thus, HCV core-mediated inhibition of cell cycle progression was based on decreased CDK4 and CDK2 activities. We next carried out an extensive investigation on the factors modulating the CDK activity, which included the expression and complex formation of CDKs and cyclins, the expression levels of various CDK inhibitors, and the phosphorylation/dephosphorylation status of CDKs. It was found that the decrease of CDK2 phosphorylation caused by suppression of CAK activity was only a factor, which was identified as a possible cause of HCV core-mediated inhibition of CDK activity.

In the CL2 core cells, the expression levels of CAK components, CDK7, cycH, and MAT1 were not decreased, but the MAT1 level bound to the CDK7-cycH complex was suppressed, compared with the mock cells. Both CAK and CTD kinase activities were also lower in the CL2 core cells than in the mock cells. To investigate whether HCV core would directly modify the CAK activity, the binding reaction of the anti-CDK7 IP product with the *in vitro* synthesized HCV core protein was carried out using both murine CL2 and human HepG2 cellular proteins. It is noteworthy that the addition of the HCV core induced the release of MAT1 from the CAK complex and led to suppression of the CAK activity in the cases of both cellular proteins. These results strongly imply that the HCV core may directly interact with CAK and act as a suppressor of CAK by disrupting the ternary CAK complex in murine and possibly human cells.

The direct interaction of the HCV core with each of the CAK components was further examined. For *in vivo* interaction of HCV core with CAK in the CL2 core-I cells, as examined by the IP/Western blot analysis, it was revealed that the HCV core targeted either CDK7 or cycH. This suggests the existence of the HCV core-CDK7-cycH complex in cells. However, when the *in vitro* binding assay was carried out using the truncated HCV core protein fused to GST, CDK7, but not cycH, was included in the bound complex in both cases using CL2 and HepG2 cellular proteins. These discrepant results may have been due to the different conditions of the two assays. Otherwise, unlike the full length of the HCV core protein, the truncated HCV core protein would disrupt the complex formation of CDK7 and cycH. In either case, our finding indicates that the HCV core may directly bind to both murine and human CDK7 proteins. The result of another binding assay supports this, because the full length of HCV core protein could bind to GST-CDK7 but not to GST-cycH.

As for the intracellular localization of CAK, expression of the HCV core did not affect the nuclear translocation of the CAK components. In the immunofluorescence analysis, the colocalization of HCV core with CDK7 was found to occur mainly in the nucleus. This indicates that only a small portion of the HCV core, which is translocated to the nucleus, may interact with CDK7. It has been demonstrated that the complete loss of CAK results in cell death caused by the inability of cell cycle progression in the previous report using the cultured $mat1^{-/-}$ blastocytes (49). Our CL2 core cells may be able to survive because of incomplete suppression of CAK by the HCV core. According to this, it would be reasonable that only a portion of the HCV core protein is involved in the direct inhibitory effect on CAK.

It has been suggested by a few investigators that the HCV core may considerably affect the $p53/p21^{CIP1}$ status. It has been reported that the HCV core binds to both p53 and $p21^{CIP1}$ (21, 22) and impairs the cell cycle regulation in association with the increased expression of $p21^{CIP1}$ (22, 35). As for the p53 status in the CL2 cells used for this study, it has been shown that the CL2 cells possess the wild-type sequence of p53 gene and do not lose its expression (50). Therefore, the differences in the $p53/p21^{CIP1}$ status between CL2 mock and core cells were also examined in this study. In contrast to these previous reports, persistent expression of HCV core did not substantially affect the expression levels of p53 and $p21^{CIP1}$. Thus, HCV core-mediated suppression of cell cycle progression was not found to be responsible for modification of the $p53/p21^{CIP1}$ status in our CL2 cells. Such conflicting results may be due to the different expression level of the HCV core or different kinds of cultured cells.

CAK components are known to also be parts of the general transcription factor TFIIH and play an essential role not only in the cell cycle progression but also in transcription. TFIIH is composed of nine polypeptides, XPB, XPD, p62, p54, p44, p34, and three CAK components, CDK7, cycH, and MAT1 (51, 52). Biochemical analysis has revealed that CAK exists in three distinct forms in cells. The major form is a "free" ternary CAK complex, but CAK is also present as a CAK-XPD complex and the nine-subunit "holo" TFIIH (51). CAK phosphorylates the CTD of the largest subunit of RNA polymerase II (53), and phosphorylation of the CTD is believed to initiate promoter clearance and transcription elongation. In this study, the HCV core was found to suppress the CTD kinase activity by CAK,

suggesting that the HCV core may affect basal transcription. Furthermore, TFIIH is an essential factor for nucleotide excision DNA repair in cells (54). It is speculated that the direct interaction of HCV core with CDK7 may disturb the formation of complete holo TFIIH complex, as well as the ternary CAK complex, leading to modification of TFIIH functions, such as transcription and nucleotide excision DNA repair. Further experimental evidence should lead to clarification of this.

Alterations between the CL2 mock and core cells may have been due to the artificial effects during establishment of the stable transfectant. To exclude this possibility, we conducted the cell-free assay using the anti-CDK7 IP product and the *in vitro* translated HCV core protein. As a result, the effects of HCV core on CAK assembly and activity in this assay were very similar to those observed in the assay using CL2 mock and core cells (Figs. 4 and 5). In addition, a difference in the E2F-mediated transcription activity between the CL2 mock and core cells was also confirmed in the cotransfection experiment using the CL2 cells (Fig. 2A). These results support that the phenotypic changes between the CL2 mock and core cells observed in this study should have been caused actually by persistent expression of the HCV core protein.

Our results conclusively showed that CAK is a novel target of the HCV core protein in the host cell. HCV core can directly bind to CDK7, and its binding causes dissociation of MAT1 from the CDK7-cycH complex and disrupts the stable ternary CAK complex. Such HCV core-mediated inhibition of CAK may prevent the activities of all cell cycle-related CDKs, including CDK2. This may be the molecular basis of HCV core-mediated suppression of cell cycle progression. It has recently been reported that a knock-out of the *mat1* gene led to embryonic lethality in mice because of the destabilization of CDK7 and cycH and the inability of cell cycle progression (49). Thus, CAK has been shown to be an indispensable factor to achieve cell cycle progression *in vivo*. According to this, it may be of great biological significance that the HCV core protein functions as an extrinsic suppressor of CAK in the host cell. In chronic HCV infection, the disease stage progresses with time accompanied by repeated liver cell injury and regeneration. The inhibitory effect on cell cycle progression caused by persistent expression of HCV core would strikingly affect this process in the pathogenesis of HCV-related liver diseases.

Acknowledgments—We thank Dr. T. Wakita (Department of Microbiology, Tokyo Metropolitan Institute of Medical Science) for providing the plasmids, pc/3EFpro and pc/3EFΔNCTH, and a mouse monoclonal antibody to HCV core and Dr. William S. Dynan (Institute of Molecular Medicine and Genetics, Medical College of Georgia) for providing the plasmid pGCTD.

REFERENCES

1. Kiyosawa, K., Sodeyama, T., Tanaka, E., Gibo, Y., Yoshizawa, K., Nakano, Y., Furuta, S., Akahane, Y., Nishioka, K., Purcell, R. H., and Alter, H. J. (1990) *Hepatology* 12, 671–675
2. Choo, Q. L., Richman, K. H., Han, J. H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, A., Barr, P. J., Weiner, A. J., Bradley, D. W., Kuo, G., and Houghton, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 2451–2455
3. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 5547–5551
4. Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., Kato, N., Tanaka, T., Kimura, K., and Shimotohno, K. (1993) *J. Virol.* 67, 4665–4675
5. Ray, R. B., Lagging, L. M., Meyer, K., and Ray, R. (1996) *J. Virol.* 70, 4438–4443
6. Yoshida, T., Hanada, T., Tokuhisa, T., Kosai, K., Sata, M., Kohara, M., and Yoshimura, A. (2002) *J. Exp. Med.* 196, 641–653
7. Moriya, K., Fujie, H., Shintani, Y., Yotsuyanagi, H., Tsutsumi, T., Ishibashi, K., Matsuura, Y., Kimura, S., Miyamura, T., and Koike, K. (1998) *Nat. Med.* 4, 1065–1067
8. Ray, R. B., Meyer, K., and Ray, R. (1996) *Virology* 226, 176–182
9. Marusawa, H., Hijikata, M., Chiba, T., and Shimotohno, K. (1999) *J. Virol.* 73, 4713–4720
10. Zhu, N., Khoshnan, A., Schneider, R., Matsumoto, M., Dennert, G., Ware, C., and Lai, M. (1998) *J. Virol.* 72, 3691–3697
11. Machida, K., Tsukiyama-Kohara, K., Seike, E., Tone, S., Shibasaki, F., Shimizu, M., Takahashi, H., Hayashi, Y., Funata, N., Taya, C., Yonekawa, H., and Kohara, M. (2001) *J. Biol. Chem.* 276, 12140–12146
12. Tsuchihara, K., Hijikata, M., Fukuda, K., Kuroki, T., Yamamoto, N., and Shimotohno, K. (1999) *Virology* 258, 100–107
13. Yoshida, H., Kato, N., Shiratori, Y., Otsuka, M., Maeda, S., Kato, J., and Omata, M. (2001) *J. Biol. Chem.* 276, 16399–16405
14. Aoki, H., Hayashi, J., Moriyama, M., Arakawa, Y., and Hino, O. (2000) *J. Virol.* 74, 1736–1741
15. Hosui, A., Ohkawa, K., Ishida, H., Sato, A., Nakanishi, F., Ueda, K., Takehara, T., Kasahara, A., Sasaki, Y., Hori, M., and Hayashi, N. (2003) *J. Biol. Chem.* 278, 28562–28571
16. Matsumoto, M., Hsieh, T. Y., Zhu, N., VanArsdale, T., Hwang, S. B., Jeng, K. S., Gorbalenya, A. E., Lo, S. Y., Ou, J. H., Ware, C. F., and Lai, M. M. (1997) *J. Virol.* 71, 1301–1309
17. Hsieh, T. Y., Matsumoto, M., Chou, H. C., Schneider, R., Hwang, S. B., Lee, A. S., and Lai, M. M. (1998) *J. Biol. Chem.* 273, 17651–17659
18. Sabile, A., Perlemuter, G., Bono, F., Kohara, K., Demaugre, F., Kohara, M., Matsuura, Y., Brechot, C., and Barba, G. (1999) *Hepatology* 30, 1064–1076
19. You, L. R., Chen, C. M., Yeh, T. S., Tsai, T. Y., Mai, R. T., Lin, C. H., and Lee, Y. H. (1999) *J. Virol.* 73, 2841–2853
20. Jin, D. Y., Wang, H. L., Zhou, Y., Chun, A. C., Kibler, K. V., Hou, Y. D., and Kung, H. (2000) *EMBO J.* 19, 729–740
21. Wang, F., Yoshida, I., Takamatsu, M., Ishido, S., Fujita, T., Oka, K., and Hotta, H. (2000) *Biochem. Biophys. Res. Commun.* 273, 479–484
22. Otsuka, M., Kato, N., Lan, K., Yoshida, H., Kato, J., Goto, T., Shiratori, Y., and Omata, M. (2000) *J. Biol. Chem.* 275, 34122–34130
23. Tsutsumi, T., Suzuki, T., Shimoike, T., Moriya, K., Shintani, Y., Fujie, H., Matsuura, Y., Koike, K., and Miyamura, T. (2002) *Hepatology* 35, 937–946
24. Weinberg, R. A. (1995) *Cell* 81, 323–330
25. Sherr, C. J., and Roberts, J. M. (1995) *Genes Dev.* 9, 1149–1163
26. Morgan, D. O. (1997) *Annu. Rev. Cell. Dev. Biol.* 13, 261–291
27. Sherr, C. J., and Roberts, J. M. (1999) *Genes Dev.* 13, 1501–1512
28. Harbor, J. W., and Dean, D. C. (2000) *Nat. Cell Biol.* 2, E65–67
29. el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Cell* 75, 817–825
30. Fesquet, D., Labbe, J. C., Derancourt, J., Capony, J. P., Galas, S., Girard, F., Lorca, T., Shuttleworth, J., Doree, M., and Cavadore, J. C. (1993) *EMBO J.* 12, 3111–3121
31. Fisher, R. P., and Morgan, D. O. (1994) *Cell* 78, 713–724
32. Fisher, R. P., Jin, P., Chamberlin, H. M., and Morgan, D. O. (1995) *Cell* 83, 47–57
33. Matsuoka, M., Kato, J. Y., Fisher, R. P., Morgan, D. O., and Sherr, C. J. (1994) *Mol. Cell. Biol.* 14, 7265–7275
34. Aprelikova, O., Xiong, Y., and Liu, E. T. (1995) *J. Biol. Chem.* 270, 18195–18197
35. Honda, M., Kaneko, S., Shimazaki, T., Matsushita, E., Kobayashi, K., Ping, L. H., Zhang, H. C., and Lemon, S. M. (2000) *Hepatology* 31, 1351–1359
36. Tokushige, K., Moradpour, D., Wakita, T., Geissler, M., Hayashi, N., and Wands, J. R. (1997) *J. Virol. Methods* 64, 73–80
37. Nakabayashi, H., Takeda, K., Miyano, K., Yamane, T., and Sato, J. (1982) *Cancer Res.* 42, 3858–3863
38. Aden, D. P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B. B. (1979) *Nature* 282, 615–616
39. Peterson, S. R., Dvir, A., Anderson, C. W., and Dynan, W. S. (1992) *Genes Dev.* 6, 426–438
40. Patek, P., Collins, J., and Cohn, M. (1978) *Nature* 276, 510–511
41. Ohkawa, K., Ishida, H., Nakanishi, F., Hosui, A., Sato, A., Ueda, K., Takehara, T., Kasahara, A., Sasaki, Y., Hori, M., and Hayashi, N. (2003) *Hepatol. Res.* 25, 396–408
42. Ishida, H., Ueda, K., Ohkawa, K., Kanazawa, Y., Hosui, A., Nakanishi, F., Mita, E., Kasahara, A., Sasaki, Y., Hori, M., and Hayashi, N. (2000) *J. Virol.* 74, 1241–1251
43. Wagner, S., Beil, W., Westermann, J., Logan, R. P. H., Bock, C. T., Trautwein, C., Bleck, J. S., and Manns, M. P. (1997) *Gastroenterology* 113, 1836–1847
44. Zhuang, S. H., and Burnstein, K. L. (1998) *Endocrinology* 139, 1197–1207
45. Yasui, K., Wakita, T., Tsukiyama-Kohara, K., Funahashi, S. I., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J. R., and Kohara, M. (1998) *J. Virol.* 72, 6048–6055
46. Schulze, A., Zerfass, K., Spitkovsky, D., Middendorp, S., Berges, J., Helin, K., Jansen-Durr, P., and Henglein, B. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 11264–11268
47. Furukawa, Y., Terui, Y., Sakoe, K., Ohta, M., and Saito, M. (1994) *J. Biol. Chem.* 269, 26249–26258
48. Hiramatsu, N., Hayashi, N., Haruna, Y., Kasahara, A., Fusamoto, H., Mori, C., Fuke, I., Okayama, H., and Kamada, T. (1992) *Hepatology* 16, 306–311
49. Rossi, D. J., Londesborough, A., Korsisaari, N., Pihlak, A., Lehtonen, E., Henkemeyer, M., and Makela, T. P. (2001) *EMBO J.* 20, 2844–2856
50. Sun, Y., Hegamyer, G., Nakamura, K., Kim, H., Oberley, L. W., and Colburn, N. H. (1993) *Int. J. Cancer* 55, 952–956
51. Drapkin, R., LeRoy, G., Cho, H., Akoulitchev, S., and Reinberg, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6488–6493
52. Reardon, J. T., Ge, H., Gibbs, E., Sancar, A., Hurwitz, J., and Pan, Z. Q. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6482–6487
53. Lu, H., Zawel, L., Fisher, L., Egly, J. M., and Reinberg, D. (1992) *Nature* 358, 641–645
54. Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H., Chambon, P., and Egly, J. M. (1993) *Science* 260, 58–63

ELSEVIER

Available online at www.sciencedirect.com
SCIENCE DIRECT

Hepatology Research 29 (2004) 142–147

Hepatology Research
www.elsevier.com/locate/ihepcom

The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan

Naoki Hiramatsu [a], Akinori Kasahara [b], Fumihiko Nakanishi [c], Takashi Toyama [c], Masahiko Tsujii [c], Shingo Tsuji [c], Tatsuya Kanto [a], Tetsuo Takehara [a], Michio Kato [d], Harumasa Yoshihara [e], Masafumi Naito [f], Kazuhiro Katayama [f], Taizo Hijioka [g], Hideki Hagiwara [h], Shinji Kubota [i], Masahide Oshita [j], Haruya Meren [j], Manabu Masuzawa [j], Yoshimichi Haruna [k], Eiji Mita [l], Kunio Suzuki [l], Norio Hayashi [a,*]

[a] Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita City, Osaka 565-0871, Japan
[b] Department of General Medicine, Osaka University Graduate School of Medicine, Osaka, Japan
[c] Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Osaka, Japan
[d] Osaka National Hospital, Osaka, Japan
[e] Osaka Rousai Hospital, Osaka, Japan
[f] Osaka Kouseinenkin Hospital, Osaka, Japan
[g] National Osaka South Hospital, Osaka, Japan
[h] Higashiosaka City Central Hospital, Osaka, Japan
[i] Kansai Rousai Hospital, Hyoga, Japan
[j] Osaka Police Hospital, Osaka, Japan
[k] Osaka Prefectural Hospital, Osaka, Japan
[l] Saiseikai senri Hospital, Osaka, Japan

Received 26 December 2003; received in revised form 16 March 2004; accepted 25 March 2004

Abstract

One hundred seventy-one patients with chronic hepatitis C were included in this study (genotype1 and high viral loads (1H), $n = 130$; non-1H, $n = 37$; N.D., $n = 4$). The combination therapy of interferon and ribavirin for 24 weeks with an additional 24 weeks of interferon monotherapy (48-week treatment) was undergone by 42 1H patients and 5 non-1H patients. The combination therapy of interferon and ribavirin was administered for 24 weeks in 67 1H patients and 22 non-1H patients. Among the 1H patients, the HCV relapse rate was significantly higher in those receiving 24-week combination treatment than in those receiving 48-week treatment (78% versus 42%, $P = 0.003$). Among the non-1H patients, no significant difference was found between them. Sustained virological response (SVR) rates were observed to decrease as the timing of HCV RNA disappearance was delayed. In spite of the small rate (16%), SVR was obtained from the patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment. In 1H patients, 24-week combination treatment followed by interferon monotherapy for 24 weeks was concluded to be the treatment offering the most hope among those that the medical insurance can be applied in Japan.
© 2004 Published by Elsevier B.V.

Keywords: Chronic hepatitis C; Interferon and ribavirin combination therapy; Combination therapy followed by IFN monotherapy

1. Introduction

Interferon is the only available treatment for patients with chronic hepatitis C since HCV was discovered in 1989 [1–4]. Thirty percent of patients with chronic hepatitis C achieved SVR by interferon therapy but the efficacy was not satisfactory. Furthermore, in the patients considered to be the most treatment-resistant, that is, the 1H patients, only 5–8% showed SVR. In Japan, 40–50% of the patients with chronic hepatitis C belong to the 1H group. Therefore, finding how to eradicate the HCV RNA

* Corresponding author. Tel.: +81-6-6879-3441; fax: +81-6-6879-3449.
E-mail address: hayashin@moltex.med.osaka-u.ac.jp (N. Hayashi).

1386-6346/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.hepres.2004.03.008

of 1H patients is most important for the treatment of chronic hepatitis C.

Recently, ribavirin, a nucleic acid analogue, exhibiting in vitro activity against various kinds of DNA and RNA viruses has been developed. The combination therapy of ribavirin and interferon has been shown to be very useful in the eradication of HCV in patients with chronic hepatitis C [5–7], although the mechanism of action of ribavirin remains speculative and ribavirin monotherapy led to no significant decrease of the amount of HCV RNA in the patients with chronic hepatitis C [8]. Most recent studies, performed with large numbers of naïve patients, have shown that the combination therapy of interferon and ribavirin can increase the SVR rate two-fold compared with interferon monotherapy for patients with chronic hepatitis C [9–12]. Especially, in the 1H patients, the combination therapy of interferon and ribavirin was more useful than in the other patients. Furthermore, Poynard et al. [10] showed that 1H patients treated by combination therapy for 48 weeks had a higher SVR rate than those treated for 24 weeks (28% versus 8%). Therefore, the combination therapy of interferon and ribavirin for 48 weeks is recommended as the standard therapy for 1H patients in Europe and the United States [13,14].

In Japan, the combination therapy of interferon and ribavirin was approved in 2001. However, the duration of the combination therapy is limited to 24 weeks in the medical insurance. As mentioned above, the SVR rate in 1H patients treated by the combination therapy for 24 weeks was clearly lower than those treated for 48 weeks. Furthermore, prolonged interferon monotherapy was reported to suppress relapse after cessation of therapy and to achieve a higher SVR rate in patients with chronic hepatitis C [15]. This study assessed the efficacy of the combination therapy of interferon and ribavirin for 24 weeks with an additional 24 weeks of interferon monotherapy compared with that of the combination therapy for 24 weeks.

2. Patients and methods

2.1. Patients

The current study was conducted at Osaka University Hospital and the institutions of the Osaka Liver Disease Study Group. The 171 patients included in this study had HCV RNA detectable in serum by the polymerase chain reaction (PCR) method, had elevated ALT (above the upper limit of the normal) and had been histologically proven to have chronic hepatitis. No patients were positive for hepatitis B surface antigen and anti-human immunodeficiency virus antibody or had other forms of liver disease (such as alcoholic liver disease and autoimmune liver disease). This study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

2.2. Determination of HCV RNA levels and HCV genotype

Serum HCV RNA levels were quantified using branched DNA (bDNA) probe assay (version 2; Chiron, Dai-ichi Kagaku, Tokyo) [16,17] or combined PCR assay (Amplicor-HCV monitor assay) [18]. In this study, a high viral load, as described previously [16,18,19], was designated as the condition of a serum HCV RNA level of more than 10^6 equivalents/ml by bDNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay. HCV genome typing was classified by serological genotyping assay [20].

2.3. Treatment schedule

Of the 171 patients with chronic hepatitis C enrolled in this study, 130 had HCV RNA with genotype 1 and high viral loads (1H group), which were difficult to eradicate by anti-viral therapy. Of the remaining 41 patients, 37 had HCV RNA with genotype 2 or low viral loads (non-1H group); genotype or viral levels could not be determined for four. One hundred thirty-six patients in whom treatment had been done without the discontinuation of interferon till the end of the scheduled duration were studied (1H, $n = 109$; non-1H, $n = 27$).

The combination therapy of interferon-α-2b and ribavirin was administered for 24 weeks in 67 patients of the 1H group and 22 patients of the non-1H group. In this protocol, interferon-α-2b was given intramuscularly every day for the first 2 weeks and then three times a week for the following 22 weeks in combination with ribavirin at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥60 kg, respectively). The combination therapy of interferon-α-2b and ribavirin for 24 weeks, followed by interferon-α-2b monotherapy three times a week for a further 24 weeks, was administered to 42 patients of the 1H group and 5 patients of the non-1H group. The pretreatment characteristics of the patients were similar (Table 1).

The starting doses of interferon-α-2b were 10 MU per day for 38, 6 MU per day for 127, and 3 MU per day for 6 patients. With ribavirin, 800 mg per day was started in 92, 600 mg per day in 77, and 400 mg per day in 2 patients. Among the 171 patients, the interferon dose was decreased in six patients during the treatment, and the interferon was stopped along with ribavirin in 33 patients (19%) due to side effects. The ribavirin dose was decreased in 43 patients (25%) during the treatment, and stopped without discontinuance of interferon in six patients. Eighty-seven patients (51%) completed treatment without discontinuance or dosage decrease of both drugs.

After the sufficient informed consent at the end of the combination therapy of interferon and ribavirin, the patients themselves decided whether to be treated for 24 or 48 weeks. The information included the results of clinical trials of the combination therapy for 24 and 48 weeks in other countries, such as the SVR rate, HCV relapse rate.

Table 1
Baseline characteristics of patients according to therapeutic protocol

	24-week treatment		48-week treatment
	1H group	Non-1H group	1H group
	67	22	42
Age (yo)	55.8 ± 10.9	55.7 ± 12.8	54.0 ± 11.7
M/F	40/27	15/7	28/14
ALT (IU/L)	107 ± 71	102 ± 45	103 ± 58
Fibrosis	1.9 ± 0.9	1.9 ± 1.2	1.8 ± 1.1
History of IFN treatment			
Naïve	34	11	17
Relapser	21	7	17
Non-responder	11	4	8
Unknown	1	0	0

Note: All comparisons are not significant. Twenty-four-week treatment, interferon plus ribavirin treatment for 24 weeks; 48-week treatment, interferon plus ribavirin treatment for 24 weeks followed by interferon monotherapy for 24 weeks. 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than 1H group. Fibrosis, Knodell's histological score (category 4).

Also, side effects were presented and the combination therapy of interferon-α-2b and ribavirin for 48 weeks was explained as not being covered by medical insurance in Japan. In the 47 patients who agreed to receive the additional 24 weeks of interferon monotherapy, the starting doses of interferon-α-2b were 10 MU per day for 10, 6 MU per day for 35, and 3 MU per day for 2 patients. All patients completed the additional treatment although interferon was decreased only in one patient from 10 to 6 MU per day.

2.4. Statistical analysis

Age, histological scores before interferon therapy, and serum ALT levels are expressed as mean ± S.D. The chi-squared test was used for statistical analysis of the comparison between group frequencies. When appropriate, the clinical and laboratory features of the two groups were compared by Student's *t*-test. Histological evaluation was substituted as a variable for Knodell's histological scores [21].

3. Results

3.1. Results of interferon and ribavirin combination therapy

Seventy-five percent of all of the patients of 1H group (82/109), including not only patients who received 24-week treatment but also those who received 48-week treatment, had no detectable HCV RNA at 24 weeks after the beginning of combination therapy of interferon and ribavirin. This was also the case for 100% of the non-1H patients (27/27). In patients given 24-week treatment of combined interferon and ribavirin, 45 out of 67 of the 1H group were negative for HCV RNA at the end of therapy, but only 22% of the patients (10/45) showed no detectable HCV RNA at 24 weeks after cessation of therapy. On the other hand, HCV RNA was negative in all non-1H patients at the end of the 24-week treatment, and the SVR rate was 86% (19/22) (Fig. 1). In patients with 48-week treatment (24-week combination treatment, followed by 24-week interferon monotherapy), HCV RNA reappeared during interferon monotherapy (break through) in 11 out of 37 patients (30%) who were negative for HCV RNA at the end of 24-week combination therapy: SVR was finally reached in 15 out of 26 patients who continued to be sero-negative for HCV RNA at the end of 48-week treatment. On the other hand, HCV RNA was not cleared even by 48-week treatment in all five patients who were positive for HCV RNA at the end of 24-week treatment (Fig. 2). In the non-1H patients who received 48-week treatment, HCV RNA was negative in all five patients at the end of the 24-week treatment, and SVR was attained by 80% (4/5).

The HCV RNA relapse rate after treatment was compared according to the duration of treatment. In all patients, 57% of those receiving 24-week treatment (38/67) had HCV RNA relapse, as compared with 39% of those receiving 48-week treatment (12/31). Among the 1H patients, a significant dif-

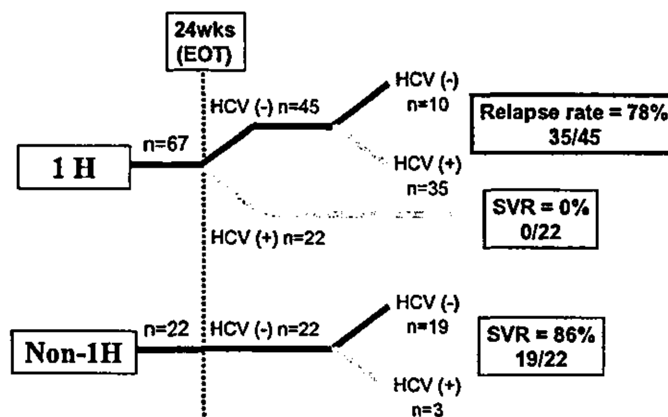

Fig. 1. Efficacy of the combination therapy (24-week treatment). 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than those of the 1H group. EOT, end of treatment. HCV, serum HCV RNA positivity by polymerase chain reaction.

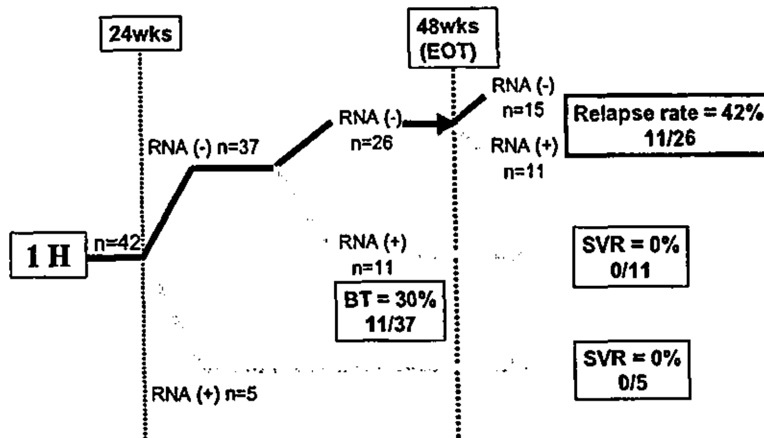

Fig. 2. Efficacy of the combination therapy followed by interferon monotherapy (48-week treatment). 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than those of the 1H group. EOT, end of treatment. HCV, serum HCV RNA positivity by polymerase chain reaction. BT, break through.

ference was found in HCV relapse rate between those receiving 24-week treatment and those receiving 48-week treatment (78% versus 42%, $P = 0.003$). Among the non-1H patients, HCV RNA relapsed in 14% (3/22) of those receiving 24-week treatment (Fig. 1) and 20% (1/5) of those receiving 48-week treatment.

3.2. Timing of HCV RNA disappearance and efficacy of treatment

The relationship between the timing of HCV RNA disappearance and SVR rate according to the duration of treatment was evaluated. As shown in Fig. 3A, in all patients receiving 24-week treatment, 71% (12/17) of the patients who had no detectable HCV RNA by week 4, 61% (11/18) by week 8 (beyond week 4), and 21% (4/19) by week 12 (beyond week 8) had SVR. Although 11 patients became negative for HCV RNA by week 24 (beyond week 12), none of them attained SVR. A tendency for a decrease in the SVR rate was observed as the timing of the HCV RNA disappearance was delayed. In the patients receiving 48-week treatment, 86% (6/7) of those who had no detectable HCV RNA by week 4, 100% (6/6) by week 8 (beyond week 4), 40% (4/10) by week 12 (beyond week 8), and 16% (3/19) by week 24 (beyond week 12) attained SVR.

Among the 1H patients, the same tendency was also observed (Fig. 3B). In the patients receiving 24-week treatment, 50% (3/6) of those who had no detectable HCV RNA by week 4, 40% (4/10) by week 8 (beyond week 4), and 18% (3/17) by week 12 (beyond week 8) attained SVR. None of the 10 patients who became negative for HCV RNA by week 24 (beyond week 12) showed SVR. In the patients receiving 48-week treatment, 80% (4/5) of those who had no detectable HCV RNA by week 4, 100% (5/5) by week 8 (beyond week 4), 38% (3/8) by week 12 (beyond week 8), and 16% (3/19) by week 24 (beyond week 12) had SVR. In spite of the small rate (16%), SVR was obtained from the patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment.

Fig. 4 shows the relationship between the timing of HCV RNA disappearance and the prediction value in 1H patients who received the combination therapy of interferon and ribavirin for 24 weeks. As the timing of the HCV RNA disappearance was late, the positive prediction value decreases and the negative prediction value increases. In particular, the negative prediction value at week 12 was 100%, that is, none

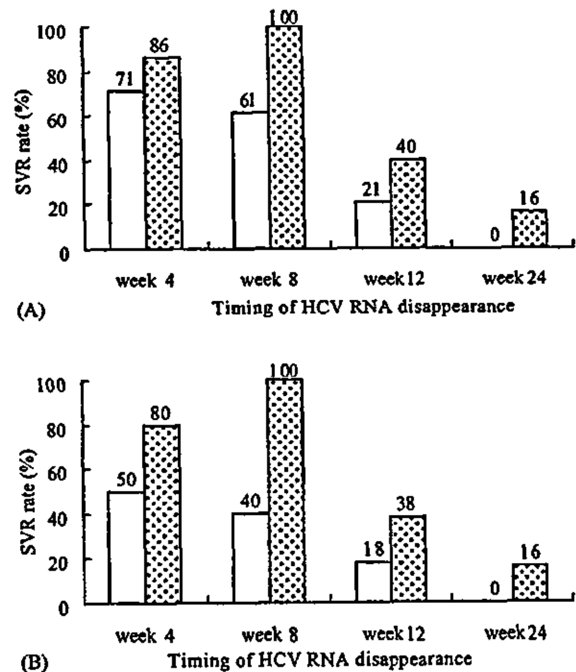

Fig. 3. Timing of HCV RNA disappearance and SVR rate (A) all patients, (B) patients with genotype 1 and high viral loads. (□) Combination therapy of interferon and ribavirin (24-week treatment); (▨) combination therapy followed by interferon monotherapy (48-week treatment).

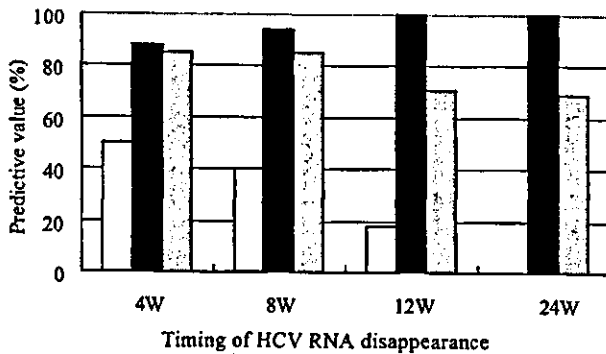

Fig. 4. Timing of HCV RNA disappearance and prediction value in patients with genotype 1 and high viral loads who received the combination therapy of interferon and ribavirin for 24 weeks. (□) Positive prediction value; (■) negative prediction value; (▨) predictive accuracy.

of the patients who were positive for HCV RNA at week 12 attained SVR.

4. Discussion

In Japan, randomized control studies were performed on the combination therapy of interferon and ribavirin for 24 weeks in patients with chronic hepatitis C, and the combination therapy was approved in November 2001. However, the duration of the combination therapy is limited to 24 weeks in the medical insurance because of the lack of clinical mega-trial evidence for the combination therapy for 48 weeks in Japan. From the results of international trials, the SVR rate in 1H patients treated by the combination therapy for 48 weeks has been shown to be higher than that of those treated for 24 weeks [10]. Moreover, for interferon monotherapy, prolonged interferon treatment was reported to suppress relapse after cessation of therapy and to lead to a higher SVR rate in patients with chronic hepatitis C [15]. Our strategy, the interferon and ribavirin combination therapy with an additional 24 weeks of interferon monotherapy, was conducted against this background.

Poynard et al. [22,23] evaluated the HCV RNA relapse rates after cessation of the combination therapy in naïve patients with chronic hepatitis C. Among patients with genotype 1, the relapse rates were 62% in those treated by interferon and ribavirin combination therapy for 24 weeks and 26% in those treated for 48 weeks: among patients with genotype 2/3, 21% in those for 24 weeks and 15% in those for 48 weeks. Among patients with genotype 1, the SVR rate increased due to suppression of the relapse rate by the combination therapy for 48 weeks. On the other hand, the patients with genotype 2/3 require only 24 weeks of therapy. In our study, patients with genotype 1 and high viral load (1H group) were evaluated, distinguishing them from others (non-1H group) since the efficacy of anti-viral therapy for the 1H patients has been known to be remarkably low. Among the 1H patients, the HCV relapse rate was significantly higher in those receiving 24-week combination treatment than in those receiving 48-week treatment, 24-week combination treatment followed by interferon monotherapy for 24 weeks (78% versus 42%, $P = 0.003$). Among the non-1H patients, no significant difference was found between those receiving 24-week treatment and those receiving 48-week treatment (14% versus 20%). These results indicate that our strategy of 48-week treatment is useful for the 1H group; the non-1H group seems to require only 24 weeks of therapy, similar to the patients with genotype 2/3 in the above-mentioned.

In the 1H patients receiving 48-week treatment, HCV RNA reappeared during interferon monotherapy in 11 out of 37 patients (30%) who were negative for HCV RNA at the end of 24-week combination therapy. The break through phenomenon should be taken into account when the efficacy of this treatment is evaluated. The SVR ratio in 1H patients receiving 48-week treatment can be calculated from the prevalence of undetectable HCV RNA at 24 weeks after the beginning of combination therapy of interferon and ribavirin (75%, 82/109), of break though (30%, 11/37) and of HCV relapse rate (42%, 11/26); the expected SVR is 30% ($(82/109) \times (1 - (11/37)) \times (1 - (11/26)) \fallingdotseq 0.30$). In the same manner, the SVR ratio in 1H patients receiving 24-week treatment is expected to be 17% $(82/109) \times (1 - (35/45)) \fallingdotseq 0.17$. In 1H patients, 48-week treatment, 24-week combination treatment followed by interferon monotherapy for 24 weeks, may be the useful treatment which can be actually performed in Japan.

The relationship between the timing of HCV RNA disappearance and the SVR rate according to the duration of treatment was evaluated. SVR rates decreased with a delay in the timing of HCV RNA disappearance in patients receiving 24-week treatment; the negative prediction value at week 12 was 100%, that is, none of the patients who were positive for HCV RNA at week 12 had SVR. In spite of the small rate (16%), SVR was attained for patients who became negative for HCV RNA by week 24 (beyond week 12) only in those receiving 48-week treatment. Accordingly, treatment withdrawal should be offered to patients who remain HCV RNA-positive after 12 weeks of therapy if the patient cannot continue treatment for 48 weeks for reasons including side effects and social issues. The patients who were positive for HCV RNA at week 24 should stop treatment because additional interferon monotherapy for 24 weeks could not clear HCV RNA in all five patients who were positive for HCV RNA at week 24.

Pol et al. [24] have reported the synergistic effect of ribavirin and interferon in 343 patients with the genotype 1b. In the study, ribavirin was administered for 4, 6, 12 months in combination with interferon-α for 12 months. A 12-month course of ribavirin achieved significantly greater virological efficacy than 6 or 4 months at the end of the 12-month course of interferon-α (59, 49, and 29%), the same trend seen at the end of follow-up duration (43, 36, and 21%). These results indicate that the maximum efficacy can be obtained

when ribavirin is administered for 12 months in combination with interferon. In our study, the break through ratio was expected to decrease with the administration of ribavirin for 48 weeks. In fact, a patient to whom ribavirin was given again after the break through, achieved marked decrease of HCV RNA (data not shown). Thus, in some patients who were negative for HCV RNA during the combination treatment, the additional ribavirin can be essential for eradicating HCV RNA. Longer duration of combination therapy with interferon and ribavirin is also most effective for suppressing HCV RNA relapse after 24 weeks of therapy [22,23]. Therefore, we would like to emphasize that combination therapy of ribavirin and interferon for 48 weeks should be permitted even in Japan. At present, 24-week combination therapy followed by 24-week interferon monotherapy is thought to be the most useful therapy that the medical insurance can be applied in Japan for suppressing the relapse rate of HCV RNA, leading to SVR.

Acknowledgements

In addition to the study authors, the following institutions and physicians were participants in the Osaka Liver Disease Study Group (Digestive Disease Study Group of Osaka Renaissance): Osaka National Hospital, R. Sakamori; Osaka Rousai Hospital, N. Kurashige, O. Nishiyama, and S. Shinzaki; Osaka Kouseinenkin Hospital, M. Kurokawa and A. Uemura; National Osaka South Hospital, T. Oze and N. Tsuda; Kansai Rousai Hospital, T. Yoshio; Osaka Police Hospital, K. Koga; Osaka Prefectural Hospital, A. Arimitsu; and Osaka University Graduate School of Medicine, S. Yamaguchi, M. Miyazaki, H. Miyatake, I. Itose, S. Egawa, and T. Nishida.

This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan.

References

[1] Davis GL, Balart LA, Schiff ER, et al. Treatment of chronic hepatitis C with recombinant interferon alpha. A multicenter randomized controlled trial. N Engl J Med 1989;321:1501–6.
[2] Di Bisceglie AM, Martin P, Kassianides C, et al. Recombinant interferon alpha therapy for chronic hepatitis C. A randomized, double blind placebo-controlled trial. N Engl J Med 1989;321:1506–10.
[3] Hiramatsu N, Hayashi N, Kasahara A, et al. Improvement of liver fibrosis in chronic hepatitis C patients treated with natural interferon alpha. J Hepatol 1995;22:135–42.
[4] Kasahara A, Hayashi N, Mochizuki K, et al. Pretreatment viral load and response to interferon therapy for liver cirrhosis caused by hepatitis C virus: a multicenter controlled study. Hepatol Res 2000;16:124–38.
[5] Kakumu S, Yoshioka K, Wakita T, et al. A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. Gastroenterology 1993;105:507–12.
[6] Brillanti S, Garson J, Foli M, et al. A pilot study of combination therapy with ribavirin plus interferon alpha for alpha-interferon resistant chronic hepatitis C. Gastroenterology 1994;107: 812–7.
[7] Lai MY, Kao JH, Yang PM, et al. Long-term efficacy of ribavirin plus interferon alfa in the treatment of chronic hepatitis C. Gastroenterology 1996;111:1307–12.
[8] Bondenheimer Jr HC, Lindsay KL, Davis GL, et al. Tolerance and efficacy of oral ribavirin treatment of chronic hepatitis C: a multicenter trial. Hepatology 1997;26:473–7.
[9] McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alpha-2b and ribavirin as initial treatment of relapse of chronic hepatitis C. N Engl J Med 1998;339:1485–92.
[10] Poynard T, Marcellin P, Lee SS, et al. Randomised trial of interferon alpha-2b and ribavirin for 48 weeks or for 24 weeks versus interferon alfa-2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998;352:1426–32.
[11] Reichard O, Norkrans G, Fryden A, et al. Randomized double-blind, placebo-controlled trial of interferon alpha-2b with and without ribavirin for chronic hepatitis C. Lancet 1998;351:83–6.
[12] Davis GL, Esteban-Mur R, Rustgi V, et al. Interferon alpha-2b alone or in combination with ribavirin for the treatment or relapse of chronic hepatitis C. N Engl J Med 1998;339:1493–9.
[13] Consensus statement. EASL international consensus conference on hepatitis C. Paris, 26–28 February 1999. J Hepatol 1999;30: 956–61.
[14] Bisceglie AM, Hoofnagle JH. Optimal therapy of hepatitis C. Hepatology 2002;36:S121–7.
[15] Kasahara A, Hayashi N, Hiramatsu N, et al. Ability of prolonged interferon treatment to suppress relapse after cessation of therapy in patients with chronic hepatitis C: a multicenter randomized controlled trial. Hepatology 1995;21:291–7.
[16] Yuki N, Hayashi N, Kasahara A, et al. Pretreatment viral load and response to prolonged interferon-α course for chronic hepatitis C. J Hepatol 1995;22:457–63.
[17] Lau YN, Davis G, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 1993;341:1501–4.
[18] Shiratori Y, Kato N, Yokosuka O, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Gastroenterology 1997;113:558–66.
[19] Hagiwara H, Hayashi N, Mita E, et al. Quantitative analysis of hepatitis C virus RNA in serum during interferon alfa therapy. Gastroenterology 1993;104:877–83.
[20] Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology 1994;19:1347–53.
[21] Knodell RG, Ishak KG, Black WC, et al. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatology 1981;1:431–5.
[22] Poynard T, Marcellin P, Lee SS, et al. Randomized trial of interferon α2b plus ribavirin for 48 weeks or for 24 weeks versus ineterferon α2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C. Lancet 1998;352:1426–32.
[23] Poynard T, McHutchison J, Goodman Z, et al. Is an "A la Carte" combination interferon alfa-2b plus ribavirin regimen possible for the first line treatment in patients with chronic hepatitis C? Hepatology 2000;31:211–8.
[24] Pol S, Nalpas B, Bourliere M, et al. Combination of Ribavirin and interferon-alfa surpasses high doses of interferon-alfa alone in patients with genotype-1b-related chronic hepatitis C. Hepatology 2000;31:1338–44.

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochemical and Biophysical Research Communications 321 (2004) 722–727

BBRC

www.elsevier.com/locate/ybbrc

Involvement of p38 signaling pathway in interferon-α-mediated antiviral activity toward hepatitis C virus

Hisashi Ishida [a], Kazuyoshi Ohkawa [a], Atsushi Hosui [b], Naoki Hiramatsu [a], Tatsuya Kanto [c], Keiji Ueda [d], Tetsuo Takehara [a], Norio Hayashi [a,*]

[a] *Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*
[b] *Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*
[c] *Department of Dendritic Cell Biology and Clinical Application, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*
[d] *Department of Microbiology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan*

Received 12 March 2004

Abstract

We studied the involvement of the p38 signaling pathway in the interferon (IFN)-α-mediated antiviral activity toward hepatitis C virus (HCV) using HCV subgenomic replicon cells. When the cells were treated with IFN-α in the presence of p38 inhibitor, the suppressive effect of IFN-α on replicon RNA was reduced. Inhibition of p38 had almost no influence on phosphorylation of signal transducer and activator transcription factor 1 (STAT1) and interferon stimulatory response element-dependent gene expression after IFN-α treatment. This indicates that the anti-HCV activity through p38 may be independent of the Janus kinase -STAT pathway. Treatment with the inhibitor of the mitogen-activated protein kinase-activated protein kinase 2 (MK2) showed the same level of reduction in the IFN-α-mediated anti-HCV activity as that with the p38 inhibitor. Thus, MK2 may also be responsible for the anti-HCV activity through p38. In conclusion, the p38-MK2 signaling pathway may be substantially involved in the IFN-α-mediated anti-HCV activity.
© 2004 Elsevier Inc. All rights reserved.

Keywords: Hepatitis C virus; Subgenomic replicon; Interferon-α; p38 signaling pathway; Mitogen-activated protein kinase-activated protein kinase 2; Replication; Antiviral activity

Hepatitis C virus (HCV) often causes persistent infection, leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1,2]. Studies on the replicative manner of HCV have been hampered due to lack of an animal model or a tissue culture system sustaining efficient viral replication. Recently, the HCV subgenomic replicon system was developed by Lohmann et al. [3]. The replicon is a bicistronic construct composed of the HCV internal ribosome entry site (IRES), the neomycin phosphotransferase gene (Neo^r), the IRES of encephalomyocarditis virus (EMCV), the HCV NS proteins (NS3 through NS5B), and the HCV 3′X region. Human hepatoma-derived Huh-7 cells [4], which are transfected with in vitro synthesized replicon RNA and selected by G418, are capable of supporting the replication of replicon RNA. This system currently becomes a new tool to investigate the HCV replication and pharmacological mechanisms of anti-HCV drugs.

Interferon (IFN)-α has been widely used as an effective antiviral agent for persistent HCV infection [5]. However, approximately 40% of patients with chronic hepatitis C did not reveal the sustained eradication of serum HCV RNA even by the most effective combination therapy of PEG-IFN-α with ribavirin [6]. To improve the efficacy of IFN-α-based therapy, more detailed mechanisms,

* Corresponding author. Fax: +81-6-6879-3449.
E-mail address: hayashin@moltx.med.osaka-u.ac.jp (N. Hayashi).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.07.015

through which the IFN-α-mediated antiviral activity is exerted in HCV-infected hepatocytes, should be clarified. It is well known that the Janus kinase (JAK)-signal transducer and activator transcription factor (STAT) pathway plays a critical role in the generation of IFN-α-mediated signal transduction and transcriptional activation [7]. In response to IFN-α stimulation, JAKs and STATs are phosphorylated, followed by the complex formation of IFN-stimulated gene factor (ISGF)-3 composed by STAT1, STAT2, and the IFN regulatory factor-9. Then, ISGF-3 translocates to the nucleus, binds to the IFN-stimulated response element (ISRE), and triggers expression of a number of IFN-stimulated genes (ISGs). Antiviral activity of IFN-α is believed to be exhibited by gene products of ISGs, including the double-stranded RNA-activated protein kinase (PKR), 2′–5′ oligoadenylate synthetase (OAS), and Mx. Recent studies demonstrated that IFN-α activates other signal-transducing molecules, such as p38 [8], extracellular-regulated kinase (ERK) [9], phosphatidylinositol 3 kinase (PI3-kinase) [10], and protein kinase C (PKC)-δ [11]. However, it has not been fully elucidated whether these molecules are involved in the antiviral activity of IFN-α.

To clarify this, we studied the IFN-α-mediated anti-HCV activity using the HCV subgenomic replicon cells. The involvement of various signal transducing molecules in the anti-HCV activity was investigated by treating the cells with IFN-α and specific inhibitors.

Materials and methods

Cells and transfection. Huh-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glucose (1 mg/ml), penicillin G (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 atmosphere. Plasmid pNNeo/3-5B, which was a kind gift of Dr. Stanley M. Lemon (Department of Microbiology and Immunology, University of Texas Medical Branch) [12], contained the sequence of HCV subgenomic replicon, which was originated from the HCV-N strain (GenBank Accession No. AF139594). Plasmid pNNeo/3-5B(SI) was constructed from pNNeo/3-5B by introducing the adaptive mutation S2005I [12] by means of site-directed mutagenesis. After pNNeo/3-5B(SI) was linearized with *XbaI*, HCV replicon RNA was prepared using the Megascript T7 kit (Ambion). Then, Huh-7 cells were transfected with the replicon RNA by electroporation, followed by the culture in the medium containing 0.8 mg/ml G418 for 3 weeks. Subsequently, individual G418-resistant colonies were picked up, and one of them (designated Huh-repS2) was used for further experiments.

Antibodies and specific inhibitors. The p38 inhibitors (SB203580 and PD169316) and the PI3-kinase inhibitors (LY294002 and Wortmannin) were purchased from Calbiochem. The JAK-specific inhibitor, 2-(1,1-dimethylethyl)-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f] isoquinolin-7-one [13], also came from Calbiochem. The inhibitor of the mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP kinase 2, MK2) (Calbiochem) is a synthetic 13-residue peptide (KKKALNRQLGVAA) corresponding to the phosphorylation site of HSP27, one of the known substrates of MK2. This peptide has been demonstrated to inhibit MK2 competitively with the substrate peptide [14]. Antibodies against whole STAT1, and phosphorylated STAT1 at Tyr^{701} and Ser^{727} residues (pY-STAT1 and pS-STAT1) were from Upstate Biotechnology. Antibodies against whole p38, phosphorylated p38 at Thr^{180}/Tyr^{182} (p-p38), whole MK2, and phosphorylated MK2 at Thr^{334} (p-MK2) were purchased from Cell Signaling Technology. An antibody against PKR was obtained from Santa Cruz Biotechnology. An antibody against NS5A was kindly provided by Dr. Y. Matsuura (Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University), and an antibody against NS3 was purchased from East Coast Biologics. Natural IFN-α was provided by Sumitomo Pharmaceuticals.

Detection of HCV replicon RNA by PCR. About 2×10^5 of Huh-repS2 cells were seeded in a 6-well culture dish and treated with various inhibitors of signal-transducing molecules for 1 h prior to the addition of IFN-α. After IFN-α treatment, total cellular RNA was extracted from the cells with the ISOGEN reagent (Nippon Gene) based on the guanidine-isothiocyanate method. Reverse transcription (RT) was performed with 1 μg of the RNA sample using a primer, 5′-GG AAATGGCCTATTGGCCTGGAGT-3′ (nt 9427–9404) and the mutated Moloney murine leukemia virus reverse transcriptase (ReverTra Ace, TOYOBO). The cDNA was subsequently amplified using the KOD polymerase (TOYOBO) and a set of primers, 5′-TCGCACG GGCTGCGTGGGAAACAG-3′ (nt 8788–8811) and 5′-GTTTAGC TCCCCGTTCATCGATTGG-3′ (nt 9403–9379) by 29 PCR cycles involving denaturation at 94 °C for 15 s, annealing at 62 °C for 30 s, and extension at 68 °C for 1 min. The PCR products were subjected to the agarose gel electrophoresis and ethidium bromide staining. The nucleotide numbering is according to the HCV-N strain. As an internal control, the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was also examined. After cDNA synthesis with the oligo$(dT)_{20}$ primer (TOYOBO), the PCR was conducted using a commercially available set of primers to detect the G3PDH mRNA (TOYOBO).

Immunoblotting. The Huh-repS2 cells after IFN-α treatment were lysed in a RIPA buffer containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 100 μg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 50 mM sodium fluoride in phosphate-buffered saline (pH 7.4). Fifty micrograms protein/lane was separated on the SDS–PAGE and transferred onto the nitrocellulose membrane (Hybond-P, Amersham–Pharmacia Biotech). Next, the membrane was incubated with the appropriate concentration of the specific antibody, followed by incubation with the horseradish peroxidase-conjugated second antibody. Then, the signals were developed by chemiluminescence (Supersignal, Pierce).

Reporter gene assay. The reporter plasmid pISRELuci, which was a kind gift of Dr. T. Hirano (Division of Molecular Oncology, Osaka University Graduate School of Medicine), carried the three tandem repeats of ISRE upstream of the minimal promoter and the firefly luciferase gene. pRLtk (Clontech) was the seapansy luciferase-expressing plasmid. For the assay, 3×10^5 of Huh-repS2 cells were seeded in a 6-well culture dish and transfected with 1 μg pISRELuci and 0.1 μg pRLtk using Lipofectin (Invitrogen). The cells were cultured for 2 days after transfection. After preincubation with the p38 inhibitors or the JAK inhibitor for 1 h, IFN-α was added at a concentration of 100 U/ml. After subsequent incubation for 6 h, the cells were lysed and subjected to the dual luciferase assay using luminometer (Lumat LB9507, EG&G Berthold). The relative light unit of the sample without IFN-α stimulation was regarded as 1, and the fold activity of each sample was determined. The assay was done three times and the mean value was calculated.

Results

HCV replicon RNA is autonomously replicated in replicon cells and susceptible to IFN-α

Huh-7 cells were transfected with in vitro transcribed replicon RNA, and four independent clones were obtained. One of them (Huh-repS2) was subjected to

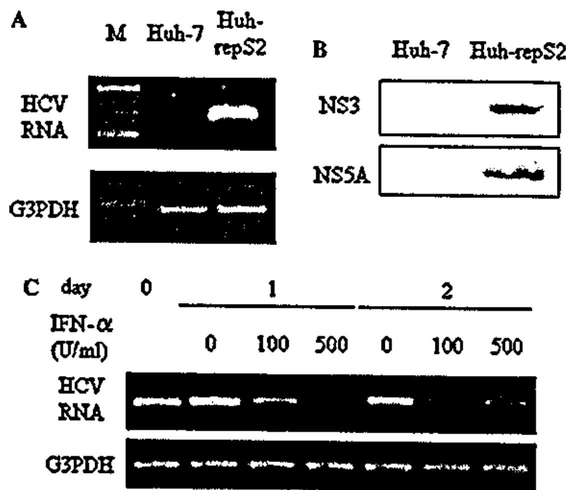

Fig. 1. Replication and susceptibility to IFN-α of HCV replicon RNA in Huh-repS2 cells. (A) HCV replicon RNA was detected by RT-PCR in Huh-7 and Huh-repS2 cells (upper panel). M, size marker. (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control. (B) Expression of HCV proteins NS3 and NS5A was examined by Western blot analyses in Huh-7 and Huh-repS2 cells. (C) Huh-repS2 cells were treated with 100 and 500U/ml IFN-α, or left untreated. Then, the replicon RNA was detected by RT-PCR at days 0, 1, and 2 (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control.

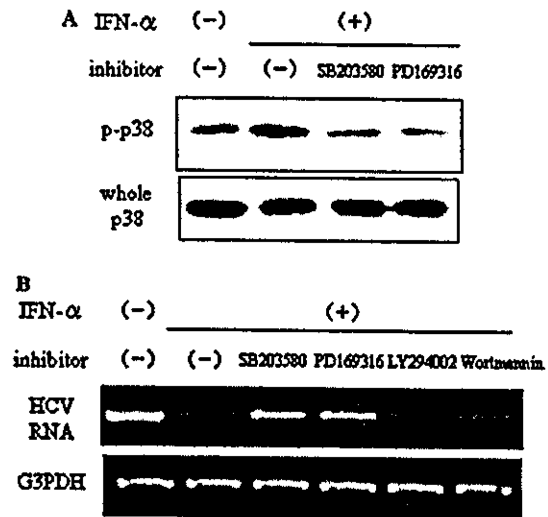

Fig. 2. Involvement of p38 in IFN-α-mediated anti-HCV activity in Huh-repS2 cells. (A) Huh-repS2 cells were treated with IFN-α (100U/ml) for 2h, or left untreated in the presence or absence of SB203580 (10μM) and PD169316 (10μM). Then, the levels of p-p38 and whole p38 were examined by Western blot analyses. (B) Huh-repS2 cells were treated with IFN-α (100U/ml) for 2 days, or left untreated in the presence or absence of SB203580 (10μM), PD169316 (10μM), LY294002 (10μM), and Wortmannin (100nM). Then, the replicon RNA was detected by RT-PCR (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control.

further experiments. The replicon RNA and HCV proteins were first detected in Huh-repS2 cells. As shown in Fig. 1A, Huh-repS2 cells specifically included the 3′ sequence of the replicon RNA, because the primers used in the PCR were located just upstream of the polyU stretch of HCV RNA. As for expression of NS3 and NS5A (Fig. 1B), these proteins were detected in the Huh-repS2 cells, but not in the parental Huh-7 cells. Next, the susceptibility of the Huh-repS2 cells to IFN-α was investigated. As shown in Fig. 1C, treatment with 100U/ml IFN-α for 2 days was enough to suppress the replicon RNA. The Huh-repS2 cells could not survive after 1 week in culture in the presence of both G418 and IFN-α (data not shown), indicating that Neo^r was not constitutively expressed in the cells. According to this, it was denied that the replicon construct was integrated into the host genome. In the Huh-repS2 cells, the HCV replicon RNA was autonomously replicated and susceptible to IFN-α.

p38 is involved in the anti-HCV activity of IFN-α

There are several reports indicating that IFN-α activates not only the JAK-STAT pathway but also other signaling pathways [8–11]. To investigate the involvement of pathways other than JAK-STAT in the anti-HCV activity under IFN-α stimulation, p38 inhibitors (SB203580 and PD169316) or PI3-kinase inhibitors (LY294002 and Wortmannin) were added to the Huh-repS2 cells prior to IFN-α treatment. Fig. 2A represents the expression level of p-p38, as a marker of p38 activity, in the Huh-repS2 cells treated with IFN-α in the presence or absence of p38 inhibitors. Phosphorylation of p38 was induced by IFN-α stimulation, as reported previously in other cells [8,15,16], and the induction was diminished by pretreatment of SB203580 and PD169316. On the other hand, the whole p38 level was not affected by IFN-α stimulation. When the effect of p38 and PI3-kinase inhibitors on expression of replicon RNA was examined in the IFN-α-treated Huh-repS2 cells (Fig. 2B), the anti-HCV effect of IFN-α was considerably reduced (but not completely abolished) by SB203580 and PD169316. In contrast, PI3-kinase inhibitors, LY294002 and Wortmannin, did not affect the suppressive effect on expression of replicon RNA by IFN-α. The similar experiments using the PKC-δ inhibitor rottlerin and the ERK inhibitor PD98059 were also carried out. The level of the replicon RNA was not changed by pretreatment of these inhibitors in the IFN-α-treated Huh-repS2 cells (data not shown). These results indicate that activation of p38 may play a substantial role in the generation of the anti-HCV activity by IFN-α. On the other hand, PI3-kinase, PKC-δ, and ERK were not involved in the antiviral activity of IFN-α in the HCV replicon cells.

IFN-α-mediated anti-HCV activity through p38 is independent of JAK-STAT signaling pathway

We further studied the anti-HCV activity of IFN-α through p38 in relation to the JAK-STAT pathway.

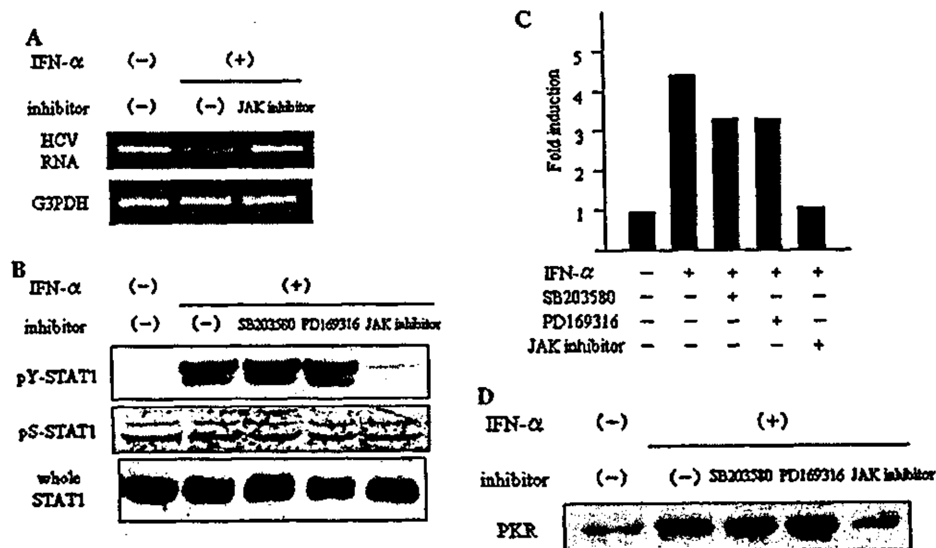

Fig. 3. Relationship of p38 with STAT1 phosphorylation and ISRE-dependent transcription in IFN-α-treated Huh-repS2 cells. (A) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 days, or left untreated in the presence or absence of JAK inhibitor (1 μM). Then, the replicon RNA was detected by RT-PCR (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control. (B) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 h, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), and JAK inhibitor (1 μM). Then, the levels of pY-STAT1, pS-STAT1, and the whole STAT1 were examined by Western blot analyses. (C) Huh-repS2 cells were cotransfected with pISRELuci and pRLtk. Two days after transfection, the cells were stimulated with IFN-α (100 U/ml) for 6 h, or left unstimulated with or without pretreatment of SB203580 (10 μM), PD169316 (10 μM), and JAK inhibitor (1 μM). Then, the cells were subjected to the dual luciferase assay. The assay was done three times and the mean value was calculated. (D) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 6 h, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), and JAK inhibitor (1 μM). Then, expression of PKR was examined by Western blot analysis.

Using the Huh-repS2 cells treated with IFN-α, the influence of the JAK inhibitor on expression of the replicon RNA was examined. As shown in Fig. 3A, the JAK inhibitor completely abolished the anti-HCV effect of IFN-α, indicating that JAK-STAT pathway is essential for the antiviral activity of IFN-α to be exhibited. STAT1 has been reported to possess two activating phosphorylation sites, Tyr^{701} and Ser^{727}. It has been shown that, under IFN-α stimulation, JAKs undergo the Tyr^{701} phosphorylation [17], whereas the Ser^{727} is phosphorylated by other kinases [18]. Especially, p38 has been suggested to be a candidate of the STAT1 kinase at the Ser^{727} [15]. Therefore, the levels of pY-STAT1 and pS-STAT1 were examined in the IFN-α-treated Huh-repS2 cells in the presence of p38 and JAK inhibitors (Fig. 3B). The strong induction of pY-STAT1 was observed by IFN-α treatment. This induction was prevented by the JAK inhibitor but not by the p38 inhibitors SB203580 and PD169316. In contrast, the pS-STAT1 was observed irrespective of IFN-α treatment, and its level was not influenced by both p38 and JAK inhibitors. Also, the whole STAT1 level was not affected by treatment with IFN-α and various inhibitors. Thus, contrary to the previous report [15], p38 did not participate in the STAT1 phosphorylation in the Hep-repS2 cells under IFN-α stimulation. As for the ISRE-dependent transcription activity, as determined by the reporter gene assay (Fig. 3C), JAK inhibitor completely decreased the activity to the baseline, whereas p38 inhibitor had a minimal influence on it. When expression of PKR, a member of ISGs, was also detected in the Hep-repS2 cells (Fig. 3D), it was induced by IFN-α stimulation. The induction of PKR expression by IFN-α was diminished by pretreatment of the JAK inhibitor but not by that of the p38 inhibitor, as was the case for the reporter gene assay. According to these results, the blockade of p38 had only a minimal effect on the JAK-STAT pathway under IFN-α stimulation. The IFN-α-mediated anti-HCV activity through p38 was almost independent of the JAK-STAT pathway.

p38-MK2 pathway is responsible for the anti-HCV activity of IFN-α

It has been demonstrated that MK2 retains in the nucleus interacting with p38, and that activation of p38 causes MK2 phosphorylation [19]. To further examine the downstream event of p38 in the anti-HCV efficacy of IFN-α, we focused on MK2 among the proteins identified as substrates of p38. The time course analyses in the expression levels of p-p38, p-MK2, whole p38, and whole MK2 were performed in Huh-repS2 cells after IFN-α stimulation. As shown in Fig. 4A, the boost of the p-p38 level was observed at 15 min after stimulation

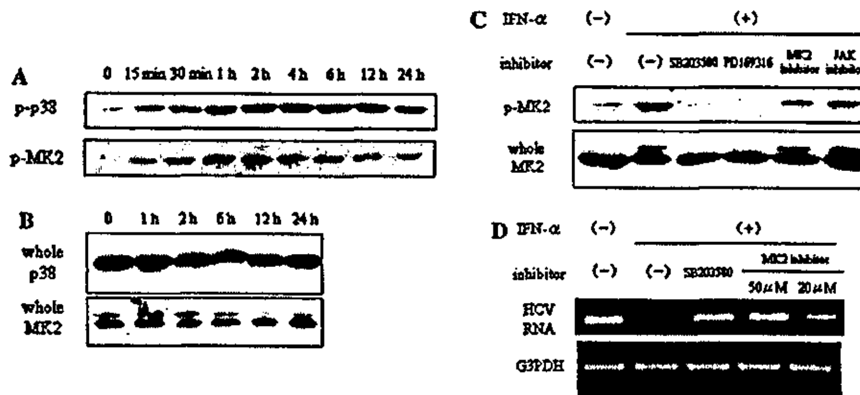

Fig. 4. Involvement of MK2 in IFN-α-mediated anti-HCV activity in Huh-repS2 cells. (A) Huh-repS2 cells were treated with IFN-α (100 U/ml), and the levels of p-p38 and p-MK2 were examined by Western blot analyses before and after the treatment. (B) Huh-repS2 cells were treated with IFN-α (100 U/ml), and the levels of whole p38 and MK2 were examined by Western blot analyses before and after the treatment. (C) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 4h, or left untreated in the presence or absence of SB203580 (10 μM), PD169316 (10 μM), MK2 inhibitor (50 μM), and JAK inhibitor (1 μM). Then, the levels of p-MK2 and the whole MK2 were examined by Western blot analyses. (D) Huh-repS2 cells were treated with IFN-α (100 U/ml) for 2 days, or left untreated in the presence or absence of SB203580 (10 μM) and the MK2 inhibitor (50 μM and 20 μM). Then, the replicon RNA was detected by RT-PCR (upper panel). (Lower panel) G3PDH mRNA examined by RT-PCR as an internal control.

and continued thereafter. Expression of p-MK2, as a marker of MK2 activity, followed this pattern, but its level showed attenuation after 12h. It is speculated that this attenuation of p-MK2 might be due to the induction of some MK2 phosphatase(s). On the other hand, the levels of whole p38 and MK2 were unchanged by IFN-α stimulation (Fig. 4B). Next, the effects of p38, JAK, and MK2 inhibitors on the p-MK2 level were investigated in the IFN-α-treated Huh-repS2 cells (Fig. 4C). The induction of p-MK2 after IFN-α treatment was blocked by the p38 inhibitors SB203580 and PD169316, but not by the JAK inhibitor. The MK2 inhibitor did not also influence the level of p-MK2, because it was a peptide competitively inhibiting MK2 activity [14]. The whole MK2 level was not affected by treatment with IFN-α and various inhibitors. These findings indicate that MK2 may function as a downstream kinase of p38, apart from the JAK-STAT pathway. The effect of the MK2 inhibitor on expression of replicon RNA was compared with that of the p38 inhibitor in the IFN-α-treated Huh-repS2 cells. As shown in Fig. 4D, the addition of MK2 inhibitor (50 μM) showed the same level of reduction in the IFN-α-mediated anti-HCV activity as that of the p38 inhibitor SB203580. According to these, the p38-MK2 pathway may be responsible for the anti-HCV activity of IFN-α in the Huh-repS2 cells.

Discussion

Under IFN-α stimulation, the signal from the specific receptor is transmitted by the JAK-STAT pathway, and the antiviral effect is believed to be achieved by a number of gene products of ISGs including PKR, OAS, and Mx [20]. It has also been demonstrated that several signal transducing molecules, such as p38, ERK, PI3K, and PKC-δ, are activated in response to IFN-α [8–11]. The aim of this study was to investigate the contribution of pathways other than JAK-STAT to the anti-HCV activity by IFN-α. It was carried out by adding various inhibitors of the signal transducing molecules to the HCV replicon Huh-repS2 cells treated with IFN-α. Among them, the activation of p38 was found to play a role in the generation of IFN-α-mediated anti-HCV activity.

p38 belongs to a mitogen-activated protein kinase (MAPK) family and is activated by various cellular stresses and cytokines [21]. A few investigators have so far suggested that the p38 activation may be an upstream event of IFN-α-mediated gene transcription driven by ISRE. Goh et al. [15] showed that p38 underwent the STAT1 phosphorylation at Ser^{727}, resulting in the ISRE-dependent transcriptional activation. It has also been revealed by Li et al. [22] that the p38 activation did not affect the STAT1 phosphorylation and ISGF3 formation but was essential for ISRE-dependent transcription. In this study, we examined the relationship of p38 with the STAT1 phosphorylation and the ISRE-dependent transcription activity in the IFN-α-treated cells. In contrast to these previous reports, the level of the STAT1 phosphorylation was not affected by p38. Also, p38 had only a minimal effect on the ISRE-dependent transcription activity. These results indicate that the anti-HCV activity of IFN-α through p38 may be exhibited independent of the JAK-STAT pathway and the ISRE-dependent transcription at least in the Huh-repS2 cells.

As for functional relevance of p38 and JAK-STAT pathways to the anti-HCV efficacy of IFN-α, the activation of JAK-STAT and subsequently occurring transcriptional activation is primarily required, because the JAK inhibitor completely blocked the anti-HCV

activity of IFN-α in the Huh-repS2 cells. However, the activation of p38 may also play a substantial role in the generation of the anti-HCV effect of IFN-α. Our findings suggest that the two independent signaling pathways, JAK-STAT and p38, may be important for the anti-HCV activity of IFN-α to be fully exerted.

In the present study, we also addressed the role of MK2, one of the substrates of p38 [20], in the exhibition of anti-HCV activity by IFN-α. It was found that MK2 worked as a downstream kinase of p38 under IFN-α stimulation and was responsible for the IFN-α-mediated antiviral activity in the Huh-repS2 cells. Very recently, Li et al. [22] reported that the antiviral response by IFN-α was decreased in the MK2-deficient mouse embryo cells with the EMCV infection. Our result agreed with this report with respect to the functional importance of MK2 in the antiviral efficacy of IFN-α.

In conclusion, we showed that the activation of the p38-MK2 pathway may be substantially involved in the generation of anti-HCV activity by IFN-α. Further studies are required to clarify the mechanisms downstream of MK2 in the antiviral effect of IFN-α.

References

[1] L.B. Seeff, Natural history of hepatitis C, Hepatology 26 (1997) 21S–28S.

[2] K. Kiyosawa, T. Sodeyama, E. Tanaka, Y. Gibo, K. Yoshizawa, Y. Nakano, S. Furuta, Y. Akahane, K. Nishioka, R.H. Purcell, H.J. Alter, Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus, Hepatology 12 (1990) 671–675.

[3] V. Lohmann, F. Körner, J.-O. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[4] H. Nakabayashi, K. Taketa, K. Miyano, T. Yamane, J. Sato, Growth of human hepatoma cells lines with differentiated functions in chemically defined medium, Cancer Res. 42 (1982) 3858–3863.

[5] H. Hagiwara, N. Hayashi, E. Mita, T. Takehara, A. Kasahara, H. Fusamoto, T. Kamada, Quantitative analysis of hepatitis C virus RNA in serum during interferon alpha therapy, Gastroenterology 104 (1993) 877–883.

[6] M.W. Fried, M.L. Shiffman, K.R. Reddy, C. Smith, G. Marinos, F.L. Goncales Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffman, J. Yu, Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection, N. Engl. J. Med. 347 (2002) 975–982.

[7] G.R. Stark, I.M. Kerr, B.R.G. Williams, R.H. Silverman, R.D. Schreiber, How cells respond to interferons, Annu. Rev. Biochem. 67 (1998) 227–264.

[8] S. Uddin, B. Majchrzak, J. Woodson, P. Arunkumar, Y. Alsayed, R. Pine, P.R. Young, E.N. Fish, L.C. Platanias, Activation of the p38 mitogen-activated protein kinase by type I interferons, J. Biol. Chem. 274 (1999) 30127–30131.

[9] T. Arora, G. Floyd-Smith, M.J. Espy, D.F. Jelinek, Dissociation between IFN-alpha-induced anti-viral and growth signaling pathways, J. Immunol. 162 (1999) 3289–3297.

[10] S. Uddin, E.N. Fish, D.A. Sher, C. Gardziola, M.F. White, L.C. Platanias, Activation of the phosphatidylinositol 3-kinase serine kinase by IFN-alpha, J. Immunol. 158 (1997) 2390–2397.

[11] S. Uddin, A. Sassano, D.K. Deb, A. Verma, B. Majchrzak, A. Rahman, A.B. Malik, E.N. Fish, L.C. Platanias, Protein kinase C-δ (PKC-δ) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727, J. Biol. Chem. 277 (2002) 14408–14416.

[12] M. Ikeda, M. Yi, K. Li, S.M. Lemon, Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells, J. Virol. 76 (2002) 2997–3006.

[13] J.E. Thompson, R.M. Cubbon, R.T. Cummings, L.S. Wicker, R. Frankshun, B.R. Cunningham, P.M. Cameron, P.T. Meinke, N. Liverton, Y. Weng, J.A. DeMartino, Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor, Bioorg. Med. Chem. Lett. 12 (2002) 1219–1223.

[14] K. Hayess, R. Benndorf, Effect of protein kinase inhibitors on activity of mammalian small heat-shock protein (HSP25) kinase, Biochem. Pharmacol. 53 (1997) 1239–1247.

[15] K.C. Goh, S.J. Haque, B.R.G. Williams, p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons, EMBO J. 20 (1999) 5601–5608.

[16] S. Uddin, F. Lekmine, N. Sharma, B. Majchrzak, I. Mayer, P.R. Young, G.M. Bokoch, E.N. Fish, L.C. Platanias, The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon α-dependent transcriptional activation but not serine phosphorylation of Stat proteins, J. Biol. Chem. 275 (2000) 27634–27640.

[17] K. Shuai, A. Ziemiecki, A.F. Wilks, A.G. Harpur, H.B. Sadowski, M.Z. Gilman, J.E. Darnell, Polypeptide signalling to the nucleus through tyrosine phosphorylation of Jak and Stat proteins, Nature 366 (1993) 580–583.

[18] X. Zhu, Z. Wen, L.Z. Xu, J.E. Darnell, Stat1 serine phosphorylation occurs independently of tyrosine phosphorylation and requires an activated Jak2 kinase, Mol. Cell. Biol. 17 (1997) 6618–6623.

[19] A. Kotlyarov, M. Gaestel, Is MK2 (mitogen-activated protein kinase-activated protein kinase 2) the key for understanding post-transcriptional regulation of gene expression?, Biochem. Soc. Trans. 30 (2002) 959–963.

[20] C.E. Samuel, Antiviral actions of interferons, Clin. Microbiol. Rev. 14 (2001) 778–809.

[21] L.C. Platanias, The p38 mitogen-activated protein kinase pathway and its role in interferon signaling, Pharmacol. Ther. 98 (2003) 129–142.

[22] Y. Li, A. Sassano, B. Majchrzak, D.K. Deb, D.E. Levy, M. Gaestel, A.R. Nebreda, E.N. Fish, L.C. Platanias, Role of p38α map kinase in type I interferon signaling, J. Biol. Chem. 279 (2004) 970–979.

ELSEVIER

Journal of Hepatology 41 (2004) 637–643

Journal of Hepatology

www.elsevier.com/locate/jhep

Ninjurin1 increases p21 expression and induces cellular senescence in human hepatoma cells

Takashi Toyama[1,*], Yutaka Sasaki[2], Masayoshi Horimoto[1], Kenya Iyoda[2], Takayuki Yakushijin[2], Kazuyoshi Ohkawa[1], Tetsuo Takehara[2], Akinori Kasahara[3], Toshiyuki Araki[4], Masatsugu Hori[1], Norio Hayashi[2]

[1]*Department of Internal Medicine and Therapeutics, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[2]*Department of Molecular Therapeutics, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[3]*Department of General Medicine, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan*
[4]*Department of Pathology and Immunology and Internal Medicine, Washington University Medical School, St. Louis, MO 63110, USA*

***Background/Aims*: Ninjurin1 is a novel adhesion molecule that has a role in promoting nerve regeneration. Although ninjurin1 is ubiquitously expressed in various human tissues, including the liver, the biologic functions of ninjurin1 in tissues other than the nervous system remain unknown. The aim of this study was to investigate the function of ninjurin1 in hepatocytes.**

***Methods*: The effect of ninjurin1 overexpression was examined in Huh-7 hepatoma cells. Ninjurin1 expression was examined by Western blot in human hepatocellular carcinoma tissues as well as their adjacent liver tissues.**

***Results*: Ninjurin1-overexpressing clones exhibited strong growth inhibition due to G1 cell cycle arrest, which is associated with a posttranscriptional increase in $p21^{WAF1/Cip1}$, a decrease of cyclin-dependent kinase 2 activity and the hypophosphorylation of Rb. The ninjurin1-overexpressing clones had increased senescence-associated β-galactosidase activity and autofluorescent pigment, characteristic features of cellular senescence. The levels of ninjurin1 expression were higher in hepatocellular carcinoma tissues than those in adjacent liver tissues.**

***Conclusions*: The present study provides the first evidence that ninjurin1 is able to induce the senescence program. Ninjurin1 may be involved in the regulation of cellular senescence in the liver during carcinogenesis.**
© 2004 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

***Keywords*: Ninjurin1; Cellular senescence; p21; G1 Arrest; Carcinogenesis; Hepatocellular carcinoma**

1. Introduction

Ninjurin1 (nerve injury-induced protein 1) is a novel adhesion molecule first isolated as a gene induced in Schwann cells after nerve injury [1]. Ninjurin1 is located on the cell surface of Schwann cells and neurons, and has an important role in neurite regeneration via homophilic interactions between neuronal axons and Schwann cells [2]. In addition, ninjurin1 is ubiquitously expressed in various human tissues originating from epithelial cells (e.g. liver, kidney, thymus, adrenal gland) [3]. However, the biological relevance of ninjurin1 on tissues other than the nervous system remains unknown.

In the present study, we explored the biologic and biochemical consequences of ninjurin1 overexpression in a human hepatoma cell line. Ninjurin1 expression in Huh-7 hepatoma cells induced p21 elevation and G1 cell cycle arrest. Furthermore, ninjurin1-overexpressing cells exhibited the characteristic features of cellular senescence: increased senescence-associated (SA) β-galactosidase activity and the presence of autofluorescent pigment. In addition, the levels of ninjurin1 expression were higher in human hepatocellular carcinoma (HCC) tissues than in their

Received 15 January 2004; received in revised form 9 June 2004; accepted 25 June 2004; available online 20 July 2004

* Corresponding author. Address: Department of Molecular Therapeutics, Graduate School of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: +81-6-6879-3636; fax: +81-6-6879-3639.

E-mail address: toyamat@moltx.med.osaka-u.ac.jp (T. Toyama).

0168-8278/$30.00 © 2004 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jhep.2004.06.027

adjacent liver tissues. The present study suggests a possible role of ninjurin1 in regulating cellular senescence pathologically arising in the liver.

2. Materials and methods

2.1. Antibodies

Anti-human ninjurin1 mouse polyclonal antibody and the anti-pRb antibody (14001A) were obtained from Pharmingen/Transduction Laboratories (San Diego, CA). Polyclonal antibodies for p21 (C-19), p27 (F-8), cyclinD1 (M-20), cyclinE (M-20), cyclinA (H-432), cyclin-dependent-kinase2 (CDK2) (H-298), CDK4 (C-22), and CDK6 (C-21) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

2.2. Establishment of ninjurin1-transfected Huh-7 cells

Huh-7 cells, a human hepatoma cell line, were obtained from the Japanese Cancer Research Resource Bank Cell Bank Center (Tokyo, Japan). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented fetal calf serum (FCS) and antibiotics. Human ninjurin1 cDNA was excised from a pT7T3 plasmid [2] and inserted into a cloning site of pcDNA3.1 plasmid (Invitrogen, Groningen, the Netherlands). The construction was verified by sequencing (ABI Prism310; Perkin–Elmer, Foster City, CA). The ninjurin1 expression plasmid was transfected into Huh-7 cells using a Lipofectin reagent (Gibco BRL/Life Technologies, Grand Island, NY). Selection was performed via the addition of 100 μg/ml Zeosin (Invitrogen, Co. Ltd., Japan). Using this method, the ninjurin1-transfected cells generated much fewer Zeosin-resistant colonies than cells transfected with vector alone (mock clones). In addition, because Zeosin-resistant clones grew slowly, it took over a month to pick the slowest growing colony. After the Zeosin-resistant clones were screened for ninjurin1 expression by immunoblotting, four positive single clones were selected. Two positive clones (Nin1 and Nin2) and mock clones were used for the experiments, but similar results were obtained using the other two clones, and the magnitude of the effects observed were proportional to the amount of ninjurin1 expressed by each clone.

2.3. Growth curves

The control cells and ninjurin1-overexpressing clones were seeded at a density of 1×10^4 cells/35 mm-diameter dish. At 1, 2, 3, and 4 days after plating, these cells were removed from the culture plate by short exposure to trypsin and EDTA (GIBCO-BRL/Life Technologies). The total cell number was determined by trypan blue staining via microscopy.

2.4. Cell-cycle analysis

Exponentially growing cultures of the control cells and ninjurin1-overexpressing clones were trypsinized and collected. Cell pellets were fixed in 70% ethanol and stored at −20 °C. On the day of the assay, the fixed cells were collected by centrifugation, and the pellets were resuspended in PBS containing 0.2 mg/ml propidium iodide, and 1 mg/ml RNase. After 30 min incubation, the cell suspension was filtered through a 60 μM Spectra mesh filter and analyzed with a FACS Caliber flow cytometer using CellQuest software (Becton Dickinson Co. Chino Hills, CA). The percentages of cells in different phases of the cell cycle were determined with the ModFit 2.0 computer program (Verity Software House, Inc., Topsham, ME).

2.5. Immunocytochemical detection of ninjurin1

Huh-7 cells were fixed with paraformaldehyde and permeabilized with 0.05% Triton X-100. After blocking with Block Ace (Snow Brand Milk Product Co., Sapporo, Japan) containing 10% normal goat serum, cells were incubated for 1 h with anti-ninjurin1 polyclonal antibody. The cells were further incubated with Alexa® 488 goat anti-mouse IgG (Molecular Probes Inc., Eugene, OR). Images were acquired by confocal laser microscopy using a Zeiss LSM510 (Carl Zeiss Co., Ltd., Germany).

2.6. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL)

Cells were fixed with neutral buffered formaldehyde on day 3 after plating. Apoptotic cells were detected using an In situ Apoptosis Detection Kit (WAKO, Tokyo, Japan) according to the manufacturer's instructions. The percentage of TUNEL-positive cells relative to total cells was determined by counting 100–300 cells in 10 randomly chosen fields per cover slide using a Nikon Microphot-FXA (Nikon, Tokyo, Japan).

2.7. Western blot analysis

Whole cell lysates were prepared in Triton lysis buffer. Protein concentration was determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL). Identical amounts of protein were separated on an acrylamide gel, and transferred to Hybond-P membranes (Amersham Pharmacia Biotech Co. Ltd., Buckinghamshire, UK). The membrane was blocked and then incubated with each antibody. The membrane was further incubated with the horseradish peroxidase-conjugated immunoglobulin. Detection was performed using the enhanced chemiluminescence (ECL) assay protocol (Pierce Chemical). The signal intensities were analyzed using an NIH image program (National Institutes of Health, Bethesda, MD).

2.8. RNA isolation and northern blot analysis

Total cellular RNA was prepared using Trizol reagent (GIBCO-BRL/Life Technologies) in accordance with the manufacturer's instructions. For Northern blot analysis, 20 μg of RNA was separated on 1.0% agarose formaldehyde gels and transferred onto Hybond-N membranes (Amersham Pharmacia Biotech Co. Ltd.), and crosslinked to the membrane with a GS GENE Linker (BioRad Laboratories, Hercules, CA). mRNAs were detected by hybridization with random-primer-labeled probes (Amersham Pharmacia Biotech Co. Ltd.).

2.9. Kinase assay

In vitro phosphorylation of Rb by CDKs was detected by the phosphoFind™ (Boston Biologicals, Inc, Bedford, MA) system following the manufacturer's instructions. Whole cell lysates were prepared in Triton lysis buffer, with 20 μg of total cell protein per sample being precleared with 15 μl of protein G-Sepharose beads (Amersham Pharmacia Biotech Co. Ltd.). Immunoprecipitation of CDKs was performed with 15 μl of each anti-CDK polyclonal antibody. For the kinase reaction, immunocomplexes were incubated in kinase buffer supplemented with ATP-γ-S and Rb kinase substrate (Boston Biologicals). Samples were then separated on an acrylamide gel and transferred to Hybond-P membranes. The membrane was blocked and then incubated with PhosphoFind antibody (Boston Biologicals). The membrane was further incubated with horseradish peroxidase-conjugated antibody. Detection of the phosphorylated Rb was performed using the ECL assay protocol (Pierce Chemical).

2.10. Senescence-associated β-galactosidase

SA-β-galactosidase activity was detected using a SA-β-galactosidase staining kit (Cell Signaling Technology, Inc., Beverly, MA) according to the manufacturer's instructions. The control cells and ninjurin1-overexpressing clones were fixed and stained at pH 6.0 with X-gal. Clear blue cytoplasmic staining was regarded as positive. The percentage of SA-β-galactosidase activity-positive cells relative to total cells was determined by counting 100–300 cells in 10 randomly chosen fields per dish using phase-contrast microscopy (ECLIPSE TS 100, Nikon).

2.11. Autofluorescence

The control cells and ninjurin1-overexpressing clones cultured on Lab-Tek® four-chamber slides (Nalge Nunc International Corp, Naperville, IL) were fixed with neutral buffered formaldehyde. Images (×200) were then obtained using a green filter (FT510, Carl Zeiss Co., Ltd.), with 60-s exposure using a fluorescence microscope (Axioplan2, Carl Zeiss Co., Ltd.).