phosphate-buffered saline once, harvested with Renilla lysis reagents (Promega), and subjected to Renilla luciferase (RL) assay according to the manufacturer's protocol.

Statistical analysis

The luciferase activities were statistically compared between the various treatment groups using Student's *t* test. *P* values of less than 0.05 were considered statistically significant.

Results

The 1b/2a chimeric replicon is less sensitive to CsA than the 1b replicon

To investigate the mechanism(s) underlying CsA's anti-HCV activity, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which the NS5B and 3′UTR regions were replaced with those of HCV JFH-1 RNA (Fig. 1a). These RNAs were transfected into OR6c cells. After 3 weeks' selection of G418, we successfully obtained a 1b replicon or 1b/2a chimeric replicon-harboring cells as polyclones (see Supplementary Material). The colony forming efficiencies of the 1b replicon and the 1b/2a chimeric replicon were 5150 ± 361 and 62 ± 10 colonies/μg RNA, respectively. Sequence analysis of HCV RNA in 1b replicon or 1b/2a chimeric replicon-harboring cells revealed that there was no conserved amino acid substitution (data not shown). The RT-quantitative PCR analysis revealed that intracellular HCV RNA copies were $3.8 \pm 0.1 \times 10^7$ and $1.1 \pm 0.1 \times 10^7$ copies/μg total RNA in 1b replicon and 1b/2a chimeric replicon-harboring cells, respectively.

Next, we examined the sensitivity of the 1b replicon and 1b/2a chimeric replicon to anti-HCV reagents (Fig. 1b). HCV RNA replication was monitored through reporter

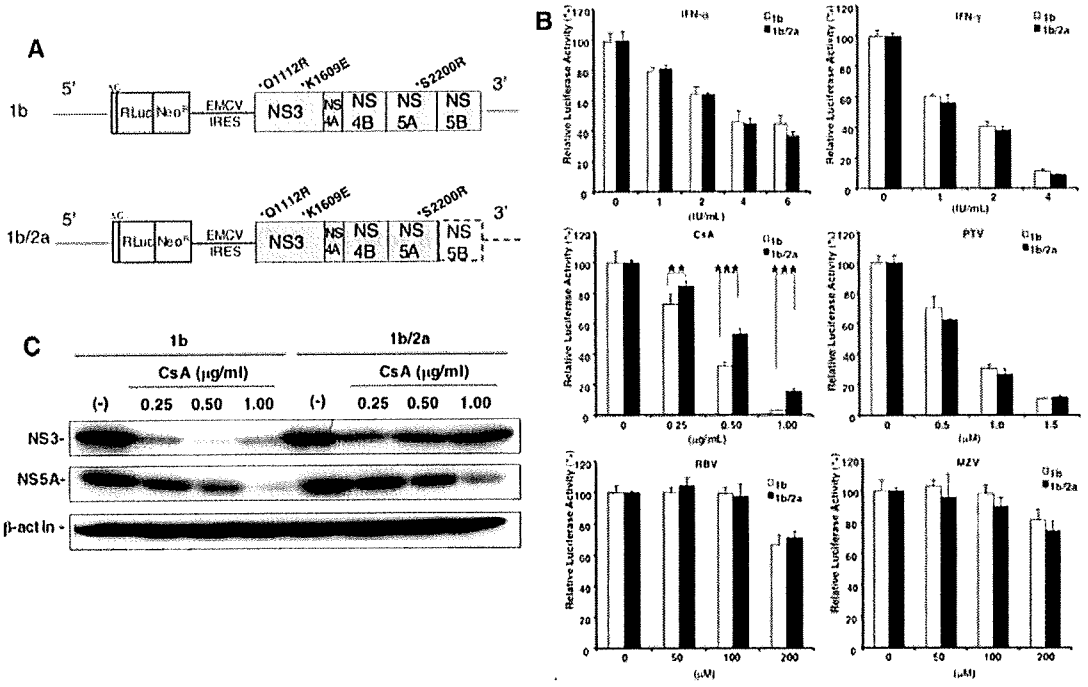

Fig. 1 1b/2a chimeric replicon-harboring cells are less sensitive to CsA. **a** Gene organization of subgenomic RNA. RLuc and DC indicate the RL gene and the 12 N-terminal amino acid residues of the core protein as a part of internal ribosomal entry site (IRES), respectively. The positions of adaptive mutations are indicated by *asterisks*. *Shaded boxes*, *dotted open boxes*, *thin lines*, *dotted lines*, *sick lines*, and *open boxes* indicate open reading frame (ORF) derived from HCV-O strain, ORF derived from JFH-1 strain, UTR of HCV-O strain, UTR of JFH-1 strain, encephalomyocarditis virus IRES, and fusion protein RL with neomycin phosphotransferase (Neo^R), respectively. **b** Effects of various anti-HCV reagents on HCV RNA replication in the 1b replicon (*open columns*) and in the 1b/2a chimeric replicon (*closed columns*) harboring cells. The cells were treated with IFN-α, IFN-γ, CsA, PTV, RBV, and MZB, respectively. After 72 h of treatment, the RL assay was performed according to the manufacturer's protocol. The relative luciferase activity is calculated when the RL activity of untreated cells was assigned as 100% (** $P < 0.01$; *** $P < 0.001$). **c** Western blot analysis of HCV proteins. The 1b replicon or 1b/2a chimeric replicon-harboring cells were treated with CsA for 72 h. After treatment, the cell lysates were subjected to Western blot analysis. The production of NS3 and NS5A was analyzed using anti-NS3 and anti-NS5A antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane

activity encoded by replicon RNAs in stable cell lines harboring these autonomously-replicating RNAs. The results revealed that the 1b/2a chimeric replicon was less sensitive to CsA than the 1b replicon. However, there were no differences in sensitivity to other anti-HCV reagents (IFN-α, IFN-γ, PTV, RBV, and MZB) between the 1b replicon and 1b/2a chimeric replicon (Fig. 1b). We also tested the expression levels of HCV proteins (NS3 and NS5A) in CsA-treated replicon-harboring cells (Fig. 1c). CsA decreased HCV protein expression levels in the 1b eplicon-harboring cells in a dose-dependent manner. On the other hand, in the 1b/2a chimeric replicon-harboring cells those levels were not changed at the higher concentration of CsA treatment. These results suggest that NS5B of JFH-1 decreased the sensitivity to CsA in 1b/2a chimeric replicon-harboring cells.

CyPA is essential for HCV RNA replication

It has been reported that CyPs are responsible for CsA's anti-HCV activity [17, 20, 21]. Therefore, we next examined the role of CyPs in HCV RNA replication and the anti-HCV activity of CsA using short-hairpin RNA (shRNA) against CyPA or CyPB. The silencing of CyPA or CyPB by shRNA was confirmed by Western blot analysis (Fig. 2a). The silencing of CyPA significantly suppressed HCV protein expression in 1b replicon-harboring cells and in 1b/2a chimeric replicon-harboring cells. The silencing of CyPB didn't suppress HCV protein expression in the former replicon-harboring cells and slightly suppressed in the latter replicon-harboring cells. We also demonstrated that the HCV RNA levels in these cells correlated with the results of Western blot analysis (data not shown). These results suggest that CyPA is essential for replication of both 1b and 1b/2a chimeric replicon. On the other hand, CyPB might partially affect HCV RNA replication of the 1b/2a chimeric replicon.

We next evaluated the sensitivity to anti-HCV reagents (CsA and IFN-α) between control cells and CyPs-knockdown cells. The results revealed that the sensitivity to CsA was drastically enhanced in both CyPA-knockdown 1b replicon-harboring cells and 1b/2a replicon-harboring cells (Fig. 2b), while the sensitivity to IFN-α was not dramatically changed in replicon-harboring either cells (see Supplementary Material). The silencing of CyPB slightly improved the sensitivity to CsA in the 1b/2a chimeric

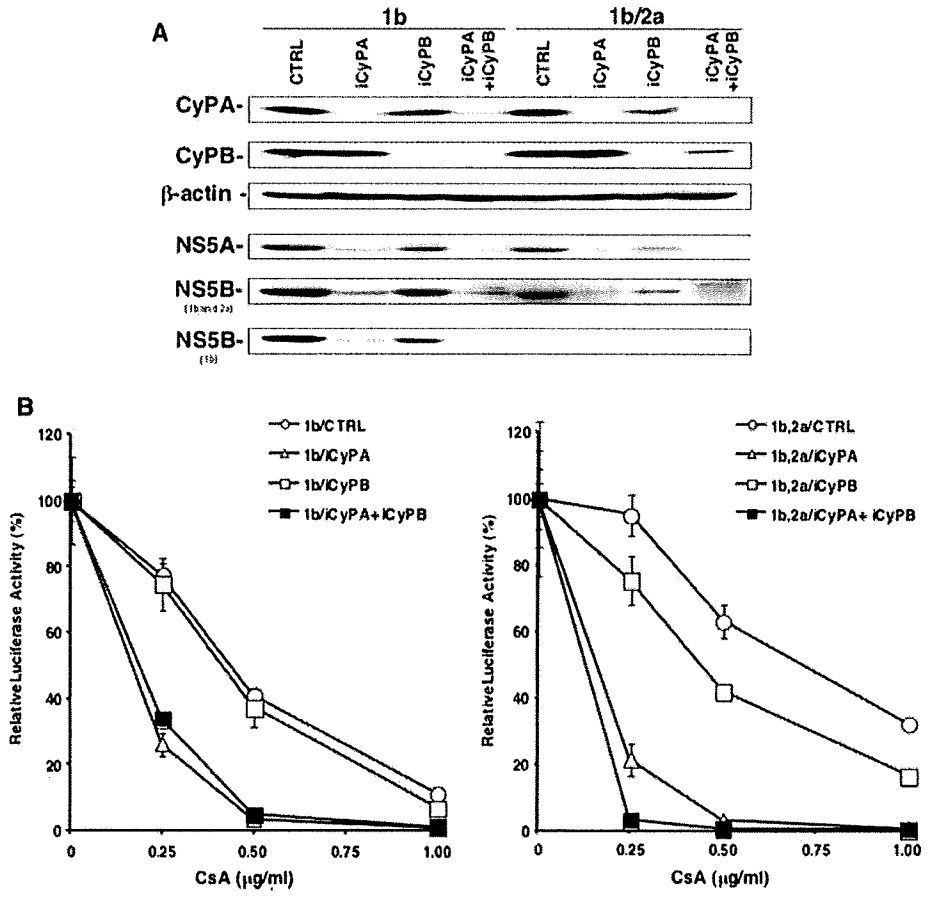

Fig. 2 CyPA is essential for HCV RNA replication and modulates the anti-HCV activity of CsA. **a** Western blot analysis of HCV proteins and CyPs. The 1b replicon and 1b/2a chimeric replicon-harboring cells were transduced with the indicated shRNA for 1 week. The cell lysates were subjected to Western blot analysis. CTRL indicates the control cells transfected with the empty vector. **b** Effects of CyPs on CsA's anti-HCV activity in the 1b replicon- (*left panel*) and 1b/2a chimeric replicon- (*right panel*) harboring cells. After 72 h treatment with CsA, the RL assay was performed. The relative luciferase activity was calculated as described in Fig. 1b. The cells transfected with CTRL, CyPA, CyPB, and both CyPA and CyPB shRNA indicate *open circles*, *open triangles*, *open squares*, and *closed squares*, respectively

replicon-harboring cells but made no improvement in the 1b replicon-harboring cells. Moreover, the silencing of CyPA and CyPB additively enhanced the sensitivity to CsA in the 1b/2a chimeric replicon-harboring cells but not in the 1b replicon-harboring cells. These results suggest that the major cellular determining factor in HCV RNA replication is CyPA rather than CyPB in the 1b/2a chimeric replicon-harboring cells.

HCV NS5B interacts more strongly with CyPB than with CyPA

Although it has been reported that the interaction between CyPs and NS5B is important for HCV RNA replication using glutathione S-transferase pull-down assay [20, 21], the binding activity of NS5B to CyPs in physiological conditions remains unclear. To evaluate the interaction between CyPs and NS5B, we performed an immunoprecipitation assay using 293FT cells transfected with the expression vectors of CyPs (CyPA or CyPB) and NS5B (HCV-O or JFH-1 strain). The obtained results revealed that both NS5Bs interacted more strongly with CyPB than with CyPA. Furthermore, NS5B of HCV-O interacted more strongly with CyPs than did NS5B of JFH-1 (Fig. 3). Since CyPA expression is important for robust HCV RNA replication, these results suggest that the interaction between CyPA and NS5B might not be important for HCV RNA replication or for the anti-HCV activity of CsA.

VE completely negates CsA's anti-HCV activity in the presence or absence of CyPs

We previously reported that VE supplementation negated CsA's anti-HCV activity [22]. To rule out the possibility that VE negates CsA's anti-HCV activity in only CyP-expressing cells, we examined whether or not VE could negate CsA's anti-HCV activity in the presence or absence of CyPs (Fig. 4). Surprisingly, VE negated CsA's anti-HCV activity in the presence or absence of CyPs. It is noteworthy that VE negated this activity more efficiently in CyPA knockdown cells than in the control or CyPB knockdown cells.

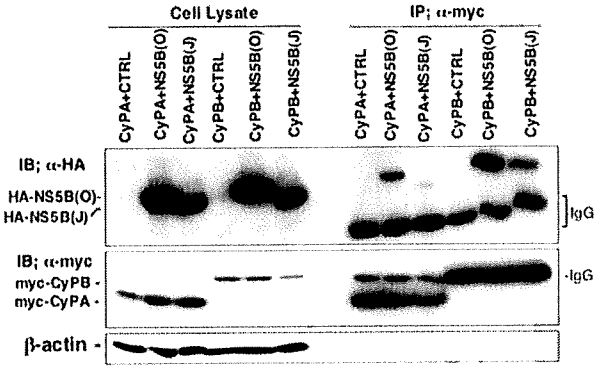

Fig. 3 CyPs interact with HCV NS5B. 293FT cells were cotransfected with plasmids expressing Myc-tagged CyP and HA-tagged HCV NS5B for 48 h. The cells were lysed and subjected to immunoprecipitation with monoclonal Myc-antibody, followed by immunoblot analysis with either anti-HA (*top*) or anti-Myc (*bottom*) antibodies. CTRL indicates empty vector

Discussion

Since it was first reported that CsA possesses anti-HCV activity, several groups have found that CsA suppresses HCV RNA replication using HCV replicon-harboring cells. In addition, the genotype 1a and 1b replicons possess high sensitivity to CsA [17, 19, 21], but the replicon of genotype 2a, JFH-1 strain, is less sensitive to CsA [12]. However, the mechanism of CsA resistance remains unclear.

Recently, Murayama et al. [16] reported that NS3 helicase and NS5B of JFH-1 were essential for robust replication using intragenotypic 2a replicon with J6 backbone. In contrast, Binder et al. [3] demonstrated that NS3 helicase from JFH-1 reduced replication efficiency of 1b/2a chimeric replicon with NS5B from JFH-1 in genotype 1b Con1 backbone. These results suggest that the effect of co-substitution of NS3 helicase with NS5B on HCV RNA replication is different between genotype 1b and 2a backbones.

In this study, we clearly demonstrated that the viral determining factor of sensitivity to CsA is NS5B, by using 1b/2a chimeric replicon-harboring cells. The homology of NS5B region between HCV-O and JFH-1 is 75% in amino acids. Fernandes et al. [5] reported amino acid change from serine to glycine at position 556 of NS5B in CsA resistant 1b replicon. Interestingly, amino acid at this position in HCV-O and JFH-1 are serine and glycine, respectively. The results indicate that the difference in amino acid sequences in NS5B between the HCV-O and JFH-1 strains contributes to the sensitivity to CsA.

Moreover, we further demonstrated that the major cellular determining factor for HCV RNA replication is CyPA rather than CyPB. CyPB is partially involved in only 1b/2a chimeric replicon RNA replication. These results suggest that decreased endogenous expression of CyPA by shRNA contributes to suppression of HCV RNA replication. Furthermore, the knockdown of CyPA enhances CsA's anti-HCV activity. Since the silencing of CyPB slightly enhanced the sensitivity to CsA in only the 1b/2a chimeric replicon-harboring cells, the expression level of CyPB might contribute to the suppression of HCV RNA replication in the case of genotype 2a, JFH-1 strain.

It has been reported that CyPB binds to NS5B and regulates its activity [20]. We also demonstrated that NS5B

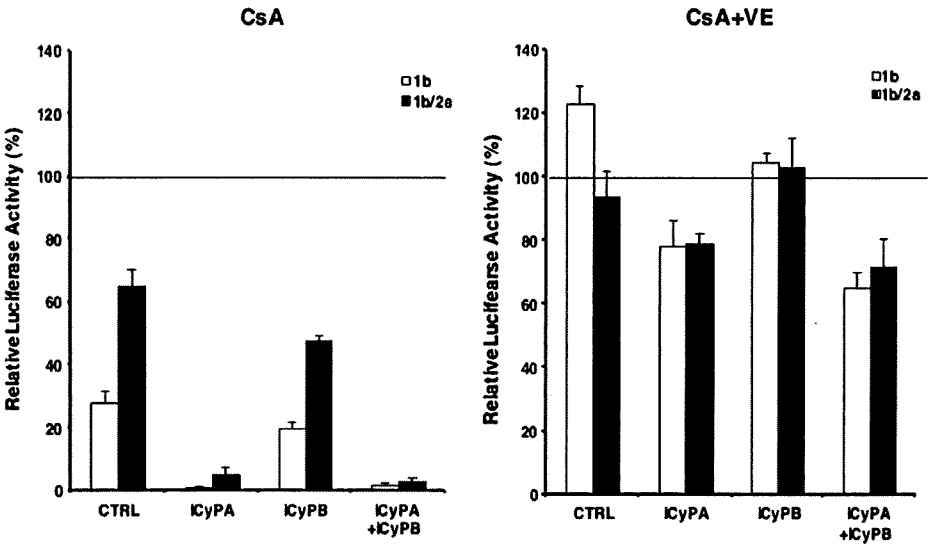

Fig. 4 The effect of CsA (0.5 μg/ml) in combination with VE (10 μM) on HCV RNA replication in the 1b replicon- (*open columns*) and the 1b/2a chimeric replicon- (*closed columns*) harboring cells. After 72 h treatment, the RL assay was performed according to the manufacturer's protocol. The relative luciferase activity was calculated as described in Fig. 1b

bound to CyPB. However, our results revealed that NS5B more strongly interacted with CyPB than with CyPA. The difference in binding activity between CyPA and CyPB may be caused by subcellular localization. It has been reported that CyPA and CyPB are localized in cytoplasm and endoplasmic reticulum (ER), respectively [7]. On the other hand, NS5B localizes with ER membranes [13]. Our data, showing that NS5B interacted more strongly with CyPB than with CyPA, might be attributable to the difference in subcellular localization between cytoplasm and ER. We also demonstrated that NS5B of HCV-O interacted more strongly with CyPs than did NS5B of JFH-1. Moreover, the expression of CyPA plays a major role in robust HCV RNA replication. On the other hand, CyPB has little impact on HCV RNA replication. Taken together, these results suggest that the interaction between CyPA and NS5B might partially affect HCV RNA replication and the anti-HCV activity of CsA.

It is noteworthy that VE can negate CsA's anti-HCV activity in the presence or absence of CyPs. We also examined whether or not the combination treatment of CsA and other antioxidants (vitamin C, sodium selenate, and coenzyme Q10) could negate CsA's anti-HCV activity. Among these antioxidants, only VE negated CsA's anti-HCV activity (data not shown). Understanding VE's involvement in CsA's anti-HCV activity may help us identify factors other than the interaction between CyPA and NS5B.

CsA derivatives that affect only CyPA and that also lack immunosuppressive function will have advantages over CsA. A combination therapy of CsA or CsA derivatives with VE should be avoided so as not to affect CsA's anti-HCV activity clinically. In conclusion, we have demonstrated that NS5B of JFH-1 contributed to the CsA-resistant phenotype of this strain using 1b/2a chimeric replicon-harboring cells and CyPA is a major cellular determining factor in HCV RNA replication.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Interferon-α-induced mTOR activation is an anti-hepatitis C virus signal via the phosphatidylinositol 3-kinase-Akt-independent pathway

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Abstract
Object The interferon-induced Jak-STAT signal alone is not sufficient to explain all the biological effects of IFN. The PI3-K pathways have emerged as a critical additional component of IFN-induced signaling. This study attempted to clarify that relationship between IFN-induced PI3-K-Akt-mTOR activity and anti-viral action.
Result When the human normal hepatocyte derived cell line was treated with rapamycin (rapa) before accretion of IFN-α, tyrosine phosphorylation of STAT-1 was diminished. Pretreatment of rapa had an inhibitory effect on the IFN-α-induced expression of PKR and p48 in a dose dependent manner. Rapa inhibited the IFN-α inducible IFN-stimulated regulatory element luciferase activity in a dose-dependent manner. However, wortmannin, LY294002 and Akt inhibitor did not influence IFN-α inducible luciferase activity. To examine the effect of PI3-K-Akt-mTOR on the anti-HCV action of IFN-α, the full-length HCV replication system, OR6 cells were used. The pretreatment of rapa attenuated its anti-HCV replication effect in comparison to IFN-α alone, whereas the pretreatment with PI3-K inhibitors, wortmannin and LY294002 and Akt inhibitor did not influence IFN-induced anti-HCV replication.
Conclusion IFN-induced mTOR activity, independent of PI3K and Akt, is the critical factor for its anti-HCV activity. Jak independent mTOR activity involved STAT-1 phosphorylation and nuclear location, and then PKR is expressed in hepatocytes.

Keywords mTOR · STAT-1 · Interferon · HCV · PKR

Abbreviations

IFN	Interferon
HCV	Hepatitis C virus
STAT	Signal transducers and activators of transcription
ISGF-3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated regulatory element
PKR	Double-stranded RNA-dependent protein kinase
Rapa	Rapamycin
PI3-K	Phosphatidylinositol 3-kinase
mTOR	Mammalian target of rapamycin
siRNA	Small interfering RNA

Introduction

Currently, a chronic hepatitis C virus (HCV) infection is the major cause of hepatocellular carcinoma worldwide [1]. Therefore, an anti-HCV strategy is important for prevention of carcinogenesis. Advancement in the treatment of HCV by a combination of pegylated interferon (IFN) and

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ribavirin is effective in 80% of HCV genotype 2 or 3 cases, but less than 50% of genotype 1 cases. To ameliorate the salvage rate of HCV infection, new anti-HCV agents have been developed to inhibit the life cycle of HCV and are combined with IFN-α [2]. Since IFN-α is the most basic agent for HCV treatment, it is necessary to improvement the salvage rate of HCV infection by clarifying the efficacy of IFN treatment.

The factors associated with a refractory response to IFN treatment are the HCV genotype, viral load, age, sex, fibrosis of the infected liver and metabolic factors such as insulin resistance and steatosis [3]. Increased hepatic expression of the suppressor of cytokine signaling (SOCS) family, known as the Jak-STAT signal inhibitors, especially SOCS-3, is associated with non-response to IFN treatment [4, 5]. It is thought that inflammatory cytokines, such as, interleukin 6, induced by HCV infection can induce SOCS-3 in hepatocyte [5]. SOCS-3 inhibits IFN-induced tyrosine phosphorylation of Jak, then intra-hepatocyte IFN signal transduction is inhibited. For HCV survival, Jak1, Tyk2 and STAT-1,-2 signaling, which is the essential pathway for type 1 IFN-induced anti-viral activity, becomes the attack targets from HCV. The relative lack of a viral response to IFN treatment is associated with blunted IFN signaling [6]. HCV coding proteins also inhibit STAT-1 tyrosine phosphorylation [7]. The cause of a refractory response to IFN treatment is thought to be HCV-induced Jak-STAT signal inhibition.

Type 1 IFN is a pleiotropic cytokine which activates various intra-cellular signal pathways other than the Jak-STAT signal [8]. Additional signaling pathways could either collaborate with STATs at the promoter level and contribute to the activation of the STATs plus transcription factor genes or function totally independent of any STAT factors, thus leading to the activation of transcription factor only genes [8]. The Jak-STAT signal alone is not sufficient to explain all the biological effects of type 1 IFN. The PI3-K and p38 kinase pathways have emerged as critical additional component of IFN-induced signaling [8–10]. p38, activated via IL-1β is enhanced STAT-1 tyrosine phosphorylation and express the anti-viral protein, PKR [9]. The IFN-induced PI3-K-Akt pathway has Jak independent activation, and it is the critical signal for cell survival and insulin action [10], but its relationship with the anti-viral action and PI3-K-Akt pathway is still unclear.

Recently, mTOR, a downstream kinase of PI3-K-Akt pathway, was shown to play a critical role in protein synthesis and anti-viral effects. Kaur and his colleagues [11] reported that the IFN activated mTOR pathway exhibits important regulatory effects in the generation of the IFN responses, including the anti-encephalo myocarditis virus effect. The IFN-induced mTOR is LY294002 sensitive and does not affect the IFN-stimulated regulatory element (ISRE) dependent promoter gene activity. Human cytomegalovirus is inhibited by 5′-AMP-activted protein kinase mediated inhibition of mTOR kinase [12]. In contrast, vesicular stomatitis virus is mTOR dependent [13]. A relationship has been reported between the replication of hepatitis virus and mTOR activity. p21-activated kinase 1 is activated through the mTOR/p70 S6 kinase pathway and regulates the replication of HCV [14]. mTOR activation is dependent upon the PI3-K-Akt and ERK pathways. Gao and colleagues reported that HCV-NS5A protein activates the PI3-K-Akt-mTOR pathway and could inhibit HBV RNA transcription and reduce HBV DNA replication in HepG2 cells [15]. The activation of the N-Ras-PI3-K-Akt-mTOR pathway by HCV is required for cell survival and HCV replication [16]. Therefore, PI3-K, Akt and mTOR activated by HCV are inhibitory signals of HCV replication and survival signals of HCV infected cells. Furthermore, the PI3-K-Akt-mTOR pathway, which is activated by HCV, is thought to be one mechanism for chronic HCV infection [14–16]. However, type 1 IFN-induced PI3-K, Akt and mTOR have not yet been fully evaluated regarding their influence on HCV replication.

This study investigated whether IFN-α induced the PI3-K-Akt-mTOR pathway, whether the Jak-STAT pathway has a relationship with the PI3-K-Akt-mTOR pathway, and, finally, whether IFN induced signal transduction, other than the Jak-STAT pathway, is associated with the anti-HCV activity.

Materials and methods

Reagents and cell culture

Recombinant human IFN-α2b was a generous gift from Schering-Plough KK (Tokyo, Japan). Wortmannin, LY 294002, Akt inhibitor and rapamycin were purchased from Calbiochem (La Jolla, CA, USA). Hc human hepatocyte cells (Applied Cell Biology Research Institute, Kirkland, WA, USA) and HuH-7 human hepatoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained in a chemically defined medium, CS-C completed (Cell Systems, Kirkland, WA, USA) and RPMI (Invitrogen, Grand Island, NY, USA), respectively, supplemented with 5% fetal bovine serum. In the pretreatment of rapamycin and chemical inhibitors for 3 h, the cells were

cultured in 5% RPMI, and then exchanged the medium and treated the cells with IFN-α2b at the indicated time.

Cell viability assay

The cells were measured using the colorimetric cell viability assay method. Cell viability was determined by the colorimetric method using a Cell Counting kit (Wako Life Science, Osaka, Japan). The absorbance of each well was measured at 405 nm with a microtiter plate reader (Multiskan JX, Thermo BioAnalysis Co., Japan). After 2 days of 100 IU/mL IFN-α and 1000 nmol/L rapamycin treatment, Cell viability is expressed as a percentage of the viability in standard media without IFN-α and rapamycin. Data were expressed as the mean ± standard deviation (SD). Statistical significance was assessed using Student's t test. Statistical difference was defined as $P < 0.05$. All numerical results were reported as the mean of four independent experiments.

Western blotting and antibodies

Western blotting with anti-PKR, anti-STAT-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tyrosine-701 phosphorylated STAT-1, anti-serine-727 phosphorylated STAT-1, anti-p48, anti-serine-437 phosphorylated Akt, anti-threonin-308 phosphorylated anti-Akt, anti-Akt, anti-serine-2448 phosphorylated mTOR, anti-serine-2481 phosphorylated mTOR, anti-mTOR, anti-JAK-1 or anti-tyrosine 1022/1023 JAK-1 (Cell Signaling, Beverly, MA, USA) was performed as described previously [9]. Briefly, Hc cells were lysed by the addition of a lysis buffer (50 mmol/L Tris–HCl, pH 7.4, 1% Np40, 0.25% sodium deoxycholate, 0.02% sodium azide, 0.1% SDS, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1 μg/mL each of aprotinin, leupeptin and pepstatin, 1 mmol/L sodium o-vanadate and 1 mmol/L NaF). The samples were separated by electrophoresis on 8–12% SDS polyacrylamide gels and electrotransferred to nitrocellulose membranes, and then blotted with each antibody. The membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and the immunoreactive bands were visualized using the ECL chemiluminescence system (Amersham Life Science, Buckinghamshire, England).

Fluorescence immunohistochemistry

The Hc cells were seeded onto 11-mm glass cover-slips in 24-well plates at 2.4×10^5 cells/well. The next day, the medium was replaced with serum-free medium, and the cells were pretreated with 10 or 100 nmol/L rapamycin, or vehicle, for 3 h and then stimulated with 100 IU/mL IFN-α for 10 min. Fluorescence immunohistochemistry was performed as described previously [17]. The cells were incubated with anti-tyrosine-701 phosphorylated STAT1 antibody for 1 h at room temperature, washed three times in PBS, incubated with rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h, washed in PBS, and mounted in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). Nuclear staining was performed using Hoechst 33258 (Invitrogen Japan K.K., Tokyo, Japan). An immunofluorescence analysis was done using an Olympus BX50 microscope (Tokyo, Japan) and the image was captured by a Nikon DXM 1200 digital camera (Tokyo, Japan).

Reporter gene assay

A pISRE-Luc cis-reporter plasmid containing five copies of the ISRE sequence and the firefly luciferase gene and pRL-SV40 containing the SV40 early enhancer/promoter and the renilla luciferase gene were obtained from Clontech (San Diego, CA, USA) and Promega (Madison, WI, USA), respectively. The HuH-7 cells were grown in 24-well multiplates and transfected with 1 μg of pISRE-Luc and 10 ng of pRL-SV40 as a standard by the lipofection method. One day later, the cells were incubated in the absence or presence of varying concentrations of chemical blockers and IFN-α, and the luciferase activities in the cells were determined using a dual-luciferase reporter assay system and a TD-20/20 luminometer (Promega). The data were expressed as the relative ISRE-luciferase activity.

HCV replicon system

OR6 cells stably harboring the full-length genotype 1 replicon, ORN/C-5B/KE [18], were used to examine the influence of the anti-HCV effect of IFN. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen) supplemented with 10% fatal bovine serum, penicillin and streptomycin and maintained in the presence of G418 (300 mg/L; Geneticin, Invitrogen). This replicon was derived from the 1B-2 strain (strain HCV-o, genotype 1b), in which the *Renilla* luciferase gene is introduced as a fusion protein with neomycin to facilitate the monitoring of HCV replication. After the treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI, USA) and then were subjected to a luciferase assay according to the manufacturer's protocol. mTOR gene knock down is used siRNA (Cell Signaling). 100 nmol/L mTOR specific and non-targeted siRNA as a control was transfected to OR6 cells in accordance with the appended manual. One day later, the cells were incubated in either the absence or presence of 10 IU/mL IFN-α.

Results

IFN-α-induced activity of STAT-1 is inhibited by rapamycin pretreatment

To attempt to clearly identify the influence of mTOR to IFN-α-induced anti-viral protein expression rapamycin (rapa), the specific inhibitor of mTOR, was added prior to treatment with IFN-α. Hc cells have been used as normal hepatocytes in previous reports [19]. The Hc cells were incubated in the absence or presence of IFN-α with or without pretreatment with rapa for 2 h the cells were then harvested for the Western blot analysis (Fig. 1). IFN-α clearly induced tyrosine and serine phosphorylation of STAT-1 at 5 (Fig. 1a, lane 4) and 10 min (Fig. 1a, lane 6), respectively, in the absence of rapa. However, when the Hc cells were pretreated with rapa before IFN-α stimulation, the levels of tyrosine and serine phosphorylated STAT-1 were clearly and rapidly lower than those induced by IFN-α alone 5 min after treatment in tyrosine (Fig. 1a, lane 5). Jak-1, an upstream protein of STAT-1, was equally phosphorylated by IFN-α with (Fig. 1b, lane3) or without (Fig. 1b, lane2) pretreatment with rapa. The viability of the Hc cells was 1 in vehicle, 0.93±0.21 in IFN-α treatment and 0.88±0.34 in rapamycin treatment. No difference in the cell viability the among vehicle, IFN-α and rapamycin treatment was not recognized in our assay. The viability of the HuH-7 and OR6 cells also demonstrated no difference between the presence of IFN-α and rapamycin treatment and the absence thereof.

IFN inducible gene products are diminished by pretreatment of rapamycin

Since pretreatment with rapa inhibited the IFN-α induced STAT-1 activity, the phosphorylation of tyrosine and serine and nuclear translocation, the effect of pretreated with rapa on the IFN-α inducible gene product was examined. The protein levels of PKR, an anti-viral protein that acts as a mRNA translation inhibiter activated by double stranded RNA [20, 21], and p48, key component of ISGF-3 with activated STAT-1 and -2 [22], were induced by IFN-α treatment for 3 h in Hc cells (Fig. 1c, lanes 1, 2). However, pretreatment with rapa had an inhibitory effect on IFN-α-induced PKR and p48 in a dose dependent manner (Fig. 1c, lanes 2–4).

The serine 473 on Akt and serine 2448 on mTOR are phosphorylated by IFN-α

Because pretreatment with rapa affected the IFN-α signaling (Fig. 1), the ability of IFN-α to activate the Akt-mTOR pathway was investigated. The phosphorylation of serine-2448 residues of mTOR and serine-473 residue of

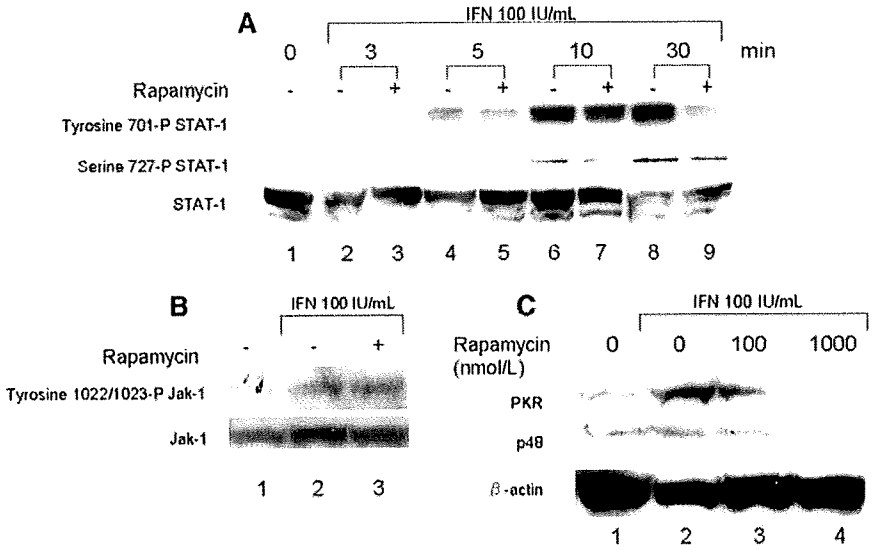

Fig. 1 Alteration in the distribution of IFN-α induced phosphorylated STAT-1 (**a**) and Jak-1 (**b**) by rapamycin and effect of rapamycin on IFN-α-induced PKR and p48 (**c**). Hc cells were pretreated without (*lanes 1, 2, 4, 6,* and *8*) or with 1 μmol/L rapa (*lanes 3, 5, 7,* and *9*). These Hc cells were stimulated by 100 IU/L IFN-α (*lane 2–9*) for 30 min. Phosphorylated STAT-1 at tyrosine-701 residue (*upper panel*) and at serine-727 residue (*lower panel*) were analyzed by Western blotting. **a** After pretreatment of 1000 nmol/L rapa (*lane 3*) for 3 h, Hc cells were untreated (*lane 1*) or treated with 100 IU/mL IFN-α (*lanes 2, 3*) for 3 min, then phosphorylated JAK-1 at tyrosine-1022/1023 residue (*first panel*), expression of JAK-1 (*second panel*) were analyzed by Western blotting (**b**). Hc cells were treated with 100 IU/mL of IFN-α in the absence (*lane 2*) or of the presence of pretreatment (*lane 3, 4*). *Lane 1* was not treated IFN-α and calcineurin inhibitors. One day latter, PKR and p48 was determined by Western blotting (**c**)

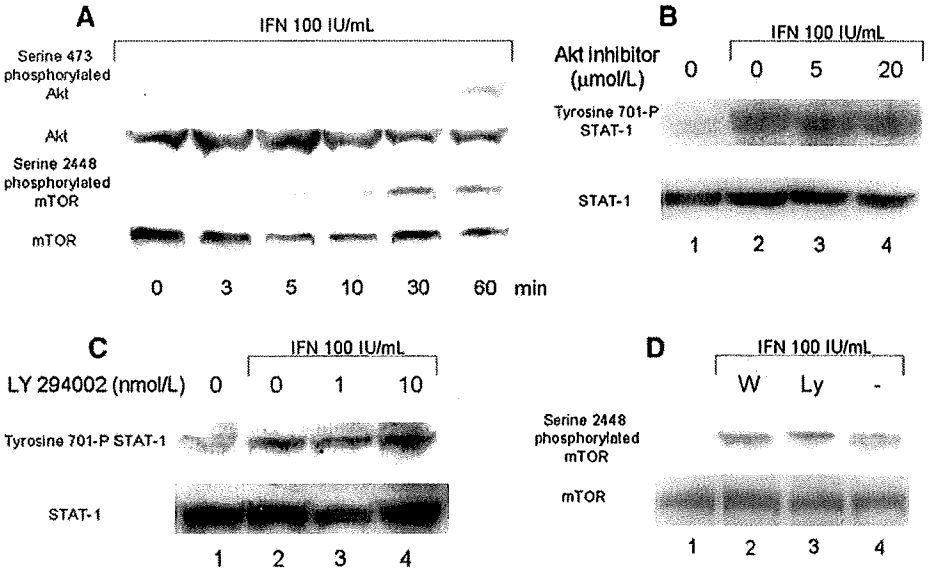

Fig. 2 Effect of IFN-α on Akt and mTOR (**a**) and effect of Akt inhibitor (**b**) and LY294002 (**c**) on IFN-α-induced tyrosine phosphorylated STAT-1 and Serine phosphorylated mTOR (**d**). Hc cells were stimulated by 100 IU/L IFN-α for 60 min. At the indicated time, the cells were harvested. Phosphorylated Akt at serine-473 residue (*first panel*), Akt (*second panel*), mTOR at serine-2448 residue (*third panel*) and mTOR (*fourth panel*) were analyzed by Western blotting. After pretreatment with 5 or 20 μmol/L Akt inhibitor (*lane 3*, and 4, respectively) (**b**) and 1 or 10 nmol/L LY294002 (*lane 3* and *4*, respectively) (**c**) for 3 h, Hc cells were untreated (*lane 1*) or treated with 100 IU/mL IFN-α (*lanes 2–4*) for 5 min and phosphorylated STAT-1 at tyrosine-701 residue (*first panel*), expression of STAT-1 (*second panel*) were analyzed by Western blotting. **d** After pretreatment with 100 nmol/L wortmannin (*lane 2*) and 1 nmol/L LY294002 (*lane 3*) for 3 h, the Hc cells were either untreated (*lane 1*) or treated with 100 IU/mL IFN-α (*lanes 2–4*) for 10 min and then were phosphorylated mTOR at Serine-2448 residue (*first panel*), the expression of mTOR (*second panel*) was analyzed by Western blotting

Akt by 100 IU/ml of IFN-α was detected at 5 min and at 60 min after IFN-α treatment, respectively (Fig. 2a). The band intensity of serine 2448 phosphorylated mTOR increased at 30 min and decreased at 60 min after IFN-α treatment. In contrast, a slight band intensity of serine phosphorylated 473 Akt was only detected at 60 min after IFN-α treatment. In addition, a Western blot analysis of phosphorylated serine 2481 of mTOR and threonine 308 Akt was conducted under the same conditions as Fig. 2a, but no bands were detected (data not shown). In Fig. 2d, IFN-α-induced Serine 2448 phosphorylated mTOR was not inhibited by PI3-K inhibitors (lanes 2, 3).

The IFN-α-induced nuclear translocation of tyrosine phosphorylated STAT-1 was inhibited by pretreatment with rapa

The location of tyrosine phosphorylated STAT-1 was evaluated by fluorescence immunohistochemistry of cultured Hc cells (Fig. 3). The IFN-α-induced nuclear translocation of tyrosine phosphorylated STAT-1 was observed (Fig. 3c), but its translocation was inhibited by pretreatment with rapa and the inhibition of the translocation of STAT-1 was more definitive at 1000 nmol/L rapa (Fig. 3e) than 100 nmol/L (Fig. 3g).

IFN-α-induced ISRE-contained promoter activity is inhibited by pretreatment of rapa, but not by wortmannin, LY294002 and Akt inhibitor

The influence of pretreatment of PI3-K-Akt-mTOR inhibitors on IFN-α inducible luciferase activity of the ISRE-containing promoter was examined. Since Hc cells were not sufficient for reporter gene transfection, HuH-7 cells were used in the transfection assay. HuH-7 cells were transfected with pISRE-Luc containing five repeats of the ISRE sequence and pRV-SV40 as a standard and then were treated with IFN-α after 3 h with or without pretreatment with rapa, wortmannin, LY294002 or Akt inhibitor. Rapa inhibited IFN-α inducible luciferase activity in a dose-dependent manner (Fig. 4, lane 2–4). However, wortmannin and LY294002, PI3-K inhibitor, and Akt inhibitor had no effect on IFN-α inducible luciferase activity (Fig. 4, lanes 2, 5–7).

The expression of IFN-α-induced tyrosine phosphorylated STAT-1 was determined after pretreatment with Akt inhibitor and LY294002 to evaluate the result of luciferase assay (Fig. 4). The Hc cells were incubated under the same conditions used in Fig. 4, but phosphorylated STAT-1 was not inhibited by the Akt inhibitor (Fig. 2b) and LY294002 (Fig. 2c).

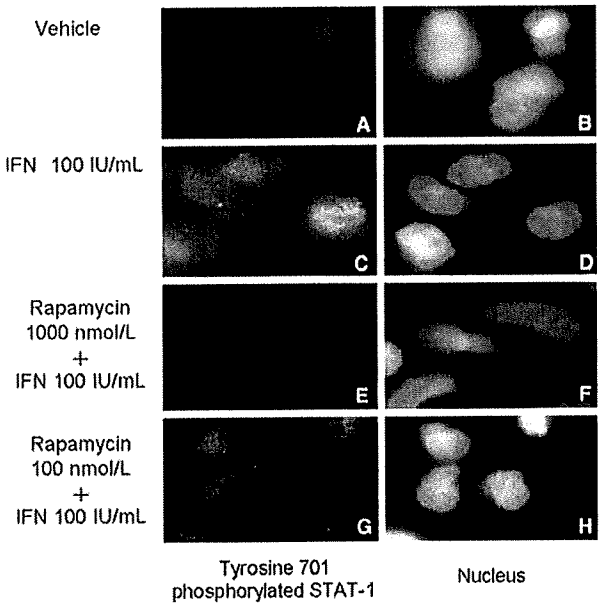

Fig. 3 Inhibition of IFN-α-induced nuclear translocation of phosphorylated STAT-1 by rapamycin. The Hc cells were pretreated without (**a–d**) or with 1000 nmol/L rapa (**e, f**) or 100 nmol/L rapa (**g, h**). After pretreatment, the Hc cells were stimulated by 100 IU/L IFN-α (**c–h**) for 30 min. Thereafter, the cells were fixed, permeabilized, processed for immunofluorescence (**a, c, e, g**) and Hoechst staining (**b, d, f, h**), and visualized by fluorescence microscopy. The results shown are from one representative experiment from a total of three performed

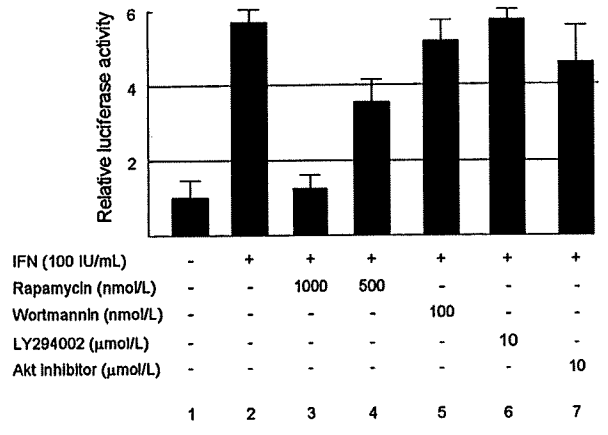

Fig. 4 Suppression effect of rapamycin, not PI3-k inhibitors and Akt inhibitor, on IFN-α-induced reporter gene assay. HuH-7 cells transfected with reporter gene (pISRE-Luc and pRL-SV40) were either untreated (*lane 1*) or pretreated with rapa (*lane 3, 4*), wortmannin (*lane 5*), LY294002 (*lane 6*) or Akt inhibitor (*lane 7*) for 3 h, followed by IFN-α 100 IU/mL (*lanes 2–7*). Six hour later, the relative ISRE-luciferase activity ($n = 4$) was determined as described in the "Materials and methods". The data are expressed as the mean ± SD and are representative example of four similar experiments

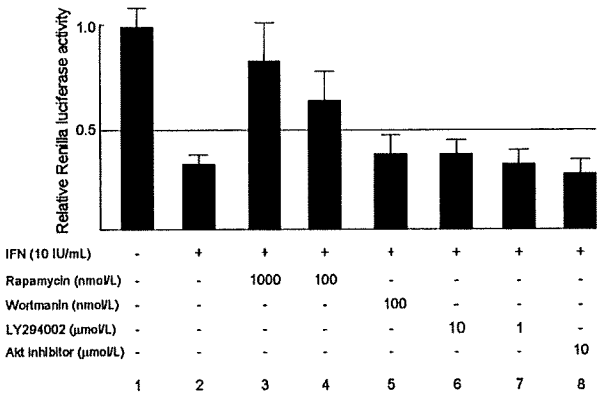

Fig. 5 Alternation of IFN-α suppressed HCV replication by rapamycin, but not PI3-K inhibitors and Akt inhibitor. OR6 cells, a full-length replicon system, were treated with 100 IU/mL of IFN-α in the absence (*lane 2*) or presence of pretreatment (*lanes 3–8*) for 3 h. *Lane 1* was not treated IFN-α alone. One day latter, *Renilla* luciferase activity was determined by luminometer ($n = 4$). The data are expressed as the mean ± SD and are representative example of four similar experiments

Rapamycin and mTOR specific siRNA, but not PI3-K inhibitor and Akt inhibitor can cancel the IFN-α-induced anti-HCV replicon activity

OR6 cells the full-length HCV replication system was used to examine the anti-viral effect of PI3-K, Akt and mTOR on IFN-α stimulation. The cells were treated with IFN-α after 3 h in the presence or absence of rapa, Akt inhibitor or PI3-K inhibitor (Fig. 5). Pretreatment with rapa attenuated its anti-HCV replication effect in comparison to IFN-α alone (Fig. 5, lanes 1–4), whereas pretreatment with PI3-K inhibitors and Akt inhibitor did not increase the *Renilla* luciferase activity (Fig. 5, lanes 1, 2, 5–8). We performed siRNA transfection for mTOR knock down (Fig. 6). Although transfection efficiency of siRNA is barely 10%, IFN-α-induced anti-HCV action was clearly inhibited in siRNA against mTOR transfected cells (lane 5) in comparison to the control cells (lane 6).

Discussion

Rapa inhibited the IFN-α-induced tyrosine and serine phosphorylation and nuclear translocation of STAT-1, the ISRE-promoter activity, the expression of PKR and the replication of HCV replicon. This suggests that the IFN-induced mTOR activity, through Jak independent STAT-1 phosphorylation, is a critical signal for IFN-induced anti-HCV action. Interestingly, mTOR activated by IFN was PI3-K-Akt independent in this study.

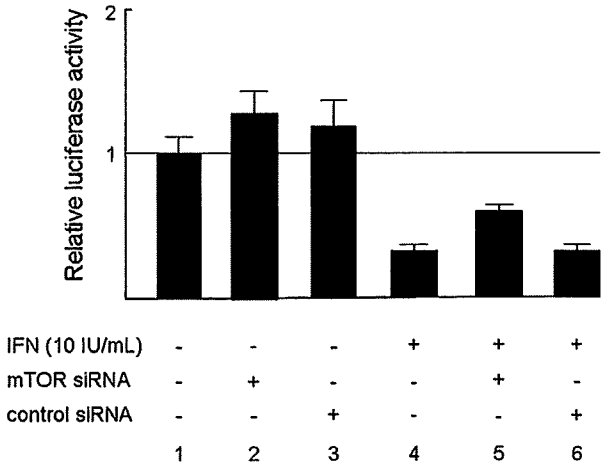

Fig. 6 Alternation of IFN-α suppressed HCV replication by siRNA against mTOR. The OR6 cells were transfected the siRNA against mTOR (*lanes 2, 5*) and the non-targeted siRNA (*lanes 3, 6*). One day later, the cells were IFN-α treatment (*lanes 4–6*). HCV replicon assay is same as Fig. 5. The data are expressed as the mean ± SD and are representative example of four similar experiments

mTOR activity may have an inhibitory action on HCV replication through STAT-1 phosphorylation, but not the translation initiation action of mTOR. This study assumed that IFN-induced PKR expression and ISRE-luciferase activity were inhibited by rapa as the result of a suppression effect on IFN inducible STAT-1 activation. IFN inducible PKR contributes the anti-HCV action [20], and anti-HCV action of ribavirin is also attributable to its ability to up-regulate PKR activity [21]. Previous reports revealed that the mTOR activity did not influence the HCV-IRES activity because the viral promoter has cap-independent translation [23]. Although mTOR is the mRNA translational regulator through phosphorylation of a downstream target such as 4E-BP and S6K [24], we think that the IFN-induced mTOR activity influences the phosphorylation of STAT-1 in our study (Fig. 1). In addition, it is thought that the alternation of STAT-1 phosphorylation by the mTOR activity influences the gene expression of anti-virus protein and IFN-induced anti-viral action.

In our study, serine-473 on Akt showed a delayed phosphorylation in comparison to that of serine-2448 on mTOR after IFN stimulation (Fig. 2a). Since serine-473 on Akt is phosphorylated by mTOR/Rictor/GβL [25, 26] and a PDK-1 independent pathway [25], IFN-induced serine-473 phosphorylated Akt may not involve the mTOR activity. Therefore, PI3-K inhibitor and Akt inhibitor had no effect on IFN inducible anti-HCV action. The pathway of mTOR activation is prismatic. PI-3Ks, upstream kinase of Akt and mTOR, are grouped into three classes (I–III), according to their substrate preference and sequence homology [27]. PI3-k inhibitor, wortmannin and LY294002, inhibit class I and III PI3-Ks, and to a lesser extent class II PI3-K, upstream kinase of Akt [27]. In our study, neither PI3-K nor Akt inhibitor inhibited IFN-induced ISRE luciferase activity and loss of HCV replication (Figs. 4, 5). These results indicate that the IFN-induced anti-HCV activity is mTOR dependent, but not PI3-K and Akt dependent. In the current report, the production of IL-1 receptor antagonist in IFN-stimulated monocytes depends on the PI3-K pathway, but not STAT-1 [28], and chronic myelogenous leukemia cells are differentially regulated by the IFN-induced PI3-K-Akt-mTOR pathway with no relation to STAT-1 phosphorylation [29]. Similar to the findings of those reports, the PI3-K-Akt pathway has been reported to be generally independent of the STAT activity [10]. Therefore, the difference in the cell type [8] may explain the discrepancy between these data and our data. We therefore speculate that in hepatocytes, unlike lymphoid cells, IFN-induced mTOR activity is not dependent on the PI3-K activity. In addition, the mTOR activity is not related to the STAT activity in lymphoid cells. However, in hepatocytes, the IFN-induced mTOR activity was closely linked to the IFN-induced STAT activity in our study.

mTOR is a serine and threonine kinase [10]. Phosphorylation of STATs by mTOR occurs also on a serine residue, but not tyrosine [10, 30]. The mTOR pathway is critical for IFN-γ-induced suppression of tyrosine phosphorylated STAT-3 in a prostate cancer cell line [31]. Although this is not consistent with the results of our study, this also showed mTOR to be associated with tyrosine phosphorylation without reference to SOCS and phosphatase. In addition, in a mouse embryo fibroblast cell line, IFN-γ-induced tyrosine and serine phosphorylation of STAT-1 is inhibited by rapa [32], while in the hepatoma cell line, HLF, IFN-β stimulated STAT-1 tyrosine phosphorylation partially decreases by LY294002, but the effect of rapa has not yet been studied [33]. In the current study [31–33], not only STAT-1 serine phosphorylation but also tyrosine was found to be downstream of the IFN induced mTOR activity; however, the mechanism controlling the tyrosine phosphorylation of STAT-1 and the mTOR activity, remains to be elucidated.

In conclusion, IFN-induced mTOR activity, independent of PI3-K and Akt, is the critical factor for anti-HCV action. The Jak independent mTOR activity is, therefore, involved in STAT-1 phosphorylation and nuclear location, thus resulting in the development of IFN-induced anti-HCV protein, especially the expression of PKR, in HCV-infected hepatocytes.

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Oxidative Stress Induces Anti-Hepatitis C Virus Status via the Activation of Extracellular Signal-Regulated Kinase

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Recently, we reported that β-carotene, vitamin D_2, and linoleic acid inhibited hepatitis C virus (HCV) RNA replication in hepatoma cells. Interestingly, in the course of the study, we found that the antioxidant vitamin E negated the anti-HCV activities of these nutrients. These results suggest that the oxidative stress caused by the three nutrients is involved in their anti-HCV activities. However, the molecular mechanism by which oxidative stress induces anti-HCV status remains unknown. Oxidative stress is also known to activate extracellular signal-regulated kinase (ERK). Therefore, we hypothesized that oxidative stress induces anti-HCV status via the mitogen activated protein kinase (MAPK)/ERK kinase (MEK)–ERK1/2 signaling pathway. In this study, we found that the MEK1/2-specific inhibitor U0126 abolished the anti-HCV activities of the three nutrients in a dose-dependent manner. Moreover, U0126 significantly attenuated the anti-HCV activities of polyunsaturated fatty acids, interferon-γ, and cyclosporine A, but not statins. We further demonstrated that, with the exception of the statins, all of these anti-HCV nutrients and reagents actually induced activation of the MEK–ERK1/2 signaling pathway, which was inhibited or reduced by treatment not only with U0126 but also with vitamin E. We also demonstrated that phosphorylation of ERK1/2 by cyclosporine A was attenuated with *N*-acetylcysteine treatment and led to the negation of inhibition of HCV RNA replication. We propose that a cellular process that follows ERK1/2 phosphorylation and is specific to oxidative stimulation might lead to down-regulation of HCV RNA replication. *Conclusion:* Our results demonstrate the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV status induced by oxidative stress in a broad range of anti-HCV reagents. This intracellular modulation is expected to be a therapeutic target for the suppression of HCV RNA replication. (HEPATOLOGY 2009;50: 678-688.)

Hepatitis C virus (HCV), which belongs to the family Flaviviridae, is a single-stranded positive-sense RNA virus of approximately 9.6 kb.[1,2] Persistent infection with HCV causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma.[3] Therefore, HCV infection is a major health problem worldwide. Interferon (IFN)-based therapies, including the combination of pegylated IFN with ribavirin, are the current standard strategies for chronic hepatitis, but their sustained virological response rates are unsatisfactory.[4,5] There is thus an urgent need for novel partners with IFN or more effective reagents that may improve the sustained virological response rate.

Following the development in 1999 of a cell culture system to support efficient HCV RNA replication,[6] numerous studies have identified reagents that inhibit HCV RNA replication and enhance the effect of IFN treatment.[7-9] Some of these reagents are already available for clinical use. Previously, we also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with Renilla luciferase (RL) as a reporter in hepatoma cell lines.[10] Using this OR6 assay system, we found that mizoribine,[11] as an immunosuppressant, and

Abbreviations: AA, arachidonic acid; BC, β-carotene; CsA, cyclosporine A; CyPA, cyclophilin A; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor: EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FLV, fluvastatin; HCV, hepatitis C virus; IFN, interferon; LA, linoleic acid: MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NS5A, nonstructural 5A; PTV, pitavastatin; PUFA, polyunsaturated fatty acid; RL, renilla luciferase; ROS, reactive oxygen species; VD2, vitamin D_2; VE, vitamin E.

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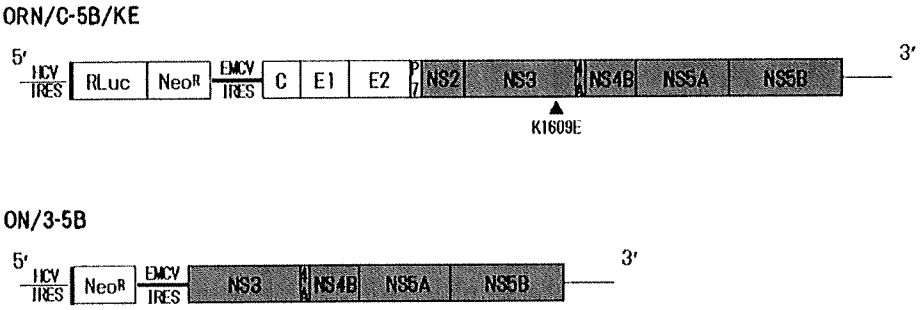

Fig. 1. Schematic gene organization of the genome-length and subgenomic HCV RNA used in this study. ORN/C-5B/KE encoding the RL gene was replicated in OR6 cells and ON/3-5B in sO cells. RL in OR6 cells was expressed as a fusion protein with neomycin phosphotransferase (Neo^R). The arrowhead indicates the position of K1609E, an adaptive mutation.

fluvastatin (FLV) and pitavastatin (PTV),[9,12] as the reagents for hypercholesterolemia, suppressed genome-length HCV RNA replication. Furthermore, in a recent study[13] in which we comprehensively analyzed the activities of ordinary nutrients on HCV RNA replication, three nutrients, β-carotene (BC), vitamin D_2 (VD2), and linoleic acid (LA), were found to suppress HCV RNA replication and enhance the antiviral activity of IFN-α or cyclosporine A (CsA) in an additive or a synergistic manner. Because the anti-HCV activities of these three nutrients, as well as CsA, were canceled by treatment with antioxidants such as vitamin E (VE) or selenium, we suggested that oxidative stress might be involved in the anti-HCV activities of these three nutrients and CsA. However, the detailed molecular mechanism via which the oxidative effects of these three nutrients and CsA suppress HCV RNA replication has not been explored.

The production of reactive oxygen species (ROS) plays a pivotal role in various cellular processes, including cell proliferation, differentiation, and apoptosis.[14] Whereas high-level production of ROS resulting from external stimuli is recognized as an important component of the pathogenesis of inflammatory and cancerous diseases, endogenously produced ROS at low concentrations are shown to function as signaling mediators of cellular responses.[15,16] Emerging evidence indicates that these ROS-triggered responses are mediated primarily via cellular signaling cascades, including a signaling pathway of extracellular signal-regulated kinase (ERK)1/2, namely p44/42 mitogen-activated protein kinase (MAPK), which belongs to the MAPK family.[17,18]

Several studies have revealed that certain viral proteins initiate activation of the MAPK/ERK kinase (MEK)–ERK1/2 signaling pathway, which may facilitate the viral replication and infectivity in the infected cells.[19,20] The HCV core protein[21] and the envelope protein[22] have also been reported to up-regulate this signaling pathway. However, another study reported that the HCV nonstructural 5A (NS5A) protein suppressed activating protein-1 activation by inhibiting the phosphorylation of ERK1/2 in replicon cells.[23] Moreover, recent studies using an inhibitor specific to the MEK–ERK1/2 signaling pathway reported that the direct anti-HCV activities of IFN-γ[24] and acetylsalicylic acid[25] are mediated in part through the induction of this cascade.

We demonstrate that the activation of MEK–ERK1/2 signaling plays a significant role in the anti-HCV activity caused by oxidative stress in a broad range of anti-HCV reagents.

Materials and Methods

Reagents and Antibodies. Dimethyl sulfoxide (DMSO), BC, VD2, VE, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and IFN-γ were purchased from Sigma Aldrich (St. Louis, MO), and CsA, FLV, U0126, PD98059, SB203580, and c-Jun N-terminal kinase inhibitor II were obtained from Calbiochem (San Diego, CA). Epidermal growth factor (EGF) was purchased from Toyobo (Osaka, Japan). PTV was purchased from Kowa Company, Ltd. (Tokyo, Japan). Anti-HCV core antibody (CP11) was purchased from the Institute of Immunology (Tokyo, Japan), and anti-HCV NS5A antibody was the generous gift of Dr. A. Takamizawa (Research Foundation for Microbial Diseases, Osaka University). Antibodies specific to ERK1/2 (p44/42 MAPK), MEK1/2, and phosphorylated (S217/221) MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA), and anti-phosphorylated (T202/Y204) ERK1/2 antibody was obtained from BD Biosciences (San Jose, CA). Anti–β-actin antibody was purchased from Sigma Aldrich.

Cell Cultures. The cell lines OR6 and sO were cloned from ORN/C-5B/KE RNA and subgenomic replicon RNA (ON/3-5B)–replicating cells, respectively (Fig. 1). These cells were derived from the hepatoma cell line HuH-7, cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), peni-

cillin, streptomycin, and 300 μg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA), and passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ON/3-5B were derived from HCV-O (strain O of genotype 1b).[10]

OR6 Reporter Assay. For the RL assay, 1.0-1.5 × 10^4 OR6 cells were plated onto 24-well plates in triplicate and precultured for 24 hours. The cells were pretreated with DMSO or a specific inhibitor for 1 hour and then were treated with each anti-HCV nutrient or compound in either the absence (DMSO) or presence of a specific inhibitor for 72 hours. After the treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

Western Blot Analysis. For analysis of the effect of a specific inhibitor on the anti-HCV activity, 6.0-6.5 × 10^4 OR6 cells were plated onto 6-well plates and precultured for 24 hours. The pretreatment with DMSO or a specific inhibitor for 1 hour and subsequent treatment for 72 hours was performed in the same manner as for the OR6 reporter assay. For analysis of the activities of each anti-HCV nutrient or reagent on the MEK–ERK1/2 signaling pathway, 1.0 × 10^5 OR6 or sO cells were plated onto 6-well plates and precultured in 10% FBS-containing medium for 24 hours. After the preculture, the culture medium was changed to FBS-free medium and the cells were cultured for 48 hours prior to treatment with each nutrient or reagent. When the effect of a specific inhibitor or VE on ERK1/2 phosphorylation was analyzed, the cells were pretreated with the specific inhibitor or VE for 1 hour prior to each treatment. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were then performed as described.[26]

Measurement of ROS. OR6 cells in 24-well plates were left untreated or were treated with hydrogen peroxide (1 mM), LA (200 μM), and CsA (15 μg/mL) for 30 minutes and then incubated with dihydrodichlorocarboxyfluorescein diacetate (Invitrogen) (5 μM) for 15 minutes. Fluorescence was measured with a FLUOROSKAN ASCENT fluorescence plate reader (Thermo Fisher Scientific, Waltham, MA) at an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Cell Growth Assay. To examine the activity of EGF on OR6 cell growth, 6.0-6.5 × 10^4 OR6 cells were plated onto 6-well plates in triplicate and were pre-cultured for 24 hours. The cells were treated with or without EGF for 72 hours, and the number of viable cells was counted after trypan blue dye treatment as described.[11]

Statistical Analysis. Statistical comparison of the luciferase activities between the various treatment groups was performed using the Student *t* test. *P* values of less than 0.05 were considered statistically significant.

Results

Effects of MEK1/2-Specific Inhibitors on the Anti-HCV Activities of BC, VD2, and LA in OR6 Cells. Our recent study suggested the involvement of oxidative stress in the suppressive mechanism of three anti-HCV nutrients: BC, VD2, and LA.[13] Because there have been reports of negative regulation of HCV RNA replication via the MEK–ERK1/2 signaling pathway,[24,25] which is one of the oxidative stress-induced cellular signaling pathways, we hypothesized that the suppression of HCV RNA replication by these three nutrients might be mediated via this cascade (Supporting Fig. 1). To test this hypothesis, we first used an OR6 assay system to examine the effects of U0126 and PD98059, inhibitors specific to MEK1/2, on the three anti-HCV nutrients at 60% inhibitory concentration. As shown in Fig. 2A, treatment with either 5 μM of U0126 or 10 μM of PD98059 slightly enhanced HCV RNA replication in comparison with the control. However, U0126 attenuated the anti-HCV activities of the three nutrients more clearly than PD98059 (Fig. 2A,B). U0126 prevented the anti-HCV activities of the three nutrients in a significant and dose-dependent manner and exerted complete inhibition against the anti-HCV activities of BC and LA (Fig. 2C,D), while the inhibitory effect of PD98059 was more mild (Fig. 2E,F). As shown in Fig. 2G, we also found that U0126 treatment restored the expressions of HCV proteins, core, and NS5A in a dose-dependent manner. We further demonstrated that knockdown of MEK1 or MEK2 by small interfering RNA negated the anti-HCV activity of LA (Supporting Fig. 2A-C). These inhibitions by U0126 against the anti-HCV activities of the three nutrients were not due to the enhancement of encephalomyocarditis virus/internal ribosomal entry site–driven RL activity, because this activity was not increased by U0126 (data not shown). Moreover, treatment with neither SB203580 (an inhibitor specific to p38 MAPK) nor c-Jun N-terminal kinase inhibitor, both of which belong to the same cascade family as MEK–ERK1/2, significantly affected the anti-HCV activities of the three nutrients (data not shown). These results imply that the activation of the MEK–ERK1/2 signaling pathway might be required for the suppression of genome-length HCV RNA replication by the three nutrients in cell culture.

Effect of U0126 on the Suppressive Effects of Polyunsaturated Fatty Acids and Anti-HCV Reagents in OR6 Cells. Previous studies using a cell culture system have shown that polyunsaturated fatty acids (PUFAs), including LA, act as anti-HCV nutrients.[27,28] A recent study reported that lipid peroxidation of PUFAs was correlated with their anti-HCV activities, which were pre-

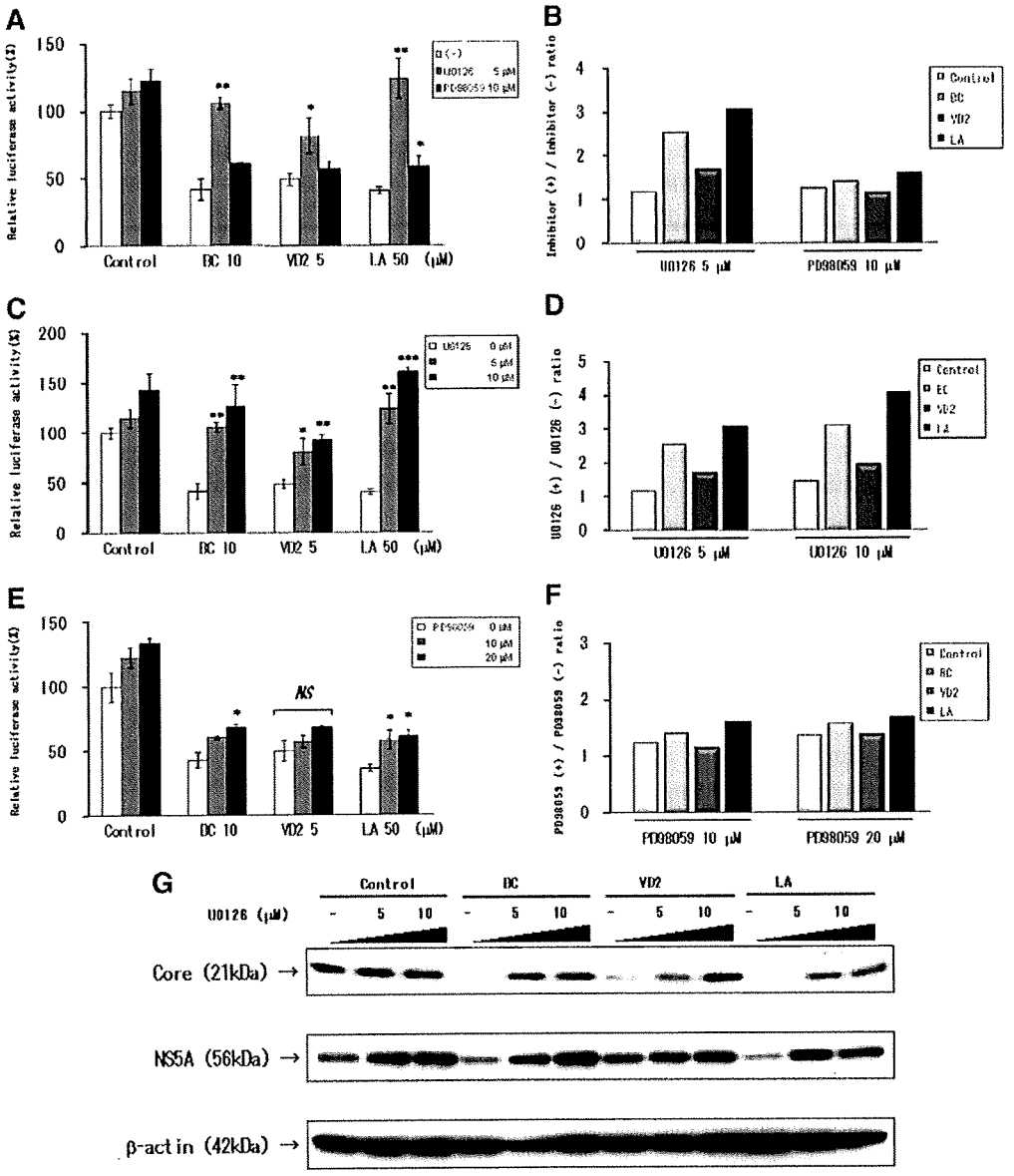

Fig. 2. U0126 strongly inhibited the anti-HCV activities of the anti-HCV nutrients BC, VD2, and LA in OR6 cells. (A,B) Effects of MEK-specific inhibitors on the three nutrients at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO, 5 μM U0126, or 10 μM PD98059 for 1 hour. The cells were then treated with control medium, 10 μM BC, 5 μM VD2, or 50 μM LA in either the absence (DMSO) or presence of each specific inhibitor for 72 hours. After treatment, RL assay was performed as described in Materials and Methods. Shown here is the relative luciferase activity (%) calculated when the RL activity of the control was assigned as 100%. Data are expressed as the mean ± standard deviation of triplicate samples from at least three independent experiments. Asterisks indicate significant difference from treatment with DMSO ($*P < 0.05$; $*P < 0.01$) (A). The ratio of the RL activity in the presence of the MEK-specific inhibitor to the RL activity in the absence of the inhibitor was then calculated (B). (C-F) OR6 reporter assays of the dose effects of MEK1/2-specific inhibitors on the three nutrients. OR6 cells were pretreated with DMSO, U0126 (C), or PD98059 (E) at the indicated concentrations for 1 hour. Treatment of the cells with control medium or each of the three nutrients in either the absence (DMSO) or presence of each specific inhibitor and the RL assay of harvested OR6 cell samples were performed as described in panels A and B. Asterisks indicate significant difference from treatment with DMSO ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant). Next, we calculated the ratio of RL activity in the presence of the MEK-specific inhibitor, U0126 (D), or PD98059 (F), to the RL activity in the absence of the inhibitor. (G) Western blot analysis of the dose effects of U0126 on three nutrients. OR6 cells were pretreated and then treated as in panel C. The production of HCV core and NS5A in the cells was analyzed by way of immunoblotting using antibodies specific to HCV core (top row) and NS5A (middle row). β-actin was used as a control for the amount of protein loaded per lane (bottom row).

vented by treatment with VE.[29] This result coincides with our previous observations on the effects of LA.[13] We proposed that the MEK–ERK1/2 signaling pathway might be involved in the anti-HCV activity of PUFAs, including LA, because lipid peroxidation is known to be a ROS-triggered cellular modification.[16] As expected, treatment with U0126 attenuated the anti-HCV activities of four representative PUFAs in a significant and dose-dependent manner (Fig. 3A,B).

Moreover, because the anti-HCV activities of BC, VD2, LA, and CsA, but not FLV, were found to be negated by VE,[13] we were also interested in the potent role of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of CsA. Furthermore, the previous study using a subgenomic replicon system had already shown the partial involvement of this cascade in the antiviral activity of IFN-γ.[24] Therefore, we examined the effects of U0126 on various anti-HCV reagents: IFN-γ, CsA, and statins (FLV and PTV). We confirmed that also in genome-length HCV RNA replication cells, U0126 significantly inhibited the anti-HCV activity of IFN-γ (Fig. 3C,D). Interestingly, consistent with the effects of treatment with VE,[13] the anti-HCV activity of CsA was completely abrogated by U0126 in a significant and dose-dependent manner, whereas statins were unaffected (Fig. 3C,D).

U0126 restored the reduced expression of HCV proteins by PUFAs, IFN-γ, and CsA in a dose-dependent manner, whereas statins were unaffected (Fig. 3E,F). These results were supported by additional real-time reverse-transcription polymerase chain reaction and immunofluorescence analyses (Supporting Fig. 3A-C). We also observed that knockdown of MEK1 or MEK2 by small interfering RNA did not affect the anti-HCV activity of PTV (Supporting Fig. 2A-C). Collectively, these findings suggest that the MEK–ERK1/2 signaling pathway may play a critical role in the negative regulation of HCV RNA replication by the anti-HCV nutrients BC and VD2, PUFAs, and the anti-HCV reagents IFN-γ and CsA, but not statins.

Activation of the MEK–ERK1/2 Signaling Pathway by Anti-HCV Nutrients and Reagents. To further ensure the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanisms of anti-HCV nutrients and reagents, we next examined whether these nutrients and reagents could actually initiate the activation of this signaling pathway. After treating the HCV RNA replicating cells with each of the nutrients and reagents, we performed immunoblotting specific to the phosphorylation of ERK1/2 and MEK1/2. In the same way as EGF, a potent activator of these kinases, the three anti-HCV nutrients (BC, VD2, and LA) enhanced the phosphorylation of ERK1/2 and MEK1/2 in both genome-length and subgenomic HCV RNA replication cells (Fig. 4A,B). IFN-γ, CsA, and all of the PUFAs also up-regulated this cascade in OR6 cells (Fig. 4C,D). The increase in phosphorylation of ERK1/2 was not observed after either statin treatment (Fig. 4D). The activation of MEK–ERK1/2 by the three anti-HCV nutrients was apparent until 1 hour after their application and subsequently attenuated, although EGF exhibited persistent enhancement of MEK–ERK1/2 phosphorylation (Fig. 4E). Because the experiments regarding ERK1/2 phosphorylation were performed in FBS-free conditions, we checked the anti-HCV activity of PTV, CsA, and LA in FBS-free medium. The results revealed that these anti-HCV reagents and nutrients also inhibited HCV RNA replication in FBS-free conditions (Supporting Fig. 4). Taken together, these findings indicate that the anti-HCV nutrients and reagents activated the MEK–ERK1/2 signaling pathway in HCV RNA replicating cells, providing further confirmation that this signaling cascade might be involved in their anti-HCV activities.

MEK1/2-Specific Inhibitors Attenuated the Increased Phosphorylation of ERK1/2 by Anti-HCV Nutrients/Reagents and EGF. We next tested whether MEK1/2-specific inhibitors could prevent not only the suppression of HCV RNA replication but also the activation of ERK1/2 by the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Consistent with the inhibitory effects on their anti-HCV activities, U0126 more markedly abrogated the increase in ERK1/2 phosphorylation by anti-HCV nutrients, reagents, and EGF than did PD98059 (Fig. 5A,B). As shown in Fig. 5C, the enhanced ERK1/2 phosphorylation by the three nutrients and EGF was reduced by U0126 in a dose-dependent manner.

VE Attenuated the Increased Phosphorylation of ERK1/2 by Anti-HCV Nutrients/Reagents and EGF. Because the suppression of HCV RNA replication by BC, VD2, LA, and CsA were completely negated by the treatment with VE in our recent study,[13] we investigated whether VE could also inhibit ERK1/2 activation by anti-HCV nutrients and reagents. As expected, VE also attenuated the enhanced phosphorylation of ERK1/2 by not only anti-HCV nutrients and CsA but also IFN-γ and EGF (Fig. 6A,B). We also demonstrated that phosphorylation of ERK1/2 by CsA was attenuated with *N*-acetylcysteine treatment and led to the negation of inhibition of HCV RNA replication (Supporting Fig. 5A-C). The anti-HCV nutrients and reagents, whose activities were negated by U0126, were also inhibited by VE. In contrast, the anti-HCV activities of statins were not negated by U0126 or VE. We also demonstrated that LA and CsA induce ROS (Fig.

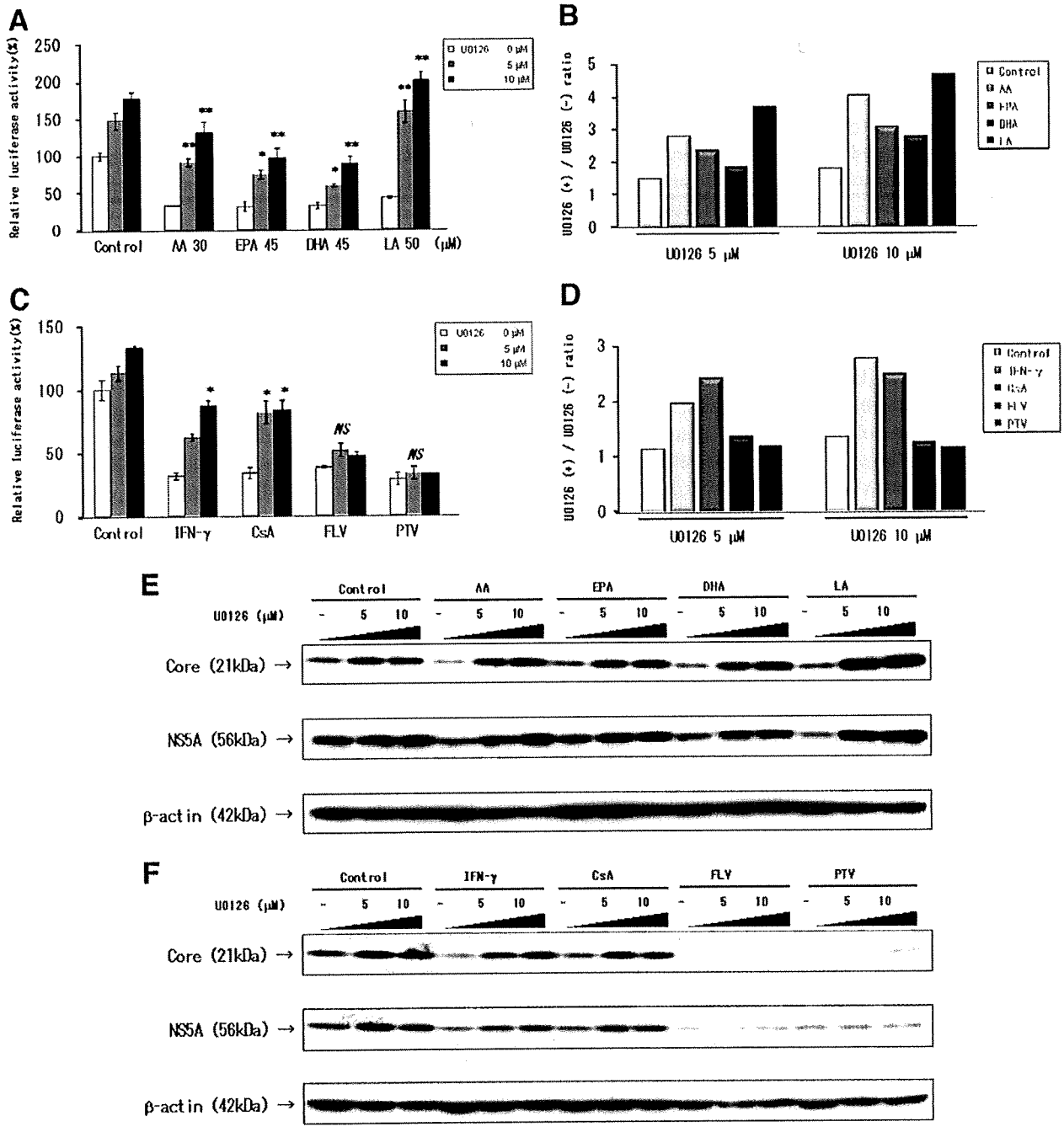

Fig. 3. U0126 dose-dependently attenuated the anti-HCV activities of PUFAs, IFN-γ, and CsA, but not the statins. (A-D) OR6 reporter assays of the dose effects of U0126 on the PUFAs and anti-HCV reagents at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO or U0126 as in Fig. 2C and then treated with control medium, 30 μM AA, 45 μM EPA, 45 μM DHA, or 50 μM LA (A) and control medium, 0.4 IU/mL IFN-γ, 0.2 μg/mL CsA, 3 μM FLV, or 1 μM PTV (C), respectively, in either the absence (DMSO) or presence of U0126 for 72 hours. After the treatment, the RL assay of harvested OR6 cell samples was performed as described in Fig. 2A and 2B. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; **$P < 0.01$; NS, not significant). The ratio of the RL activity in the presence of U0126 to the RL activity in the absence of U0126 was then calculated (B, D). (E, F) Western blot analysis of the dose effects of U0126 on the PUFAs and anti-HCV reagents. The production of HCV core (top row) and NS5A (middle row) in the cells treated as in panel A (E) and panel C (F) was analyzed as described in Fig. 2G. β-actin was used as a control for the amount of protein loaded per lane (bottom row).

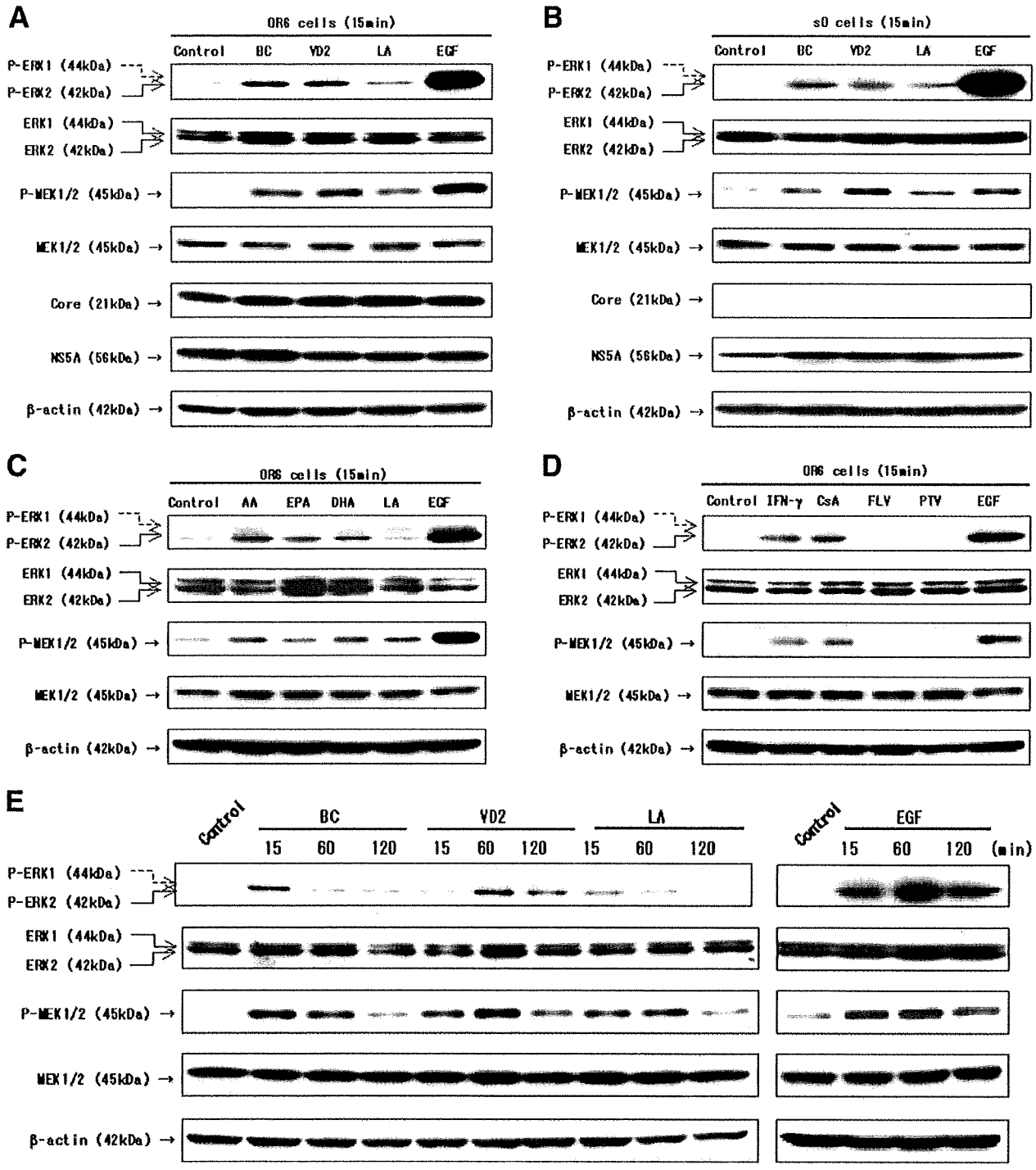

Fig. 4. U0126 attenuated the MEK-ERK1/2 signaling pathway activated by anti-HCV nutrients and reagents. (A, B) Three anti-HCV nutrients—BC, VD2, and LA—increased the phosphorylation of MEK-ERK1/2 in both full-length and subgenomic HCV RNA replication cells. OR6 cells (A) or s0 cells (B) were maintained in FBS-free medium for 48 hours and then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF for 15 minutes. After treatment, cell lysates underwent western blot analysis using antibodies specific to phosphorylated ERK1/2, ERK1/2, phosphorylated MEK1/2, and MEK1/2. The appropriate expression of HCV core and NS5A was determined by way of immunoblotting with their respective antibodies. (C, D) IFN-γ, CsA, and the PUFAs, but not the statins, increased the phosphorylation of MEK-ERK1/2 in OR6 cells. OR6 cells were precultured as described in panels A and B, then treated with control medium, 100 μM AA, EPA, DHA, or LA, or 50 ng/mL EGF (C) and control medium, 2 IU/mL IFN-γ, 2 μg/mL CsA, 5 μM of FLV or PTV, or 50 ng/mL EGF (D), respectively, for 15 minutes. (E) Time-course western blot analysis of the increase of MEK-ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. Samples for analysis were harvested prior to treatment with the control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (0 time point) and at 15, 60, and 120 minutes posttreatment. After all of the treatments (C-E), cell lysates were subjected to western blot analysis of the activation of the MEK-ERK1/2 signaling pathway as described in panels A and B. β-actin was used as a control for the amount of protein loaded per lane in all analyses.