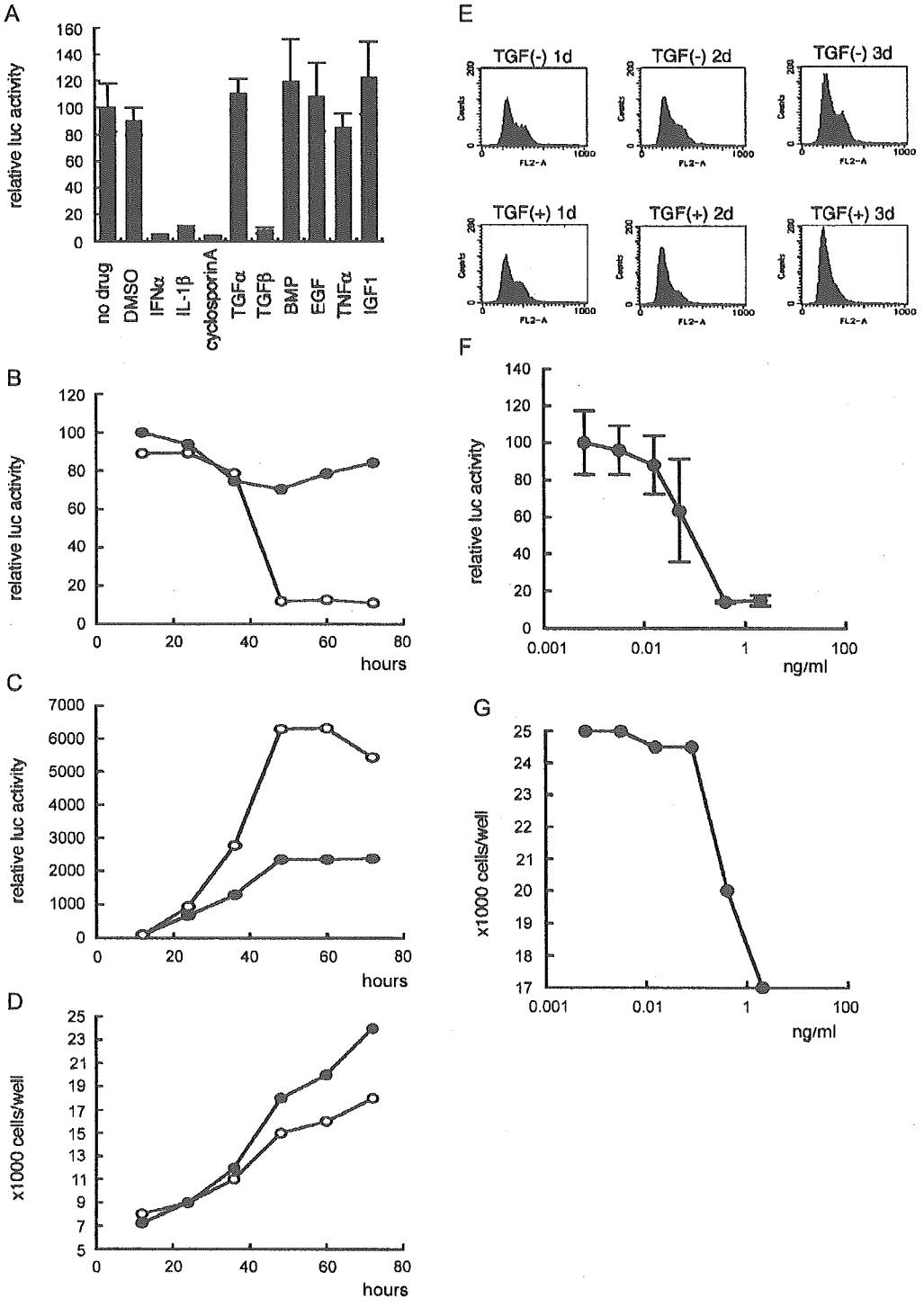
approximately 0.1 ng/ml TGF-β (Fig. 2F). Cell growth started to be suppressed at similar concentrations (Fig. 2G).

Smad-dependent suppression of HCV replicon

The cellular effects of TGF-β are mediated by both type I and type II serine/threonine kinase receptors. Receptor ligation is followed by the activation of both the canonical Smad and the MAPK signaling pathways, which include p38 MAPK, JNK, and ERK (Derynck and Zhang, 2003; Miyazono et al., 2000). To clarify the signaling pathways crucial for the suppression of HCV replication, we utilized two constitutive active receptors. One constitutively active TGF-β type-I receptor, TβR-I(T/D), evokes both Smad and



MAPK pathways, even in the absence of the TGF-β ligand (Imamura et al., 1997). The other constitutively active type-I receptor, TβR-ImL45(T/D), only activates the MAPK pathways, lacking the ability to excite Smad signaling (Yu et al., 2002). Three days after transfection of curedMH14 cells with the combination of the luciferase-replicon (LMH14) RNA and the TβR-I(T/D) expression plasmid, luciferase activity was reduced to 37% of the control levels, even in the absence of TGF-β (Fig. 3A). In contrast, co-transfection with TβR-ImL45(T/D) produces 71% of the control levels of luciferase activity, suggesting that the second constitutively active receptor had little effect on HCV replication (Fig. 3A). As summarized in Figs. 4B and C, the TβR-I(T/D) receptor likely activated transcription from promoters containing either AP-1-or Smad-responsive elements, while the TβR-ImL45(T/D) evoked transcription from the AP-1-dependent promoter alone. These data serve as evidence that the antiviral activity of TGF-β is dependent on Smad signaling.

Upon TGF-β stimulation, R-Smads specific for this cytokine, Smad2 and Smad3, are activated, forming complexes with the Co-Smad, Smad4, to activate transcription from the corresponding promoters. As co-expression of Smad2 with Smad4 or Smad3 with Smad4 mimics the effects of TGF-β stimulation (Yingling et al., 1997), we tested if such the co-expression of these molecules would also suppress HCV replicon production. Transfection of either Smad2/4 or Smad3/4 reduced the luciferase activity to 16% or 13%, respectively, even in the absence of TGF-β (Fig. 3A). Expression of an inhibitory-Smad (I-Smad), Smad7, which inhibits TGF-β mediated Smad signaling (Derynck and Zhang, 2003), only modestly reduced (81% of the control) the luciferase activity (Fig. 3A). The enhancement of transcription from AP-1-responsive (Fig. 3C), as well as TGF-β-responsive (Fig. 3B), promoters by co-transfection of either Smad2/4 or Smad3/4 was expected, as Smad4 itself has been reported to elicit transcription from AP-1 binding site-containing promoters (Liberati et al., 1999; Yingling et al., 1997).

To verify whether the Smad but not MAPK signaling is crucial for the suppression of HCV replicon by TGF-β,we used several specific MAPK inhibitors to examine if the antiviral effect of TGF-β is associated with the activation of specific MAPKs. The addition of inhibitors of ERK, p38, or JNK, U0126, SB20350, or dexamethasone, respectively, did not cancel TGF-β suppression of luciferase activity (Fig. 4A), despite effective inhibition of kinase activity by the inhibitors (Figs. 4B–D). The phosphorylation of ERK by TGF-β was not observed under these conditions (Fig. 4B), as ERK was already activated by growth factors contained within the bovine serum supplementing the culture medium.

Suppression of G418-resistant replicon by TGF-β

We then investigated the effect of TGF-β on G418-resistant replicon RNA or protein levels. MH14, G418-resistant subgenomic replicon cells, were treated with IFN-α, TGF-β, or BMP-4. BMP-4 was used here because it is a member of TGF-β superfamily cytokines and it does not induce inhibition of cell proliferation at least in Huh-7 cells. Total RNA and protein were collected at various time points. RNAs were subjected to Real-Time RT-PCR (Fig. 5A) or Northern blotting (Fig. 5B), while proteins were examined by Western blotting (Fig. 5C). Replicon RNA levels gradually decreased following treatment with 2 ng/ml TGF-β to 0.6% of the levels observed in mock-treated samples on the 7th day. This inhibition was similar to that seen following treatment with 100 IU/ml IFN-α. The NS5A protein was virtually undetectable by the 5th day after transfection (Fig. 5C). The suppressive effect of TGF-β on viral protein production was also observed by indirect immunofluorescence (not shown).

Because the replicon RNA of MH14 cells has the EMCV IRES to produce NS proteins, one could not deny the possibility that the EMCV IRES might cause the inhibition by TGF-β. Therefore, we next used full-genome replicon cell line SNC#2 (Fig. 6), which has the HCV IRES instead of the EMCV IRES, and tested if TGF-β would affect the replication. As shown in Fig. 6B, replicon RNA in SNC#2 cells treated with TGF-β decreased clearly while BMP-4 did not suppress the RNA levels.

Simultaneous suppression of viral RNA replication and protein synthesis by TGF-β

The suppression of HCV replication by TGF-β treatment was associated with the inhibition of cell growth. We examined the kinetics of the suppression of HCV replication

Fig. 2. Suppression of luciferase-replicon by TGF-β. (A) curedMH14 cells transfected with the luciferase-replicon RNA construct (LMH14), were administered with DMSO (0.1%), IFN-α (100 IU/ml), IL-1β (10 ng/ml), cyclosporin A (1 μg/ml), TGF-α (1 ng/ml), TGF-β (2 ng/ml), BMP-4 (10 ng/ml), mEGF (100 ng/ml), TNF-α (10 ng/ml), or IGF-1 (1 ng/ml). Three days later, cellular luciferase activity was measured. Bars represent the mean and SD of three independent experiments. (B) curedMH14 cells transfected with LMH14 luciferase-replicon RNA construct were mock-treated (black circle) or treated with TGF-β (2 ng/ml, white circle). At the indicated times, cells were harvested for determination of luciferase activity. The activity was normalized to cell number. (C) In parallel with the experiments in Fig. 3B, cells transfected with pCMV-Luc were mock-treated (black circle) or treated with TGF-β (2 ng/ml, white circle). At the indicated times, cells were harvested for determination of luciferase activity. The activity was normalized to cell number. (D) In parallel with the experiments in Fig. 3B, cells were mock-treated (black circle) or treated with TGF-β (2 ng/ml, white circle). Cell numbers were counted at the indicated time points. (E) Flow cytometric analysis of cell cycle progressing in curedMH14 cells transfected with the S2204R luciferase-replicon RNA construct. Cells were incubated in the presence or absence of TGF-β (2 ng/ml) for 1, 2, or 3 days. The DNA content of these cells was analyzed as described in Materials and methods. Dose-dependence of luciferase-replicon (F) and cell growth inhibition (G). curedMH14 cells transfected with the LMH14 luciferase-replicon RNA construct were treated with varying concentrations of TGF-β for 3 days. Luciferase activity (F) and cell number (G) were subsequently determined. The luciferase activity was normalized to cell number and shown with the SD value of three experiments in F.

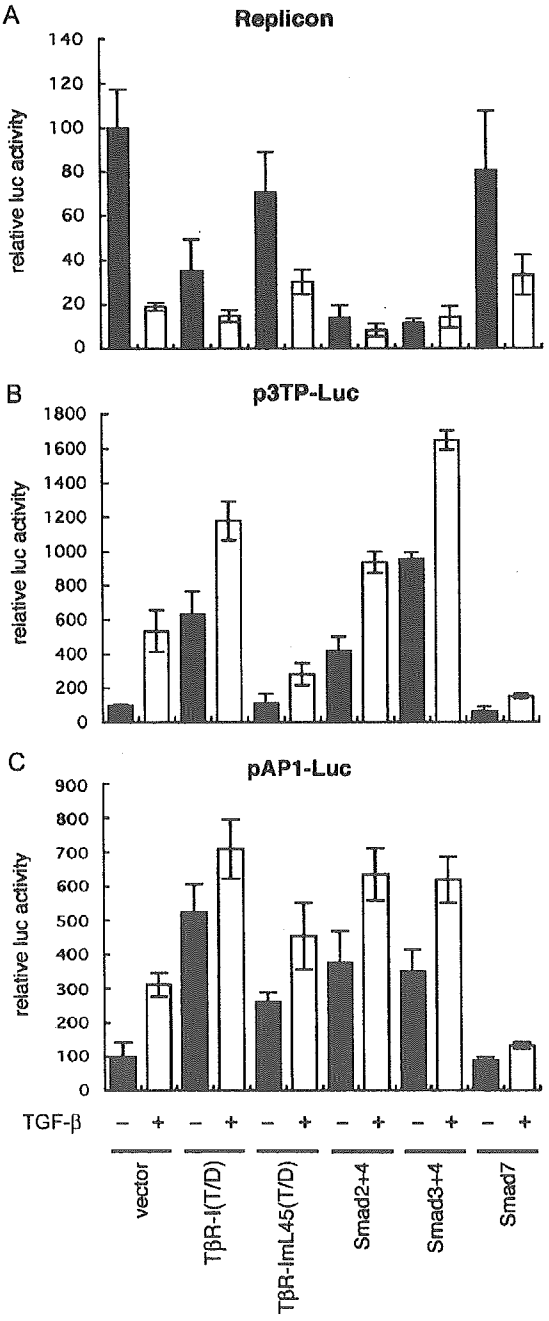


Fig. 3. Expression of TGF-β signaling-related proteins affected HCV replicon. curedMH14 cells were transfected with the expression plasmid designated below panel C together with (A) the luciferase-replicon construct with the mutations (LMH14), (B) p3TP-Luc, to monitor Smad-dependent transcription, or (C) pAP1-Luc, used to monitor AP-1-dependent transcription. Four hours after transfection, TGF-β (2 ng/ml) was added. Cells were incubated with or without the cytokine for 3 days (A) or 12 h (B, C) and then cellular luciferase activity was measured. The luciferase activity in A was normalized to cell number and then, the mean and SD value of three transfections are shown.

by examining viral RNA and protein synthesis rates at various times after the inoculation of replicon cells with the cytokine (Fig. 7). Aphidicolin and IFN-α were used as controls. The inhibition of viral protein synthesis by 100 IU/ml IFN-α began 12 h after treatment, while viral RNA synthesis was not affected until 24 h after cytokine addition. These results suggest that IFN-α first represses protein synthesis, thereby blocking RNA replication. In contrast, both aphidicolin and TGF-β inhibited protein synthesis and RNA replication concurrently, by 48 h after treatment. As both aphidicolin and TGF-β have growth inhibitory effects on cells, it is likely that both prevent HCV replicon in similar manners.

Anti-HCV activity of TGF-β was not mediated by IFN-induced signaling pathway

Although the antiviral activity of TGF-β was dependent on Smad signaling, the possibility remains that TGF-β may exert an antiviral activity via the same mechanisms as IFN-α and -γ. The binding of IFN-α or -γ to cellular receptors activates the JAK tyrosine kinase, which in turn phosphorylates effector Stat proteins. These proteins stimulate transcription from promoters with the specific sequences, ISRE or GAS, respectively. We prepared reporter plasmids that produce firefly luciferase following IFN-α or -γ stimulation by placing the gene under the control of a promoter containing either ISRE or GAS sequence. While 100 IU/ml IFN-α stimulation enhanced transcription from the ISRE-promoter 3.5-fold, enhancement of promoter activity was not observed following TGF-β treatment (Fig. 8A). The addition of 1000 IU/ml IFN-γ activated the GAS-dependent promoter by 5.8-fold, while TGF-β had little effect (Fig. 8B). The results suggest that TGF-β exerts its antiviral activity in a manner independent of IFN signaling.

Discussion

First, we have developed an efficient HCV subgenomic replicon system in this study. When maintained in cells, HCV replicon RNA often acquires cell culture-adaptive mutations. We found that the replicon RNA in MH14 cells carries two mutations, L1882L and S2204R. Among them, the S2204R, but not the L1882L, mutation was necessary and sufficient for the high efficiency (not shown). Although the mechanism by which the adaptive mutation produce high replication efficiency is not known, the interaction of the NS5A protein with a co-factor, such as hVAP-A (Gao et al., 2004; Tu et al., 1999), might explain the phenomenon.

As cytokines can play major roles in pathogenetic process during the courses of viral diseases, the relationships between viruses and cytokines, such as TGF-β, are of great importance. In this study, we found that TGF-β inhibits viral RNA replication and protein expression in the HCV replicon system.

The mechanism by which IFN-α suppresses HCV replicon is not well understood. Here, we showed that

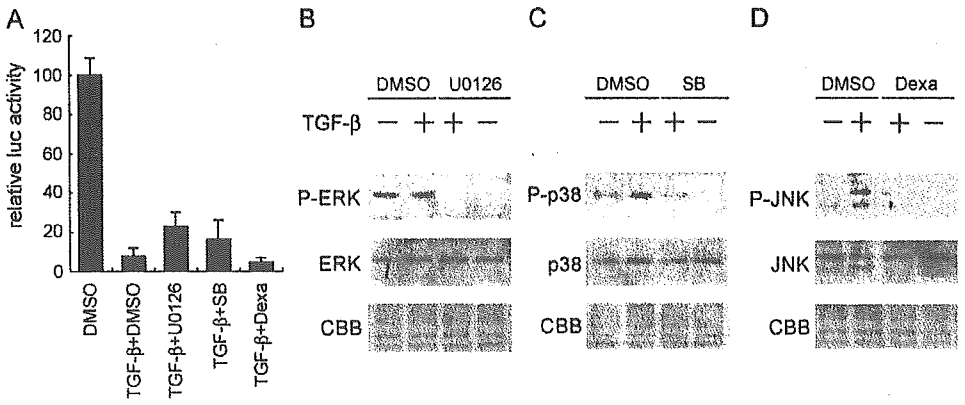


Fig. 4. Effect of MAPKs on the HCV replicon. (A) curedMH14 cells transfected with the luciferase-replicon construct (LMH14) were treated with DMSO, TGF-β (2 ng/ml) plus DMSO, or TGF-β (2 ng/ml) with either U0126 (3 μM), SB203580 (30 μM), or dexamethasone (1 μM). Inhibitors were added 1 h prior to the addition of TGF-β . After a 3-day treatment, cellular luciferase activity was measured. The mean and SD of three independent transfections are depicted after normalization to cell number. (B–D) The effect of inhibitors on the phosphorylation of ERK (B), p38MAPK (C), and JNK (D). Western blotting examined the phosphorylation of these molecules in cells treated with or without the designated reagents. The upper panels depict phosphorylated MAPKs, while the middle panels display the total amount of the MAPKs. CBB staining pattern of the same blot is used as a loading control (lower panel).

IFN-α repressed protein synthesis first, then suppressed RNA replication of the HCV replicon (Fig. 7). These data seem to support the previous report (Guo et al., 2004) and may aid our understanding of the suppression mechanism by IFN-α. IFN-α might suppress HCV translation trough the La (Shimazaki et al., 2002)-, ISG56 (Sumpter et al., 2004) or PKR (Wang et al., 2003)-dependent manner.

In contrast, either aphidicolin or TGF-β simultaneously inhibited both protein synthesis. and RNA replication from the replicon de novo (Fig. 7). While the mechanism of simultaneous suppression by aphidicolin or TGF-β remains unknown, both reagents arrest cell cycle progression at G(1)/S, suggesting a common target in the repression of HCV replicon expression. Recently, Scholle et al. (2004) reported that the replication of the HCV replicon RNA depends on host cell growth. Our results clearly correspond with that report, in which viral RNA levels remained unchanged during a 24-h period of cell cycle arrest followed by drops by 48 h.

It has been demonstrated that cell cycle arrest by TGF-β is mainly caused in the Smad pathway-dependent manner and the MAPK signaling serves as an accessory modifier of the arrest (Ten Dijke et al., 2002). Therefore, our result is convincing in that Smad, but not the MAPK pathway, played an essential role in the suppression of HCV replication.

Broadly speaking, serological investigations of HCV in chronically infected patients imply an inverse relationship between viral RNA load and TGF-β levels. Increased TGF-β expression significantly correlates with the degree of hepatic fibrosis (Calabrese et al., 2003; Nelson et al., 1997). A 3-year follow-up study demonstrated that TGF-β levels were elevated in patients with fibrosis that was

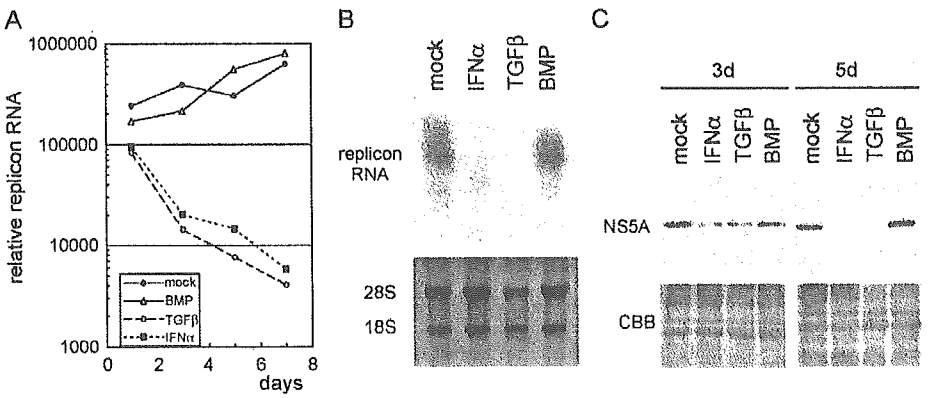


Fig. 5. Suppression of G418-resistant subgenomic replicon by TGF-β. (A) MH14, a G418-resistant subgenomic replicon cell line, was mock-treated or treated with BMP-4 (10 ng/ml), TGF-β (2 ng/ml), or IFN-α (100 IU/ml) for 1, 3, 5, or 7 days. Following the extraction of total RNA, the quantity of HCV replicon RNA was determined by real-time RT-PCR analysis. (B) Total RNA was also subjected to Northern blot analysis (upper panel). The ethidium bromide-staining pattern of ribosomal RNA is shown as an internal control (lower panel). (C) Total protein from cells prepared as above was harvested after either 3 or 5 days of cytokine treatment. Western blot analysis was performed using an antibody against NS5A. CBB staining pattern of the same blot is shown as a loading control (lower panel).

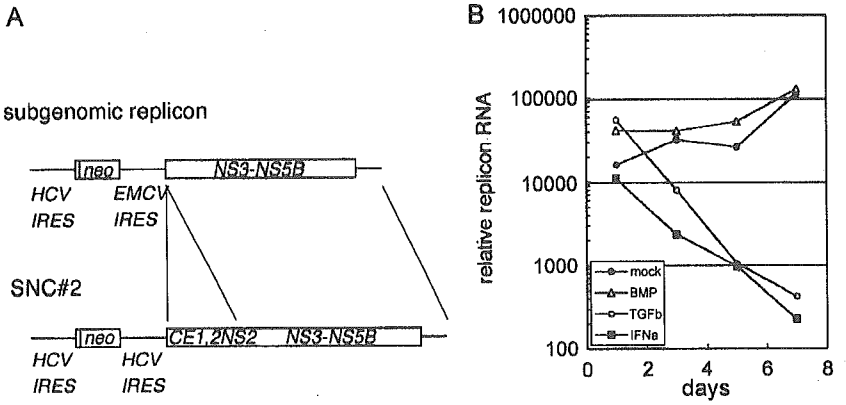


Fig. 6. Suppression of G418-resistant replicon without EMCV IRES by TGF-β. (A) Schematic representation of an RNA construct in typical subgenomic replicon cells (upper) and that in SNC#2 replicon cells (lower). RNA in SNC#2 carries the sequence for the whole HCV ORFs, driven by the HCV IRES instead of the EMCV IRES. The ORFs are depicted as open boxes. (B) SNC#2 cells were mock-treated or treated with BMP-4 (10 ng/ml), TGF-β (2 ng/ml), or IFN-α (100 IU/ml) for 1, 3, 5, or 7 days. Following the extraction of total RNA, the quantity of HCV replicon RNA was determined by real-time RT-PCR analysis.

increasing in severity (Neuman et al., 2002), which correlated with lower levels of viremia in patients than those with less progressed fibrosis (Adinolfi et al., 2001). These reports suggest that the presence of TGF-β, which may be induced by HCV core protein (Taniguchi et al., 2004), has a suppressive influence on viral RNA load. Gewaltig et al. (2002) demonstrated that polymorphisms in the TGF-β gene were associated with progression of HCV-induced liver fibrosis, suggesting again that the cytokine and the cytokine signaling have a certain influence on the virus.

The precise molecular mechanism of the anti-HCV activity of TGF-β remains to be clarified. Additional studies, including clinical studies, may reveal a novel mechanism of HCV replication regulation, potentially providing a target for novel anti-HCV therapies in the future.

Materials and methods

Cell culture, antibodies, and reagents

Huh-7, curedMH5, and curedMH14 cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL) supplemented with 10% fetal bovine serum, 100 units/ml nonessential amino acids (Invitrogen), and 100 μg/ml of both penicillin and streptomycin sulfate (Invitrogen). MH5, MH14, and SNC#2 replicon cells were cultured in the above medium supplemented with 300 μg/ml G418 (Geneticin, Invitrogen). Cured cells were prepared by treating cells with 5000 IU/ml of IFN-α for 2 weeks. Absence of replicon RNA and viral proteins was checked by Northern blotting, Western blotting, and RT-PCR.

Rabbit antisera raised against p38, JNK, ERK, and phospho-ERK were purchased from Cell Signaling Tech-

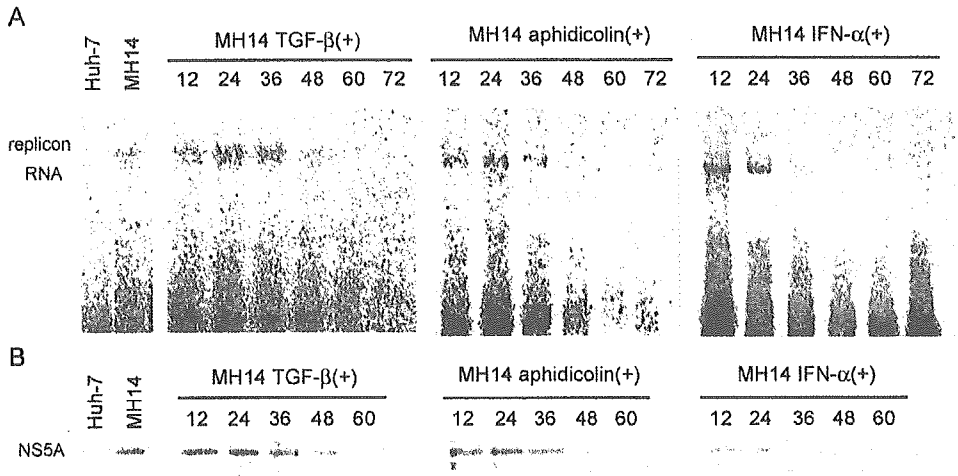


Fig. 7. Simultaneous suppression of viral RNA replication and protein synthesis by TGF-β. (A, B) MH14, a G418-resistant subgenomic replicon cell line, was mock-treated or treated with TGF-β (2 ng/ml), aphidicolin (5 μg/ml), or IFN-α (100 IU/ml) for 12, 24, 36, 48, 60, or 72 h. Cells were then subjected to semi-intact replication assay (A) or [^{35}S]-methionine metabolic labeling and immunoprecipitation using an anti-NS5A antibody (B) as described in Materials and methods.

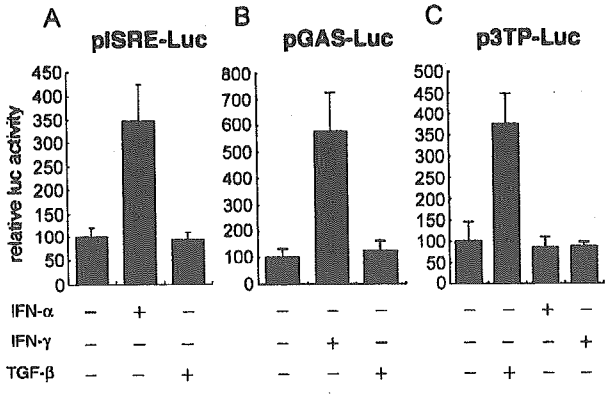


Fig. 8. TGF-β exerts an anti-HCV activity independent of the signal transduction pathway activated by IFN-α or -γ. curedMH14 cells were transfected with either pISRE-Luc (A), pGAS-Luc (B) or p3TP-Luc (C) and treated with TGF-β (2 ng/ml), IFN- (100 IU/ml), or IFN-γ (1000 IU/ml). Luciferase activities were measured 12 h after transfection. Data represent the means and SD values of relative luciferase activities in three independent experiments.

nology. Mouse antibodies specific for phospho-p38 and phospho-JNK were acquired from BD Biosciences and SIGMA, respectively. Horseradish peroxidase-conjugated goat antibodies to mouse and rabbit IgG were procured from Amersham Biosciences. TGF-β, BMP-4, and SB203580 were obtained commercially from Calbiochem. U0126 and Dexamethasone were purchased from SIGMA.

Northern and Western blot analysis

RNA was extracted from cells using Sepasol RNAI super reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. Northern blot analysis was performed as described (Kishine et al., 2002). The 1.5-kb *Eco*RI fragment of pNNRZ2 was used, which corresponds to the C-terminal half of the NS5A gene and the N-terminal half of the NS5B gene as a probe.

Plasmid construction

pNNRZ2 was used to prepare G418-resistant prototype NN replicon RNA (Kishine et al., 2002). To generate pMH14, L1882L and S2204R mutations in NS4B and NS5A were inserted into pNNRRZ2 by PCR-based site-directed mutagenesis. The following primers were used for mutagenesis: 5′-CTGGTCAATCTACTTCCTGCC-3′ and 5′-GGCAGGAAGTAGATTGACCAG-3′ (Bold letters in the primers denote the substituted nucleotides for L1882L). 5′-CTTCAGCTAGACAGTTGTCTGC-3′ and 5′-GCAGACAACTGTCTAGCTGAAG-3′ (same for S2204R). In addition, 5′-CACCCAAATGTACACC-AATG-3′ and 5′-CGATCCTCATGGAACCGTTC-3′, 5′-GAACGGTTCCATGAGGATCG-3′, and 5′-TGATGGG-CAGCTTGCTTTCC-3′ were used for amplification of appropriate fragments. The *neo* genes in pNNRZ2 and pMH14 were replaced with the luciferase gene from the pGL3 vector (Promega, Tokyo, Japan) to create pLNNRZ2 and pLMH14. To prepare the NS5B (RNA polymerase)-defective luciferase-replicon constructs, we inserted a GHD motif into either pLNNRZ2 or pLMH14 by replacing the corresponding sequence with pNNRZ2GHD (Kishine et al., 2002) to create pLNNRZ2GHD or pLMH14GHD, respectively. pSNC was generated to prepare G418-resistant full-genome replicon cell line, SNC#2. To prepare the plasmid, the sequence from NS3 to the end of the NS5B was cloned from I377NS3-3′ (Lohmann et al., 1999) with the S2204I adaptive mutation and other parts were from pM1LE (Kishine et al., 2002).

The coding region for NS5A in the pNNRZ2 plasmid was cloned into the *Sma*I site of pCALNL5/pBR (kindly provided by Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science). The neomycin-resistance gene was removed by *Xho*I digestion to prepare the expression vector pcNS5A.

pcDNA3 (Invitrogen)-based plasmids expressing FLAG-tagged human Smad2 or Smad4, HA-tagged constitutively active TGF-β type I receptor (TβR-I[T/D]), and p3TP-Luc have been described previously (Ohshima and Shimotohno, 2003; Imamura et al., 1997). The combined mutant type I receptor, R-ImL45(T/D), which possesses a constitutively active kinase domain, but lacks the ability to phosphorylate Smad, was generated by PCR-based mutagenesis as described (Ohshima and Shimotohno, 2003; Yu et al., 2002). The pAP1-Luc reporter plasmid was obtained commercially (PathDetect Reporter System; Stratagene, LaJolla, CA). Two additional reporter plasmids, pISRE (IFN-α-stimulated response element)-Luc and pGAS (gamma activation site)-Luc, were based on pGL3-Promoter Vector (Promega, Tokyo, Japan) that contains the SV40 basal promoter sequence without an enhancer sequence. To create pISRE-Luc, the ISRE consensus sequence (ACTTT-CAGTTTCAT) was repeated five times in tandem and inserted between the *Mlu*I and *Xho*I cloning sites of the pGL3-Promoter vector. For pGAS-Luc, three tandem repeats of the GAS sequence (TTTCCCCGAAA) were cloned into the pGL3-Promoter Vector at the *Kpn*I–*Bgl*II cleavage site.

RNA synthesis

HCV subgenomic RNA was transcribed in vitro using a MEGAscript T7 kit (Ambion) according to the manufacturer's instructions. Following DNase treatment, RNA was purified by lithium chloride precipitation.

Transfection and luciferase assay

For the luciferase assay to monitor luciferase-replicon, curedMH14 or other cells seeded on 48-well plate (5×10^3 cells/well) were transfected with 0.25 μg of the luciferase-replicon RNA using DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions. Proteins in cells were

extracted in a lysis buffer supplied in a Dual-Luciferase Reporter Assay System (Promega) kit and the luciferase activity was measured using the kit. Assays were performed in triplicate; standard deviations are denoted by bars in the figures. Plasmid DNA was transfected into cells using FuGENE6 reagent (Roche).

Real time RT-PCR analysis

To monitor the effect of cytokines on neo-resistant replicon RNA, TGF-β, IFN-α, or BMP-4 was added in the media of replicon cells seeded on 6-well plate (4 × 10 4 cells/well). At various times, total cellular RNA was collected and subjected to Real time RT-PCR analysis. The 5′-UTR of HCV genomic RNA was quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems) as described (Watashi et al., 2003) using the 5′-CGGGAGAGCCATAGTGG-3′ (forward) and 5′-AGTACCACAAGGCCTTTCG-3′ (reverse) primers and the fluorogenic probe 5′-CTGCGGAACCGGTGAGTACAC-3′. As an internal control, ribosomal RNA was quantified using TaqMan ribosomal RNA control reagents (Applied Biosystems).

Flow cytometry analysis Cells were trypsinized, fixed in formaldehyde, washed with PBS, and treated with staining solution containing 50 μg/ml propidium iodide (PI) (Sigma), 50 μg/ml of RNaseA (Wako), and 0.1% Triton X-100 (nacalai tesque) in PBS for 15 min. PI fluorescence was analyzed using a FACScalibur flow cytometer (Becton Dickinson). Twenty thousand events were collected and analyzed using CellQuest software (Becton Dickinson).

Semi-intact cell replication assay

A semi-intact cell replication assay was performed as described (Miyanari et al., 2003). In brief, cells were permeabilized by incubation in reaction buffer containing digitonin. Following two washes, samples were incubated for 4 h in the labeling reaction mixture containing 10 μCi of [^{32}P]UTP (Amersham Biosciences) in reaction buffer at 27 °C. Total cellular RNA was collected and fractionated by denaturing agarose gel electrophoresis. Radioactivity incorporated into newly synthesized replicon RNA was visualized using a Fujix Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film, Japan).

Metabolic labeling and immunoprecipitation of NS5A protein

Cells were washed in PBS and incubated in methionine-free DMEM (ICN biomedicals) containing 10% dialyzed FBS and 100 μC i/ml of [^{35}S]-methionine (Tran 35S label, ICN biomedicals) for 4 h. After washing in PBS, cells were lysed in RIPA buffer (10 mM Tris–HCl pH 7.4, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) for 1 h. Cellular debris was removed by centrifugation at 15,000 rpm for 10 min. Following preclearing, lysates were incubated with a monoclonal antibody against NS5A in the presence of protein-G sepharose. After extensive washing, immune complexes were recovered by low-speed centrifugation and subjected to SDS-PAGE. Radioactivity incorporated into newly synthesized NS5A protein was visualized using a Fujix Bio-Imaging Analyzer BAS2000 System (Fuji Photo Film, Japan).

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Enhancement of internal ribosome entry site-mediated translation and replication of hepatitis C virus by PD98059

Takayuki Murata, Makoto Hijikata, Kunitada Shimotohno*

Department of Viral Oncology, Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606-8507, Japan

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Abstract

Translation initiation of hepatitis C virus (HCV) occurs in an internal ribosome entry site (IRES)-dependent manner. We found that HCV IRES-dependent protein synthesis is enhanced by PD98059, an inhibitor of the extracellular signal-regulated kinase (ERK) signaling pathway, while cellular cap-dependent translation was relatively unaffected by the compound. Treatment of cells with PD98059 allowed for robust HCV replication following cellular incubation with HCV-positive serum. Though the molecular mechanism underlying IRES enhancement remains elusive, PD98059 is a potent accelerator of HCV RNA replication.

Keywords: HCV; IRES; ERK; Replication

Introduction

Hepatitis C virus (HCV), a member of the family Flaviviridae, is an enveloped virus with a positive-, single-stranded, 9.6-kb RNA genome (Murphy et al., 1995). The virus is the major causative agent of non-A, non-B hepatitis (Choo et al., 1989) and an estimated 170 million people throughout the world are persistently infected. Although acute phase HCV infection, in most cases, is asymptomatic, the virus frequently establishes a persistent infection, which is associated with serious clinical diseases such as chronic hepatitis followed by liver cirrhosis and hepatocellular carcinoma (Goodman and Ishak, 1995).

Like other positive-stranded RNA viruses, the 5′-untranslated region (UTR) of HCV RNA genome functions as an internal ribosomal entry site (IRES) and mediates translation initiation in a cap-independent manner (Tsukiyama-Kohara et al., 1992). Nearly the entire 5′-UTR (340 nt) and a short sequence of the coding region downstream of the initiator AUG codon of the HCV genome serve as an IRES (Honda et al., 1996). Unlike encephalomyocarditis virus (EMCV) or poliovirus, the 5′ end of the HCV genome is modified by neither cap structure nor VpG but bears a phosphate residue (Takahashi et al., 2005).

Molecular biological investigations of HCV have been hampered for a long time because of the lack of cell culture system that efficiently supports HCV replication. However, establishment of an HCV subgenomic replicon cell culture system in 1999 (Lohmann et al., 1999) allowed for such studies to be undertaken. The subgenomic replicon RNA is composed of, in this order, the HCV 5′-UTR containing an IRES, neomycin phosphate transferase or luciferase gene, HCV nonstructural (NS) proteins 3 through 5B directed by an EMCV IRES and the HCV 3′-UTR. As the replicon RNA replicates autonomously in cultured cells, the system provides a unique tool to analyze the molecular mechanisms governing viral genome replication and protein synthesis. Additionally, this system facilitates the screening of anti-HCV compounds.

PD98059 was identified as a potent inhibitor of mitogenic-extracellular signal-regulated kinase (MEK)–extracellular signal-regulated kinase (ERK) signaling pathway and has been widely used as a specific inhibitor of the pathway. The MEK–ERK pathway is elicited by broad

* Corresponding author. Fax: +81 75 751 3998.
E-mail address: kshimoto@virus.kyoto-u.ac.jp (K. Shimotohno).

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range of growth factors or hormones and plays a crucial role in various events including cell growth promotion, differentiation, cell death and morphogenesis in eukaryotic cells (reviewed in Robinson and Cobb, 1997).

In this study, we demonstrate that PD98059 enhances HCV IRES-dependent translation. Because several lines of evidence suggest that IRES-mediated translation regulates replication in cultured cells (Lerat et al., 2000; He et al., 2003) and in vivo (Lott et al., 2001; Laporte et al., 2003; Forton et al., 2004), we examined the effect of PD98059 on viral replication. Although the RNA levels in replicon cells were relatively unaffected, PD98059 increased viral RNA levels in cultured cells infected with HCV-positive serum. Our results provide insight into the mechanisms of HCV IRES-dependent translation initiation and, in addition, suggest a simple infection system in cultured cells that supports HCV replication very efficiently.

Results

Enhancement of luciferase-replicon or HCV IRES by PD98059

We previously developed a highly efficient subgenomic HCV replicon system (Murata et al., 2005). Briefly, we used cured cells (curedMH14) as a host cell line, and the adaptive mutations were introduced into the subgenomic replicon construct for efficient replication. The luciferase gene was then placed under the control of the HCV IRES for rapid, quantitative and sensitive detection (Fig. 1, LMH14RNA). We have used this system to screen for compounds that inhibit HCV IRES-mediated translation. Treatment with IFN-α, IL-1β, cyclosporin A (CsA) or TGF-β, all factors known to repress HCV replication (Blight et al., 2000; Zhu and Liu, 2003; Watashi et al., 2003; Murata et al., 2005), reduced the observed luciferase activity (Fig. 2A), demonstrating the effectiveness of this system. Conversely, the compound PD98059 increased the luciferase activity by 348% compared to vehicle (DMSO)-treated control (Fig. 2A). The increase in luciferase activity induced by PD98059 was not apparent at 6 h after compound addition, but the activity was significantly elevated by 12 h and remained high for at least 3 days (Fig. 2B). Since PD98059 is an inhibitor of the MEK–ERK pathway, we examined its effects on ERK phosphorylation (Fig. 2C). PD98059 treatment blocked ERK phosphorylation, but a clear band of phospho-ERK was seen in DMSO-treated cells due to growth factors present in the growth medium. Luciferase activity increased in a dose-dependent manner following PD98059 treatment (Fig. 2D). Treatment with the inhibitor at 30 μM slowed cell growth (Fig. 6B) but did not put cells to death, while >30 μM of the chemical resulted in a high degree of toxicity (not shown). We next wished to examine whether PD98059 specifically affected HCV IRES-mediated translation. Using a plasmid based di-cistronic vector

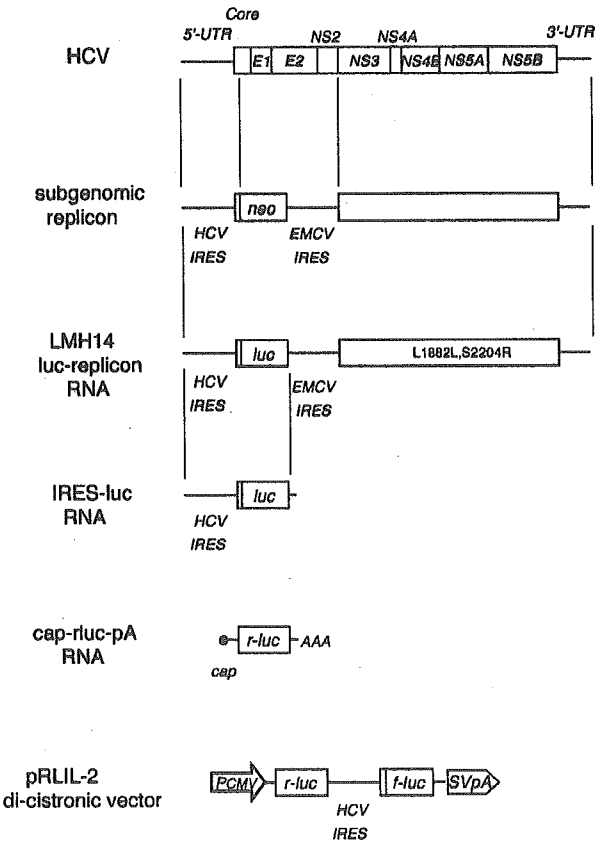

Fig. 1. Constructs used in this study. Schematic representation of the HCV RNA genome (HCV), G418-resistant subgenomic replicon (subgenomic replicon), subgenomic luciferase-replicon (LMH14), mono-cistronic luciferase expression vector with HCV IRES (IRES-luc), mono-cistronic renilla luciferase expression vector with cap and polyA (cap-rluc-pA) or di-cistronic plasmid-based vector construct. The ORFs are depicted as open boxes.

(Fig. 1 pRLIL-2), we found that PD98059 increased the ratio of IRES-dependent translation to cap-dependent translation (Fig. 3B, 247 and 278% at 30 and 10 μM, respectively). Translation downstream of a mono-cistronic mRNA was also enhanced, while cap-dependent translation was not affected (Figs. 3C,D). These results suggest that the positive response of the luciferase-replicon is primarily explained by increased IRES activity. Similar results were obtained when another inhibitor of the MEK–ERK signaling pathway, U0126, was used (Figs. 3E–H).

Effect of CGP57380 on HCV IRES

It has been known that either mitogen-activated protein kinase (MAPK)-interacting protein kinase (MNK) or eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP) regulates translation initiation downstream of the MEK–ERK pathway (Raught and Gingras, 1999). We first examined the involvement of MNK in the IRES activation using CGP57380, a specific inhibitor of MNK (Knauf et al., 2001). ERK interacts with and phosphorylates MNK in

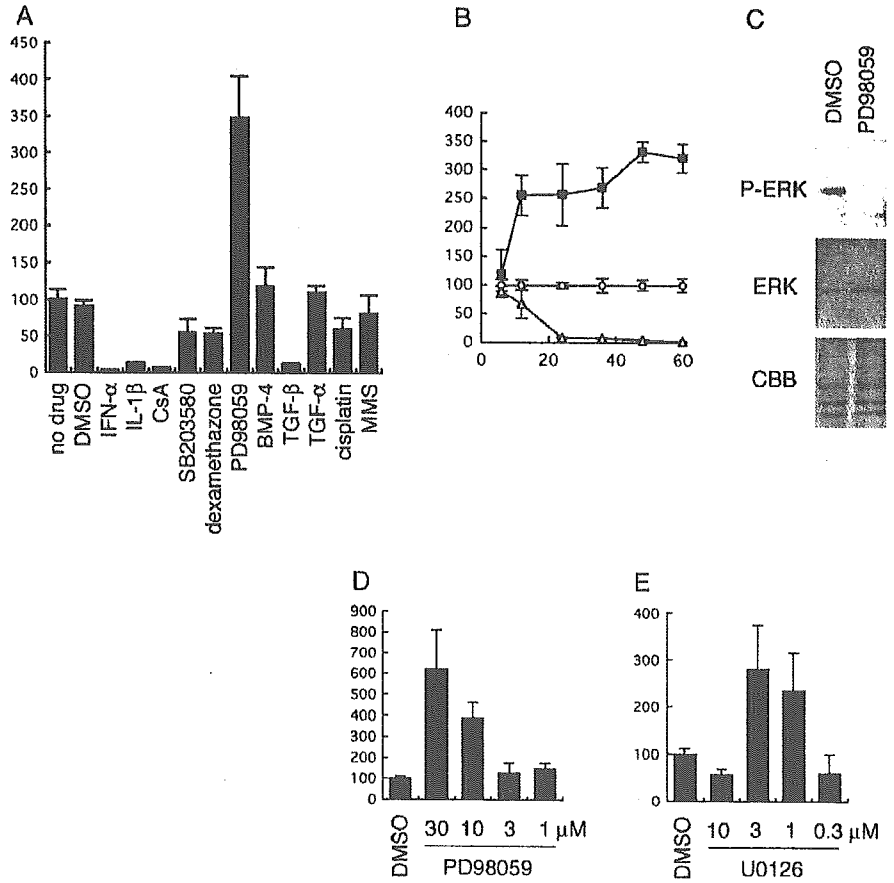

Fig. 2. Increased luciferase-replicon by PD98059 or U0126. (A) curedMH14 cells transfected with the luciferase-replicon RNA construct (LMH14) were treated with DMSO (0.1%), IFN-α (100 IU/ml), IL-1β (10 ng/ml), cyclosporin A (CsA, 1 μg/ml), SB203580 (10 μM), dexamethazone (1 μM), PD98059 (10 μM), BMP-4 (10 ng/ml), TGF-β (2 ng/ml), TGF-α (1 ng/ml), cisplatin (1 μg/ml) or MMS (methyl methanesulfonate; 0.1 mM). Three days later, cellular luciferase activity was measured. (B) curedMH14 cells transfected with the luciferase-replicon RNA construct were treated with DMSO (white circle), PD98059 (10 μM, black box) or IFN-α (100 IU/ml). At the indicated times, cells were harvested for determination of luciferase activity. The activity of DMSO-treated cells was set to 100%. (C) Western blotting analysis of phospho-ERK (upper panel) and total ERK (middle panel) in cells treated with or without PD98059 (10 μM) for 10 h. CBB staining pattern of the same blot is used as a loading control (lower panel). (D,E) Dose-dependence of MEK–ERK inhibitors on the activity of the luciferase-replicon. Cells transfected with the luciferase-replicon RNA construct were treated with varying concentrations of PD98059 (D) or U0126 (E), and luciferase activity was subsequently determined. The luciferase activity was shown with the SD value of three independent experiments.

response to growth or stress signals, respectively, and MNK phosphorylates eIF4E (Raught and Gingras, 1999).

Cell treatment with 20 μM CGP57380 decreased the luciferase-replicon to 34% (Fig. 4A) and the ratio of IRES-dependent over cap-dependent value to 64% when dicistronic vector (Fig. 4B) was used. There was little to no effect of lower inhibitor concentrations on translation (Figs. 4A,B,C). In order to verify the effectiveness of CGP57380, we examined the activation of eIF4E by blotting with an anti-phospho-eIF4E antibody. Treatment with CGP57380 clearly eliminated eIF4E phosphorylation, and a partial reduction in eIF4E phosphorylation was seen following treatment with PD98059, even though total eIF4E levels were unchanged (Fig. 4D).

These data, combined with accumulating evidence (Scheper and Proud, 2002), suggest that eIF4E phosphorylation does not play a positive role in cap-dependent translation, and, moreover, it may limit cap-dependent translation in cultured cells, although the physiological significance of eIF4E phosphorylation remains controversial. Nevertheless, drug-induced reduction in eIF4E phosphorylation did not enhance IRES-dependent translation compared to cap-dependent translation.

Effect of 4EBP on HCV IRES

An additional key translation regulator downstream of the MEK–ERK pathway is the eIF4E-binding protein 4EBP. When eIF4E is bound by 4EBP, ribosomes are not recruited to the cap structure and translation is blocked. Among the three isoforms, 4EBP1 is the best characterized. The binding of 4EBP1 with eIF4E is controlled by the phosphorylation state of 4EBP1, where the hypo/basal-phosphorylated form of 4EBP1 interacts tightly with eIF4E, but upon hyper-phosphorylation, 4EBP1 binding to eIF4E is inhibited (Gingras et al., 2001). mTOR has been reported to

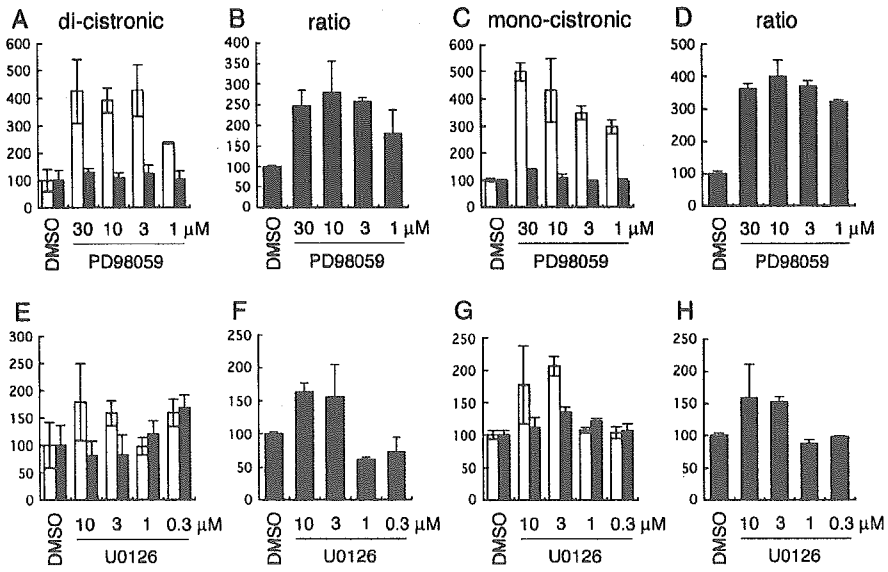

Fig. 3. Increased HCV IRES by PD98059 or U0126. Dose-dependence of MEK–ERK inhibitors on the activity of the di-cistronic pRLIL-2 vector (A,B,E,F) and mono-cistronic expression vectors (C,D,G,H). (A,E) Cells transfected with di-cistronic pRLIL-2 were incubated with varying concentrations of PD98059 (A) or U0126 (E), and luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (B,F) The results in panels (A) and (E) are shown as the ratio of IRES-dependent over cap-dependent value, respectively. (C,G) Cells transfected with mono-cistronic IRES-luc (white bar) and cap-rluc-pA (black bar) were incubated with varying concentrations of PD98059 (C) or U0126 (G), and luciferase activity was subsequently determined. (D,H) The results in panels (C) and (G) are shown as the ratio of IRES-dependent over cap-dependent value, respectively. The luciferase activity was shown with the SD value of three independent experiments.

phosphorylate 4EBP1 (Gingras et al., 1999), and, recently, Herbert et al. (2002) proposed that ERK is involved in the hyper-phosphorylation of 4EBP1.

We investigated a possible role for 4EBP1 in the observed IRES activation by PD98059. Exogenous expression of wild type or dominant active form of 4EBP1 (T46A, Mothe-Satney et al., 2000) elevated the luciferase-replicon to 420 and 325% of control levels, respectively, and PD98059 treatment enhanced these effects (Fig. 5A). A mutant form of 4EBP1 unable to interact with eIF4E (mBD, Mader et al., 1995), however, did not affect the luciferase-replicon activity (Fig. 4A). Luciferase expression driven by a di-cistronic vector resulted in a similar trend (Fig. 5C). Both the wild type and mBD forms of 4EBP1 were hyper-phosphorylated (Fig. 5D). In the cell line used, Huh-7, endogenous 4EBP1 was not detected (Fig. 5D, vec). The expression levels of wild-type and T46A were reduced compared to mBD, likely as a result of the auto-suppression of cap-dependent translation by the wild type or T46A 4EBP1.

We next tried to eliminate endogenous 4EBP. Knock-down of 4EBP was confirmed following individual siRNA (Fig. 6A) or all siRNAs treatment (Fig. 6B). Among the different 4EBP isoforms, knock-down of 4EBP2 led to the strongest reduction in the luciferase-replicon (Figs. 6C,D) and the IRES/cap-translation in the di-cistronic vector (Figs.

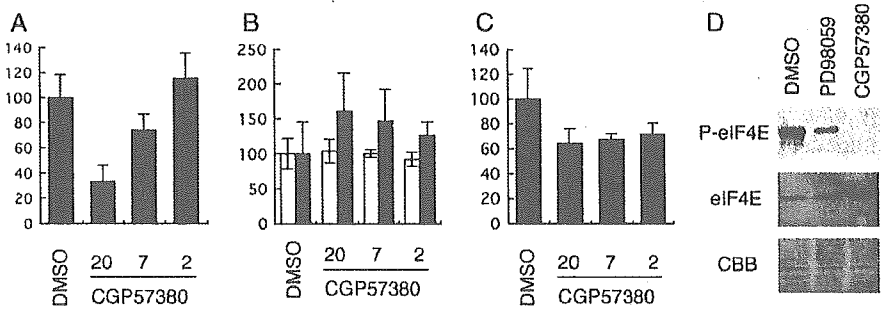

Fig. 4. Effect of CGP57380 on luciferase-replicon or HCV IRES. (A) Cells transfected with the luciferase-replicon RNA construct were treated with CGP57380 (μM). Three days after transfection, cells were harvested for determination of luciferase activity. The activity of DMSO-treated cells was set to 100%. (B) Cells transfected with di-cistronic pRLIL-2 were incubated with CGP57380, and luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (C) The results in panel (B) are shown as the ratio of IRES-dependent over cap-dependent value. The luciferase activity was shown with the SD value of three independent experiments. (D) Western blotting was performed to examine the phosphorylation of eIF4E (upper panel) and total eIF4E (middle panel) in cells treated with or without CGP57380 (20 μM). CBB staining pattern of the same blot is used as a loading control.

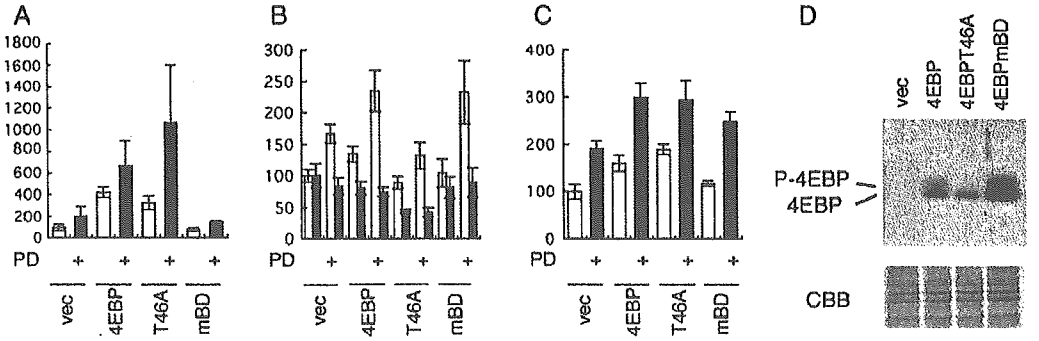

Fig. 5. Effect of exogenous expression of 4EBP on luciferase-replicon or HCV IRES. (A) Cells were transfected with the luciferase-replicon RNA construct together with empty vector (vec), vector for 4EBP1, 4EBP1T46A or 4EBP1mBD. 4EBP1T46A is a dominant-active form of 4EBP1, and 4EBP1mBD lacks the ability to bind with eIF4E. Cells were harvested for determination of luciferase activity after 3 days incubation with PD98059 (10 μM). White and black bars indicate absence and presence of PD98059, respectively. The data are normalized by cotransfection with the pRL-TK. (B) Cells were cotransfected with dicistronic pRLIL-2 together with empty vector (vec), vector for 4EBP1, 4EBP1T46A or 4EBP1mBD. Luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (C) The results in panel (B) are shown as the ratio of IRES-dependent over cap-dependent value. White and black bars indicate absence and presence of PD98059, respectively. The luciferase activity was shown with the SD value of three independent experiments. (D) Western blotting was performed to examine the expression and phosphorylation state of 4EBP in cells using anti-4EBP antibody. CBB staining pattern of the same blot is used as a loading control.

6G,H). Huh-7 cells express higher levels of 4EBP2 compared to the other isoforms, and we hypothesize that this may account for the observed effect.

The above results suggest that 4EBP proteins, particularly 4EBP2 in this cell line, play an important role in HCV IRES-mediated translation. However, no evidence implicated 4EBP in the ERK-mediated modification of IRES activity because, even in the presence of the mBD mutant or the elimination of 4EBP isoforms, PD98059-mediated activation of IRES-dependent translation still occurred.

Effect of PD98059 on G418-resistant subgenomic replicon

Since IRES-mediated translation can regulate RNA replication in cultured HCV replicon cells (He et al., 2003), we tested the effect of PD98059 on G418-resistant replicon RNA replication. When monitored by either real-time RT-PCR (Fig. 7A) or Northern blotting (Fig. 7C), replicon RNA was increased up to 210% of vehicle-treated control by the administration with 30 μM of PD98059 for 24 h. The replicon RNA levels decreased at 48 h or later probably because of the cell growth suppression (Fig. 7B). Additionally, PD98059 induced the production of viral protein NS5A (Fig. 7D). Although replicon RNA levels can fluctuate and are not the most stringent test, as Zhu and Liu (2003) also observed, the observed up-regulation of HCV replicon RNA and a viral protein at 24 h strongly suggests an effect of PD98059.

PD9805 promotes HCV multiplication in a model of HCV infection

To examine the effects of PD98059 on HCV replication, we infected curedMH14 (Fig. 8A), Huh-7 (B), OUMS-29 H-11 (C) or PH5CH8 (D) cells with HCV-positive serum for 1 day and incubated cells with either PD98059 or vehicle. curedMH14 had been prepared by curing an HCV replicon cell line of replicon RNA (Murata et al., 2005). OUMS-29/H-11 is a human hepatocyte cell line, in which SV40 large T antigen and hepatocyte nuclear factor 4 (HNF4) had been introduced by stable transfection (Inoue et al., 2001), and PH5CH8 is a human hepatocyte line that hat had been immortalized with SV40 large T antigen (Ikeda et al., 1998).

HCV replication efficiency is highly dependent on the cell culture conditions, and poor infectivity can lead to little or no replication. However, HCV infectivity was dramatically improved by the addition of 30 μM PD98059 (Fig. 8). With 30 μM PD98059, virus RNA levels on day 5 were 162, 113 and 146% of the levels of day 1, whereas they were 0, 33 and 0% in curedMH14, OUMS-29 H-11 and PH5CH8 cells treated with DMSO, respectively. Thus, HCV replication was increased by 100-fold or more in curedMH14 and PH5CH8 cells on the fifth day. Huh-7 cells were not as permissive for viral infection under these conditions.

Discussion

In this study, we found that the addition of PD98059, an inhibitor of the MEK–ERK pathway, enhanced HCV IRES-dependent translation and HCV replication in cultured cells.

Multiple cellular factors bind directly to the HCV IRES including eIF3 (Sizova et al., 1998), the 40S ribosome (Otto et al., 2002), polypyrimidine tract-binding protein (PTB, Ali and Siddiqui, 1995), La autoantigen (Ali and Siddiqui, 1997) and heterogeneous nuclear ribonucleoprotein L (hnRNP L, Hahm et al., 1998). Some of these molecules may play a role in the PD98059-mediated activation of HCV IRES-dependent translation.

Several reports have suggested that translation driven by the HCV IRES (Honda et al., 2000), as well as other IRESes (Pyronnet et al., 2000; Cornelis et al., 2000), is highest in

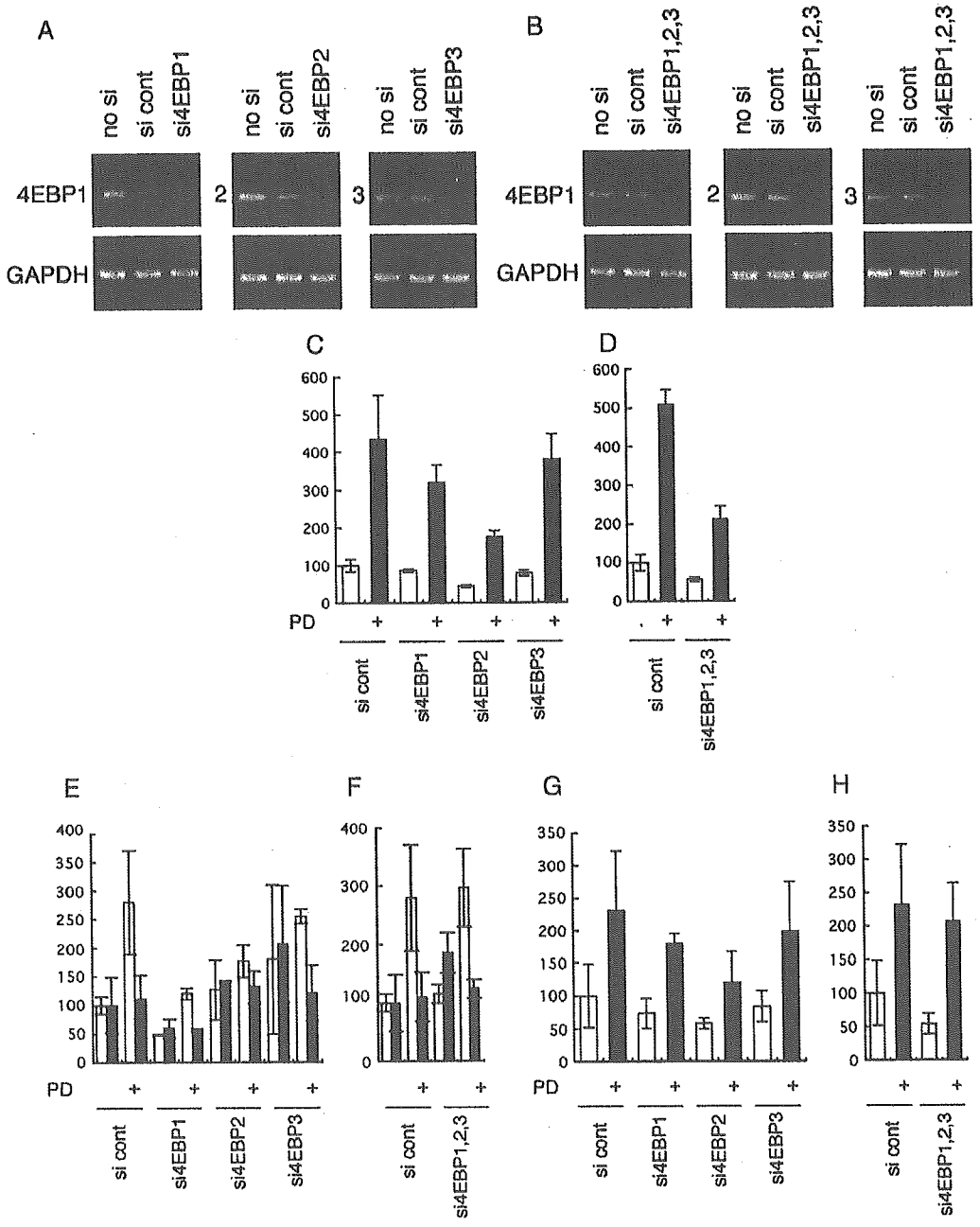

Fig. 6. Effect of RNAi knock-down of 4EBPs on luciferase-replicon or HCV IRES. (A) Cells were transfected with control siRNA (si cont) or si4EBP1, 2 or 3, independently. Total RNA was collected to examine the levels of relevant 4EBP RNA by RT-PCR. (B) Cells were transfected with control siRNA (si cont) or si4EBP1, 2 and 3, altogether. Total RNA was collected to examine the levels of every isoform of 4EBP RNA by RT-PCR. (C,D) Cells were cotransfected with LMH14 luciferase-replicon RNA construct and various siRNAs, independently (C) or together (D). Luciferase activity was determined after 3 days incubation with or without 10 μM PD98059. White and black bars indicate absence and presence of PD98059, respectively. The data are normalized by cotransfection with the pRL-TK. (E,F) Cells were cotransfected with di-cistronic pRLIL-2 together with various siRNAs, independently (E) or together (F). Luciferase activity was subsequently determined. IRES-dependent firefly luciferase activity is shown in white, and cap-dependent renilla luciferase activity is in black. (G,H) The results in panels (E) and (F) are shown as the ratio of IRES-dependent over cap-dependent value. White and black bars indicate absence and presence of PD98059, respectively. The luciferase activity was shown with the SD value of three independent experiments.

the mitotic phase (G2/M) and relatively lower in other phases of the cell cycle. Since the MEK–ERK signaling pathway is largely suppressed in the G2 phase (Tamemoto et al., 1992), MEK–ERK signaling may also be a key regulator of this phenomenon.

In addition to ERK signaling, p38 MAPK and JNK signaling pathways are also involved in translation regulation. Cellular stress negatively affects cap-dependent protein synthesis (Patel et al., 2002), while EMCV (Hirasawa et al., 2003) or c-*myc* (Subkhankulova et al., 2001) IRES-

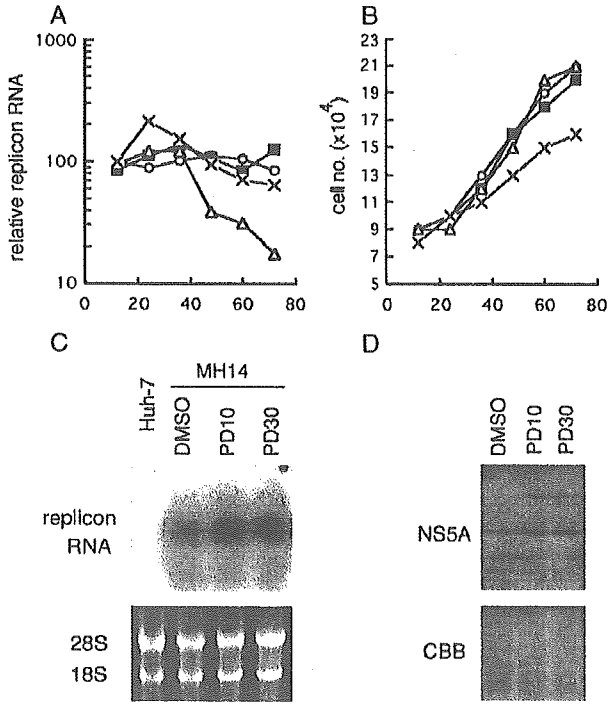

Fig. 7. Effect of PD98059 on G418-resistant subgenomic replicon. (A) MH14, a G418-resistant subgenomic replicon cell line, was treated with DMSO (white circle), 30 μM PD98059 (X), 10 μM PD98059 (black box) or 100 IU/ml IFN-α (white triangle) for 1, 3, 5 or 7 days. Following the extraction of total RNA, the quantity of HCV replicon RNA was determined by real-time RT-PCR analysis. (B) In parallel with the experiments in Fig. 5A, cells were treated with DMSO (white circle), 30 μM PD98059 (X), 10 μM PD98059 (black box) or 100 IU/ml IFN-α (white triangle). Cell numbers were counted at the indicated time points. (C) Total RNA of cells treated for 1 day was also subjected to Northern blot analysis (upper panel). The ethidium bromide staining of ribosomal RNA is shown as an internal control (lower panel). (D) Total protein of cells treated for 1 day was harvested to examine the amount of NS5A (upper panel). CBB staining pattern of the same blot is shown as a loading control (lower panel).

dependent translation is elevated by these signals. Therefore, these signaling pathways may also affect HCV IRES-dependent translation.

A cell culture system supporting HCV replication has not existed for some time. When immortalized hepatocyte cell lines are infected with HCV, viral replication efficiency is not high despite high replication rates in patients. Many researchers have attempted to solve this problem. Ikeda et al. (1998) demonstrated that incubation of cells at lower temperature helps virus replication. Aizaki et al. (2003) used a three-dimensional hepatocyte culturing system. Others varied the bovine serum levels, vitamins, lipids or amino acid composition or the pH of the culture medium. We observed that freshly thawed cells with lower viability supported replication better than rapidly growing cells. We now propose a simple infection system that supports highly efficient HCV replication in cultured cells by adding PD98059 in the medium.

Cells isolated from human liver are cultured in conditions that substantially differ from the in vivo environment and are often immortalized by oncogene expression. Consequently, many signaling pathways are likely aberrantly regulated in vitro. Among these pathways, it seems likely that ERK signaling is responsible for regulating HCV replication in cultured cells, and PD98059 may help mimic the in vivo environment and facilitate HCV replication by enhancing IRES-dependent translation.

Although treatment with PD98059 increased the replication of viral RNA in various cell lines when infected with HCV-positive serum (Fig. 8), replicon RNA levels were not increased under similar conditions (Fig. 7). The RNA copy number may explain these differences. PD98059 may not enhance the replication of replicon RNA because, in these systems, viral RNA and proteins are abundant even in the absence of the inhibitor. In cells infected with patient serum, highly efficient IRES-dependent translation may be essential for viral replication due to the low copy number of viral RNA per cell.

Mutations of serine residues within NS5A that affect the protein hyper-phosphorylation enhance replication of the virus replicon (Blight et al., 2000), and inhibitors of NS5A kinase(s) activate replication (Neddermann et al., 2004). Since the CMGC group of serine–threonine kinases has been implicated in the phosphorylation of NS5A (Reed et al., 1997), PD98059 might affect the

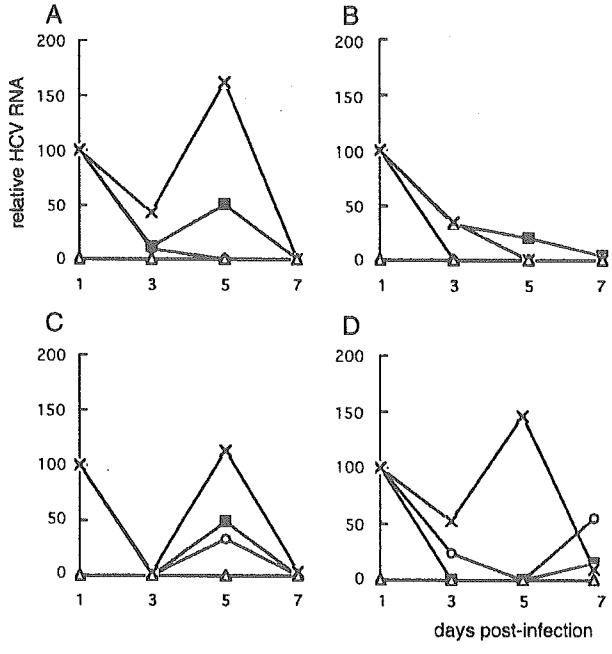

Fig. 8. Increased HCV multiplication by PD9805 in cells infected with HCV-positive serum. curedMH14 (A), Huh-7 (B), OUMS-29 H-11 (C) or PH5CH8 (D) cells were infected or mock-infected (white triangle) with HCV-positive serum for 1 day. After extensive washing with PBS, the cells were cultured with fresh medium supplemented with DMSO (white circle), 30 μM PD98059 (X), 10 μM PD98059 (black box). At the indicated times, total RNA was extracted, and the quantity of HCV RNA was determined by real-time RT-PCR analysis.

phosphorylation of the NS5A protein and thereby elevate replication. When we treated cells with PD98059, however, the levels of hyper-phosphorylated NS5A were not affected (not shown). This suggests that PD98059 activates viral replication through the enhancement of IRES-mediated translation but not through a reduction in phosphorylation state of NS5A.

Multiplication of influenza virus (Pleschka et al., 2001), borna disease virus (Planz et al., 2001), coxsackievirus (Luo et al., 2002), visna virus (Barber et al., 2002), human immunodeficiency virus (Montes et al., 2000), vaccinia virus (de Magalhaes et al., 2001), Epstein–Barr virus (Gao et al., 2001), cytomegalovirus (Rodems and Spector, 1998) and human herpesvirus-8 (Akula et al., 2004) are promoted by MEK–ERK signaling pathway activation. Activation of this pathway results in efficient cell cycle promotion, high cellular or viral gene production and increased availability of biomaterials, such as nucleotides or amino acids. Many of these viruses, therefore, likely exploit the cellular environment created through the activation of the MEK–ERK pathway. Interestingly, replication of the hepatitis B virus (HBV) is negatively regulated by the MAPK signaling pathway (Zheng et al., 2003). Because both HBV and HCV infect the same target organ, it is possible that both viruses have evolved similar means to exploit host signaling pathways. Much research is needed to identify the factors conferring organ specificity to HCV, however.

Materials and methods

Cell culture, antibodies and reagents

Huh-7 or curedMH14 cells (Murata et al., 2005) were maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 units/ml nonessential amino acids (Invitrogen, Carlsbad, CA) and 100 μg/ml penicillin and streptomycin sulfate (Invitrogen, Carlsbad, CA). MH14 replicon cells (Miyanari et al., 2003) were cultured in the same medium with 300 μg/ml G418 (Geneticin, Invitrogen, Carlsbad, CA). OUMS-29/H-11 cells (Inoue et al., 2001, Fukaya et al., 2001) were maintained in ASF-104 medium (Ajinomoto, Tokyo, Japan) with 100 μg/ml penicillin and streptomycin sulfate (Invitrogen, Carlsbad, CA), and PH5CH8 cells were cultured as described (Ikeda et al., 1998).

Rabbit anti-ERK, rabbit anti-phospho-ERK, rabbit anti-eIF4E and mouse anti-phospho-eIF4E antibodies were purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-4EBP antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish-peroxidase-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences (Piscataway, NJ). PD98059 and other inhibitors were obtained commercially from Calbiochem-Novabiochem (San Diego, CA).

Plasmid construction

The pLMH14, used to synthesize the luciferase-replicon LMH14 RNA and mono-cistronic IRES-luc RNA, has been described previously (Murata et al., 2005). The di-cistronic plasmid vector, pRLIL-2, was based on the pRL-CMV Vector (Promega, Madison, WI) and contains HCV IRES sequence (complete 5′-UTR sequence and initial part of the Core gene) plus the firefly luciferase sequence obtained from pGL2 Vector (Promega, Madison, WI).

The human 4EBP1 gene was cloned by RT-PCR into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) to obtain pcDNA4EBP. Primers used to clone the gene were 5′-cggaattcgatgtccgggggcagcagctgc-3′ and 5′-ctgactcgagttaaatgtccatctcaaactgtg-3′. To generate pcDNS4EBPT46A and pcDNA4EBPmBD plasmids, mutations were inserted into pcDNA4EBP by PCR-based site-directed mutagenesis using the primers 5′-ctggtacc-tcccg**ggg**cggtgctgaagagcgtg-3′ for T46A and 5′-gaggtac-caggatcatc**tat**gaccggaaa**ttc**gcggcggagtgtcggaactc-3′ for mBD. Bold letters in the primers denote the substituted nucleotides.

RNA synthesis in vitro

In order to synthesize the LMH14 luciferase-replicon RNA or mono-cistronic IRES-luc RNA, pLMH14 was digested with *Xba*I or *Kpn*I, respectively, and subjected to in vitro transcription using a MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions. Following DNase treatment, RNA was purified by lithium chloride precipitation. For production of mono-cistronic cap-rluc-pA RNA, the pRL-TK Vector (Promega, Madison, WI) was cut with *Xba*I and transcribed in vitro using mMESSAGE mMACHINE T7 Kit (Ambion, Austin, TX) for capping. Poly(A) Tailing Kit (Ambion, Austin, TX) was then used for polyadenylation of the RNA.

Luciferase assay

Lipofection with RNA was performed using DMRIE-C reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Plasmid DNA, including pRLIL-2, was transfected into cells using FuGENE6 reagent (Roche, Indianapolis, IN). Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Assays were performed in triplicate; standard deviations are denoted by bars in the figures.

Real-time RT-PCR analysis

Total RNA was extracted from cells using Sepasol RNAI super reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The 5′-UTR of HCV genomic RNA was quantified with the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City,

CA) as described (Watashi et al., 2003) using the 5′-CGGGAGAGCCATAGTGG-3′ (forward) and 5′-AGTAC-CACAAGGCCTTTCG-3′ (reverse) primers and the fluorogenic probe 5′-CTGCGGAACCGGTGAGTACAC-3′. As an internal control, ribosomal RNA was quantified using TaqMan ribosomal RNA control reagents (Applied Biosystems, Foster City, CA).

Northern and Western blot analysis

Total RNA was extracted from cells using Sepasol RNAI super reagent (Nacalai Tesque, Kyoto, Japan). Northern or Western blot analysis was performed as described previously (Kishine et al., 2002). The 1.5-kb *Eco*RI fragment of pNNRZ2 was used as the probe, which corresponds to the C-terminal half of the NS5A gene and N-terminal half of the NS5B gene.

In vitro HCV infection

The in vitro HCV infection experiment was carried out as described previously (Watashi et al., 2003). In short, cells were infected with the serum which was prepared from an HCV-positive blood donor. At 24 h post-inoculation, the cells were washed three times with PBS and maintained with fresh medium with DMSO or PD98059 until the extraction of the RNA sample.

siRNA

Sequences of siRNAs (Invitrogen, Carlsbad, CA) were as follows: 5′-aactcacctgtgaccaaaaca-3′ for 4EBP1, 5′-aagactccaaagtagaagtaa-3′ for 4EBP2 and 5′-aagctggagtg-caagaactca-3′ for 4EBP3. Before using, the siRNAs were dissolved in RNase-free water, denatured once at 98 °C for 1 min and annealed at 37 °C for 1 h. For electroporation of siRNA, 4×10^5 cells and 0.8 μg siRNA were suspended in 400 μl of OPTI-MEM (Invitrogen, Carlsbad, CA) and pulsed at 250 V and 950 μF using GenePulser (Bio Rad, Hercules, CA) at 4 °C. To evaluate the silencing effects of siRNAs, RT-PCR was performed using One-Step RT-PCR Kit (TaKaRa, Ohtsu, Japan) according to the manufacturer's instruction. Primer sequences used were as follows: 4EBP1, 5′-cggaattcgatgtccgggggcagcagctgc-3′ and 5′-ctgactcgagt-taaatgtccatctcaaactgtg-3′, 4EBP2, 5′-cggaattcatgtcctcgt-cagccggcag-3′ and 5′-ctgactcgagtcagatgtccatctcgaac-3′, 4EBP3, 5′-cggaattcatgtcaacgtccactagctg-3′ and 5′-ctgactc-gagttagatgtccatttcaaattg-3′, GAPDH, 5′-tttctcgagatggg-gaaggtgaaggtcg-3′ and 5′-ccggaattctggagggatctcgctcctg-3′.

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Short Communication

Interferon resistance of hepatitis C virus replicon-harbouring cells is caused by functional disruption of type I interferon receptors

Kazuhito Naka,[1] Kazunori Takemoto,[1] Ken-ichi Abe,[1] Hiromichi Dansako,[1] Masanori Ikeda,[1] Kunitada Shimotohno[2] and Nobuyuki Kato[1]

Correspondence
Nobuyuki Kato
nkato@md.okayama-u.ac.jp

[1]Department of Molecular Biology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan

[2]Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawara-cho Shogo-in, Sakyo-ku, Kyoto 606-8507, Japan

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Hepatitis C virus (HCV) replicon-harbouring cell lines possessing interferon (IFN)-resistant phenotypes have recently been established. These were divided into two classes: partially IFN resistant and highly IFN resistant. Here, the viral and cellular factors contributing to the IFN resistance of HCV replicon-harbouring cells were evaluated. The results revealed that cellular factors rather than viral factors contributed to a highly IFN-resistant phenotype. The possibility of genetic abnormality of the factors involved in IFN signalling was investigated. As a result, nonsense mutations and deletions in type I IFN receptor genes (IFNAR1 and IFNAR2c) were found in replicon-harbouring cells showing a highly IFN-resistant phenotype, but rarely appeared in cells showing a partially IFN-resistant phenotype. Furthermore, similar genetic alterations were also found in IFN-resistant phenotype, replicon-harbouring cell lines obtained additionally by IFN-β treatment. Moreover, it was shown that ectopic expression of wild-type IFNAR1 in IFN-resistant phenotype, replicon-harbouring cells possessing the IFNAR1 mutant restored type I IFN signalling.

Persistent infection by hepatitis C virus (HCV) is a major cause of chronic hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989), which can progress to liver cirrhosis and hepatocellular carcinoma (Saito *et al.*, 1990). Since at least 170 million people are currently infected with HCV worldwide, this infection constitutes a global health problem (Thomas, 2000). HCV is an enveloped RNA virus belonging to the family *Flaviviridae*, the genome of which consists of a positive-stranded RNA encoding an approximately 3000 aa polyprotein precursor (Kato *et al.*, 1990). This precursor protein is processed by the host and viral proteases to generate at least 10 proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (Kato, 2001).

Since 1998, combined treatment with interferon (IFN)-α and ribavirin has been standard clinical therapy for patients with chronic hepatitis C; however, the effectiveness of IFN is limited to approximately 50 % (Hadziyannis *et al.*, 2004). This clinical result suggests that HCV directly or indirectly attenuates the antiviral actions of IFN (Pawlotsky, 2000).

Although an HCV replicon system carrying autonomously replicating HCV subgenomic RNA containing the NS3–NS5B regions (Lohmann *et al.*, 1999) was considered to be useful in studies on the mechanism(s) of IFN resistance of HCV, all HCV replicons established to date have been highly sensitive to IFN-α, -β and -γ (Frese *et al.*, 2001, 2002; Kato *et al.*, 2003). This seems to contradict the fact that half of the patients with chronic hepatitis C are resistant to current IFN therapy. Therefore, we assumed that prolonged IFN treatment might change HCV replicons from an IFN-sensitive phenotype to an IFN-resistant phenotype.

Based on this assumption, we recently established nine HCV replicon cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series: 1αR, 3αR, 4αR, 5αR and αRmix) and a highly resistant phenotype (βR series 1βR, 3βR, 4βR and 5βR) obtained by IFN-α and -β treatment, respectively (Namba *et al.*, 2004). Genetic analysis of these replicons found one common amino acid substitution (Q1737H) in the NS4B region and several additional amino acid substitutions (such as M2174V and T2242N) in the NS5A region of the βR series (Namba *et al.*, 2004). To examine which viral and cellular factors contribute to the IFN resistance of HCV replicons, we evaluated the IFN sensitivity of replicon-harbouring cells (6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc) established by transfection of total RNAs isolated from an IFN-sensitive clone (6M) and from highly IFN-resistant clones (1βR and 4βR) into cured 6Mc cells, from which 50-1 replicons (Kishine *et al.*, 2002) had been eliminated by IFN-γ treatment (500 IU ml^{-1} for

Supplementary material is available in JGV Online.