CYP2E1; the effect could be suppressed by NAC, leading to the conclusion that the increase was due to ROS generation by CYP2E1. In contrast, we have consistently found that ROS suppresses HCV replication whereas antioxidants tend to counter this suppression (10–14) (Fig. 4). In particular, our BSO studies clearly demonstrate that endogenous ROS are sufficient to suppress HCV replication in cell culture (10, 11). Also, NAC and vitamin E either enhanced or had no significant effect on the potentiation of HCV replication by ethanol (Fig. 4*G*) as well as acetaldehyde, isopropyl alcohol, acetone, and acetate (data not shown). The reason for this discrepancy is unclear. However, CYP2E1 generates acetaldehyde as well as ROS, both of which can react with thiols, such as cysteine and GSH, which are generated from NAC, and the study by McCartney *et al.* did not differentiate whether the potentiation of HCV replication by ethanol was due to ROS, acetaldehyde, or other variables (7, 28). NAC can also have other effects on cells, including alteration of the pH and acting as a pro-oxidant, and careful monitoring of the pH and comparison with other antioxidants and pro-oxidants, therefore, are necessary. Indeed, our findings have been recently corroborated by other studies that show that HCV RNA replication is enhanced by antioxidants (*e.g.* vitamins E and C) and suppressed by lipid peroxidation products and ROS (12–14, 40). The mechanism by which ROS suppresses HCV replication is still not completely clear but it is likely to involve calcium and the dissociation of HCV replication complex from the membranes (10, 11). Detailed understanding of the mechanism by which ROS suppresses HCV replication and how acetaldehyde, NADH, acetyl-CoA, and ROS affect HCV *in vivo* will require additional *in vitro* and animal studies.

Therefore, we show that physiological levels of ethanol, acetaldehyde, and acetone promote HCV replication in the context of the complete HCV replication, and that the response is likely mediated by the modulation of host lipid metabolism requiring elevated NADH/NAD^+. Further study into the precise mechanisms of this regulation may lead to the development of novel treatments that target both the virus and its pathogenic interactions with ethanol in chronic hepatitis C patients.

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ORIGINAL ARTICLE

HCV genotype 1b chimeric replicon with NS5B of JFH-1 exhibited resistance to cyclosporine A

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Abstract Cyclosporine A (CsA) is a well-characterized anti-HCV reagent. Recently it was reported that the genotype 2a JFH-1 strain was more resistant than genotype 1 HCV strains to CsA in a cell culture system. However, the JFH-1 responsible region for the resistance to CsA remains unclear. It was also demonstrated that in genotype 1b HCVs, NS5B interacts with cyclophilin (CyP). To clarify whether or not NS5B of JFH-1 is significant for CsA resistance, we developed a chimeric replicon with NS5B of JFH-1 in the genotype 1b backbone. The chimeric replicon was more resistant to CsA than the parental genotype 1b replicon. Furthermore, reduction of CyPA had a greater effect on HCV RNA replication and sensitivity to CsA than reduction of CyPB. Here, we demonstrated that NS5B of JFH-1 contributed to this strain's CsA-resistant phenotype. NS5B and CyPA are significant for determining HCV's sensitivity to CsA.

Introduction

The combination of a pegylated interferon (IFN) with ribavirin (RBV) is the current standard therapy for chronic hepatitis C and yields a sustained virological response (SVR) rate of about 55% [6]. This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma. To find a more effective therapy, several anti-HCV reagents have been reported using HCV replicon systems [11, 14]. Especially, cyclosporine A (CsA), which is widely used as an immunosuppressive reagent, and its derivatives, which lack immunosuppressive activity, possess anti-HCV activity [8, 18, 19]. These reagents will help to improve the SVR rate.

Cyclophilins (CyPs), CsA ligands, are a family of cellular enzymes possessing peptidyl-prolyl isomerase activity. CyP family members play significant roles in numerous cellular processes, including transcriptional regulation, immune response, protein secretion and mitochondrial function [7]. CsA possesses three major cellular targets: CyP, the calcineurin-nuclear factor of activated T-cells pathway and P-glycoprotein [7]. The mechanism of anti-HCV activity of CsA is through disassociation between CyP and HCV nonstructural protein 5B (NS5B), an RNA-dependent RNA polymerase [20]. Fernandes et al. [5] also reported that NS5A was significant in the sensitivity of HCV to CsA. However, the role of CyPs as a cellular target of CsA in HCV RNA replication remains controversial [17, 20, 21]. While genotype 1a and 1b HCV strains were highly sensitive to CsA, a genotype 2a strain, JFH-1, was less sensitive to CsA [12, 21]. Moreover, in genotype 1b HCV, interaction between CyPB and HCV NS5B is required for robust HCV RNA replication [10].

To investigate whether or not NS5B of JFH-1 is an important factor for determining sensitivity to CsA, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which NS5B and a 3′-untranslated region (UTR) were replaced with

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those of HCV JFH-1 RNA. This replicon system enables to investigate the direct effect of NS5B on the CyPs.

We report here that NS5B of JFH-1 contributes to the CsA-resistant phenotype of this strain. Furthermore, CyPA but not CyPB is essential for HCV RNA replication in 1b and 1b/2a chimeric replicon-harboring cells. Finally, supplementation with vitamin E (VE) negates the anti-HCV activity of CsA in the presence or absence of CyPs. These results contribute to our understanding of the mechanism(s) that mediate the efficacy of CsA's anti-HCV activity.

Materials and methods

Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum. The HuH-7-derived OR6c cells were cultured as previously described [10]. The cells harboring the subgenomic replicon were maintained in the culture medium containing G418 (0.3 mg/ml; Promega, Madison, WI).

Reagents

IFN-α, IFN-γ, and VE were purchased from Sigma-Aldrich (St. Louis, MO). CsA was purchased from Calbiochem (San Diego, CA). Pitavastatin (PTV) was purchased from KOWA Co., Ltd. (Tokyo, Japan). Mizoribine (MZB) and RBV were kindly provided by Asahi Kasei Pharma (Tokyo, Japan) and Yamasa Corporation (Choshi, Japan), respectively.

Plasmid construction

The plasmid of pORN/3-5B/QR,KE,RS/5B(J) was based on pORN/3-5B/QR,KE,RS [1] and was constructed by replacing the NS5B coding region and 3′UTR with the corresponding JFH-1 sequence. The NS5A/NS5B junction was set after amino acid 2419 of HCV-O and generated by polymerase chain reaction (PCR). The sequence numbering for coding and non-coding regions was based on a sequence from GenBank: HCV-O (accession no. **AB191333**) and JFH-1 (accession no. **AB047639**). Retroviral vector pCX4bsr [2] was used as an expression vector. To obtain full-length CyPA and CyPB cDNAs, reverse transcription (RT)-PCR with KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was performed as previously described [4]. The pCX4bsr/Myc-CyPA and pCX4bsr/Myc-CyPB plasmids expressing Myc-tagged CyPA and CyPB, respectively, were obtained by inserting the PCR products of full-length CyPA and CyPB into the MluI-NotI sites of the pCX4bsr/Myc vector. Expression plasmids for HA-tagged NS5B (HCV-O and JFH-1) were generated by insertion of PCR fragments encoding each HCV protein into the MluI-NotI sites of the pCX4bsr/HA vector. The sequences of all constructed plasmids were confirmed by the sequencing analysis as described previously [1].

RNA synthesis, RNA transfection, and selection of G418 cells

Plasmid DNAs were linearized by XbaI and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). In vitro transcribed RNA was transfected into OR6c cells by electroporation [9]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks.

RNA interference, lentiviral vector construction

A detailed description of methods of RNA interference and lentiviral vector construction is available in Supplementary Materials.

Western blot analysis

Western blot analysis was performed as described previously [1]. The antibodies used in this study were those against NS3 (Novocatra Laboratories, UK), NS5A (a generous gift from Dr. A Takamizawa, Research Foundation for Microbial Diseases, Osaka University), NS5B (1b, a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), NS5B (1b/2a) [15], CyPA (BIOMOL, Plymouth Meeting, PA), CyPB (Affinity BioReagents, Rockford, IL), and β-actin (AC-15; Sigma).

Immunoprecipitation

Immunoprecipitation was performed as described previously [10]. Briefly, pre-cleared cell lysates were incubated with an anti-Myc antibody (PL14; MBL, Nagoya, Japan). Immunocomplexes were recovered by adsorption to protein G-Sepharose resin (GE Healthcare Bioscience, Uppsala, Sweden). After three washes with lysis buffer, the immunoprecipitates were analyzed by immunoblot analysis using anti-Myc and anti-HA (3F10; Roche, Mannheim, Germany) antibodies.

Evaluation of sensitivity of anti-HCV reagents

The cells were plated onto 24-well plates ($1.5–2 \times 10^4$ cells/well). After 24 h culture, the culture medium was replaced with anti-HCV reagent containing medium. After 72 h additional culture, the cells were washed with

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

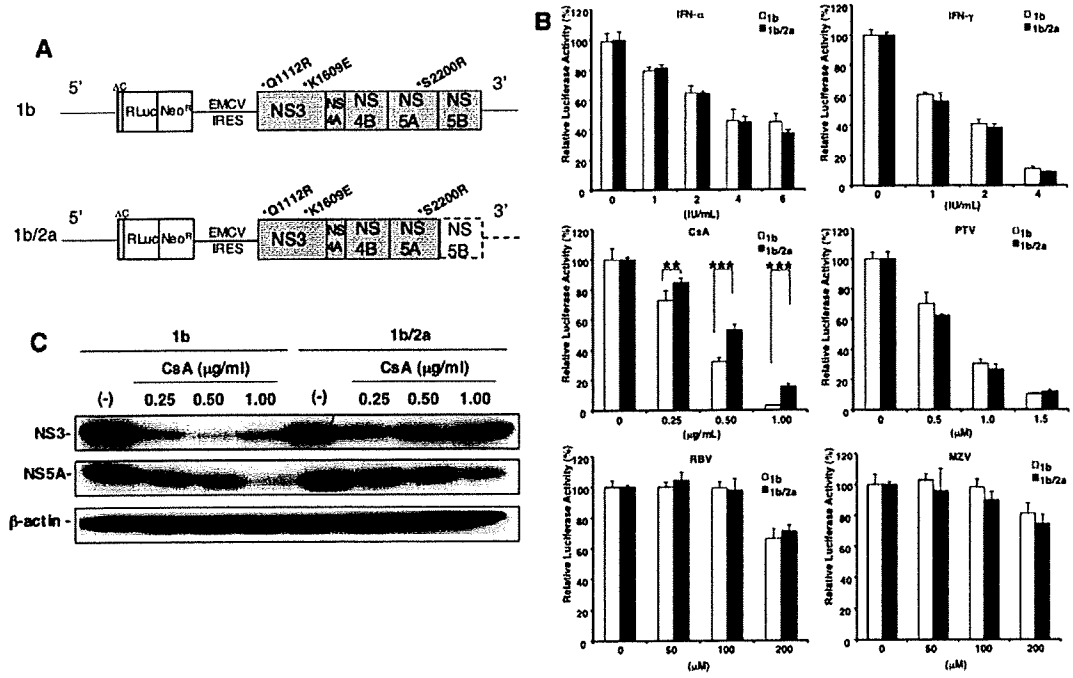

Fig. 1 1b/2a chimeric replicon-harboring cells are less sensitive to CsA. **a** Gene organization of subgenomic RNA. RLuc and DC indicate the RL gene and the 12 N-terminal amino acid residues of the core protein as a part of internal ribosomal entry site (IRES), respectively. The positions of adaptive 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

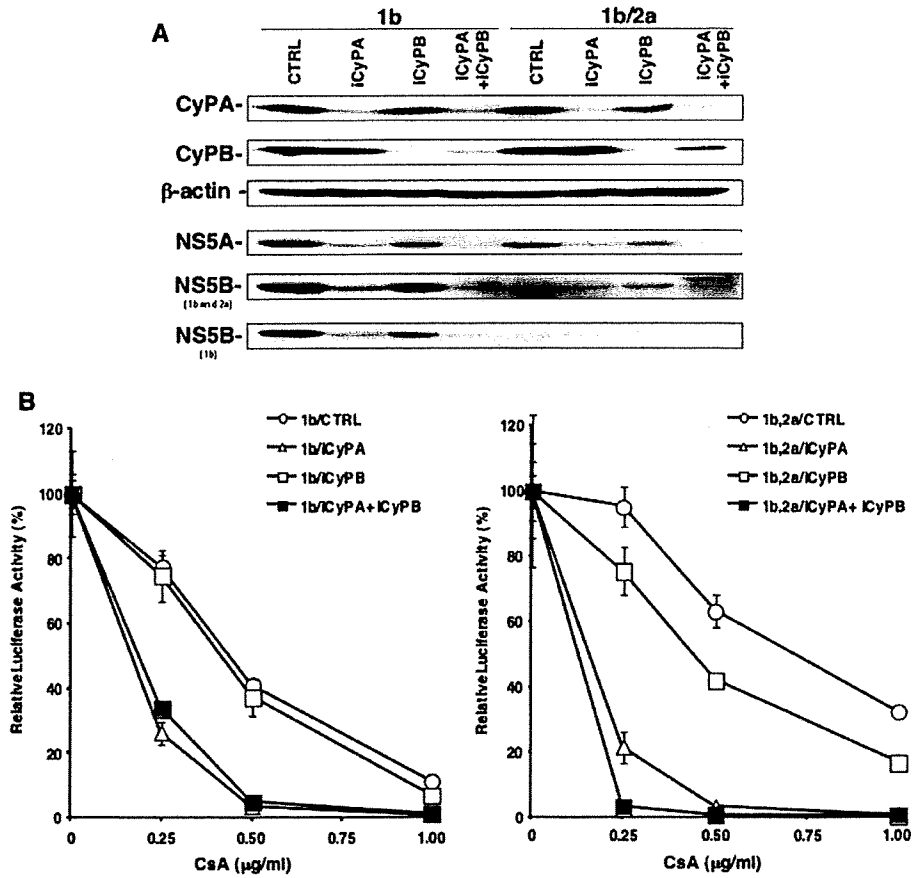

Fig. 2 CyPA is essential for HCV RNA replication and modulates the anti-HCV activity of CsA. **a** Western blot analysis of HCV proteins and CyPs. The 1b replicon and 1b/2a chimeric replicon-harboring cells were transduced with the indicated shRNA for 1 week. The cell lysates were subjected to Western 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.

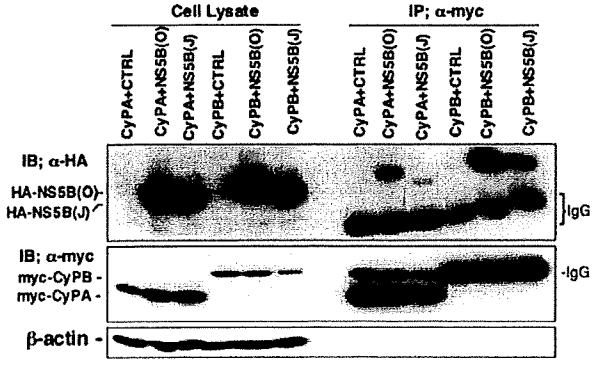

Fig. 3 CyPs interact with HCV NS5B. 293FT cells were cotransfected with plasmids expressing Myc-tagged CyP and HA-tagged HCV NS5B for 48 h. The cells were lysed and subjected to immunoprecipitation with monoclonal Myc-antibody, followed by immunoblot analysis with either anti-HA (*top*) or anti-Myc (*bottom*)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

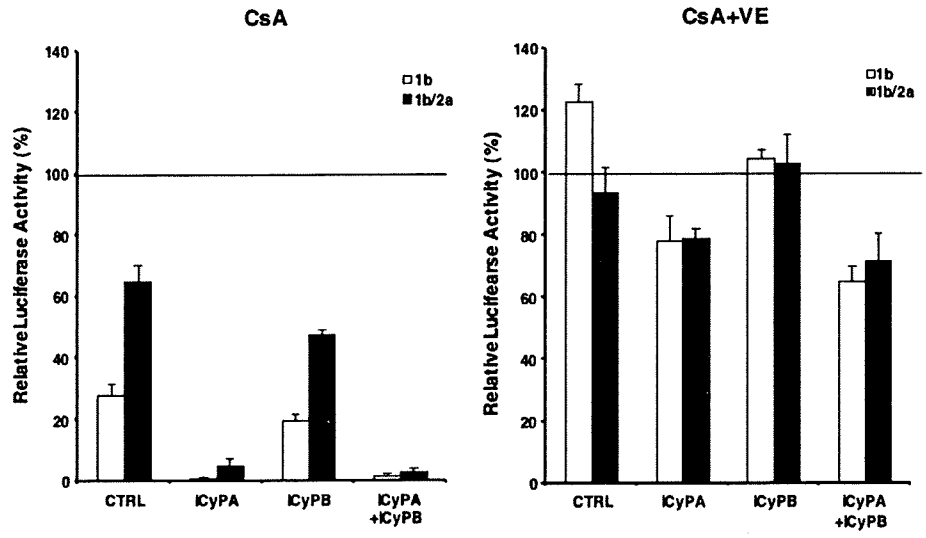

Fig. 4 The effect of CsA (0.5 μg/ml) in combination with VE (10 μM) on HCV RNA replication in the 1b replicon- (*open columns*) and the 1b/2a chimeric replicon- (*closed columns*) harboring cells. After 72 h treatment, the RL assay was performed according to the manufacturer's protocol. The relative 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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Evaluation of Hepatitis C Virus Core Antigen Assays in Detecting Recombinant Viral Antigens of Various Genotypes[▽]

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A single substitution within the hepatitis C virus core antigen sequence, A48T, which is observed in ~30% of individuals infected with genotype 2a virus, reduces the sensitivity of a commonly used chemiluminescence enzyme immunoassay. Quantitation of the antigen is improved by using a distinct anticore antibody with a different epitope.

Hepatitis C virus (HCV) is a major cause of chronic liver disease throughout the world. Accurate diagnosis of HCV infection is important due to the morbidity associated with the virus, and determining the level of viral replication is important in predicting and monitoring the effect of antiviral treatment. Although quantifying viral RNA represents the standard method for identifying active infection (5, 8, 13), several sensitive immunoassays that detect the viral core antigen (Ag) have now been developed as an alternative to HCV RNA testing (3, 4, 6, 9, 10, 12, 16). The amino acid sequence of the core Ag is largely conserved among different viral isolates (14); however, genetic variability of the virus constitutes one of the major challenges to using core Ag assays for diagnosis. In this study, we examined the effects of sequence heterogeneity on the sensitivity of diagnostic kits for detection of the core Ag by using recombinant Ag derived from each of the major HCV genotypes. Expression plasmids for epitope-tagged core Ag were generated by inserting cDNA for the full-length core region of genotype 1a (17; GenBank accession no. AF011751), 1b (1; D89815), 2a (7; AB047639), 2b (AB030907), or 3a virus, with a FLAG tag sequence attached at its 5′ end, into the EcoRI site of the pCAG mammalian expression vector (11). HEK293T cells transiently transfected with the expression plasmids were harvested 48 h after transfection using a passive lysis buffer (Promega, Madison, WI). Centrifugation was performed to remove the debris after ultrasonication. Total protein was quantified in aliquots of cell lysate by using the bicinchoninic acid method (Pierce, Rockford, IL) and then used for determining the concentrations of HCV core Ag.

Figure 1A shows comparable levels of core Ag in each sample of cell lysate, as determined by immunoblotting with anti-FLAG antibody (Ab). The ability of HCV core Ag assays to detect five different HCV genotypes were compared using a commercially available chemiluminescence enzyme immunoassay (CLEIA) (Lumipulse II HCV core assay [assay detection range, approximately 50 to 50,000 fmol/liter]; Fujirebio, Japan) (15) and enzyme-linked immunosorbent assay (ELISA) (Ortho HCV Ag ELISA test [assay detection range, approximately 44.4 to 3,600 fmol/liter]; Ortho-Clinical Diagnostics, Japan) (2) to detect HCV core Ag in cell lysate. As shown in Fig. 1B,

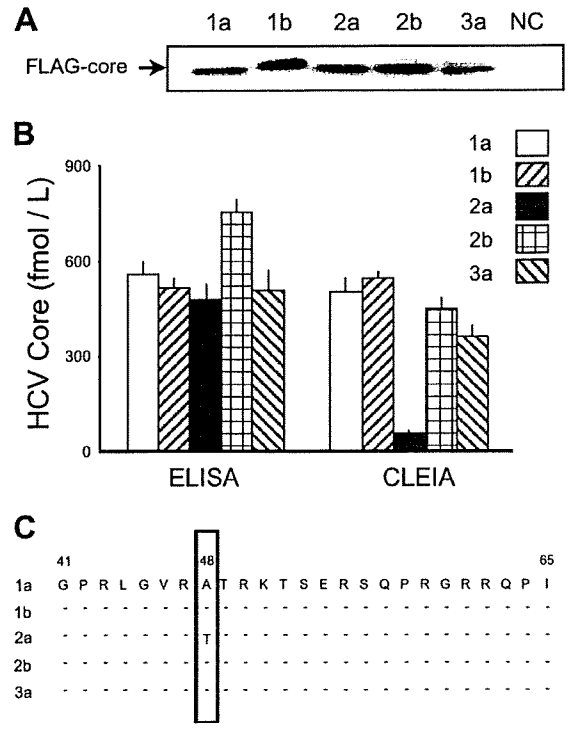

FIG. 1. Detection of recombinant HCV core Ag derived from genotype 1a, 1b, 2a, 2b, and 3a isolates by immunoblotting using an anti-FLAG Ab (A) as well as ELISA and CLEIA (B). The data shown in panel B represent the mean values and standard deviations ($n = 3$). NC, negative control. (C) The amino acid sequence from amino acids 41 to 65 of the core Ag used in this study. Key residues at the 48th position are boxed. Hyphens indicate conservation.

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TABLE 1. Comparison of the 48th residues of HCV core Ags of genotypes 1a, 1b, 2a, 2b, and 3a

Genotype	No. of isolates	No. (%) of isolates with residue at 48th position		
		T	A	Other
1a	263	9 (3.5)	254 (96.5)	0 (0)
1b	298	2 (0.7)	294 (98.6)	2 (0.7)
2a	17	5 (29.5)	12 (70.5)	0 (0)
2b	17	0 (0)	17 (100)	0 (0)
3a	23	0 (0)	23 (100)	0 (0)
Total	618	16 (2.6)	600 (97.1)	2 (0.3)

although the ELISA measured similar concentrations of core Ag in all samples, apparent low levels of the genotype 2a core Ag, originally from an isolate known as the JFH-1 isolate (7), were detected using the CLEIA method, suggesting that some differences in the amino acid sequences corresponding to particular HCV genotypes or isolates may influence the sensitivity of core Ag detection. A comparison of the core Ag sequences, including the monoclonal Ab epitopes used in the development of CLEIA, revealed conservation of alanine at the 48th position in four clones, of genotypes 1a, 1b, 2b, and 3a, but not genotype 2a, for which there is a threonine at this position (Fig. 1C). Based on our analysis of sequences available from the HCV database (http://hcv.lanl.gov/content/sequence/NEWALIGN/align.html), alanine is highly conserved at the 48th residue of the core Ag for HCV isolates of genotypes 1a, 1b, 2b, and 3a (Table 1). In contrast, alanine and threonine occur in this position in 70.5% and 29.5%, respectively, of genotype 2a isolates. To examine whether the low sensitivity of the CLEIA method might be due to this particular amino acid change, we next replaced threonine with alanine at the 48th position of the JFH-1 core Ag (for

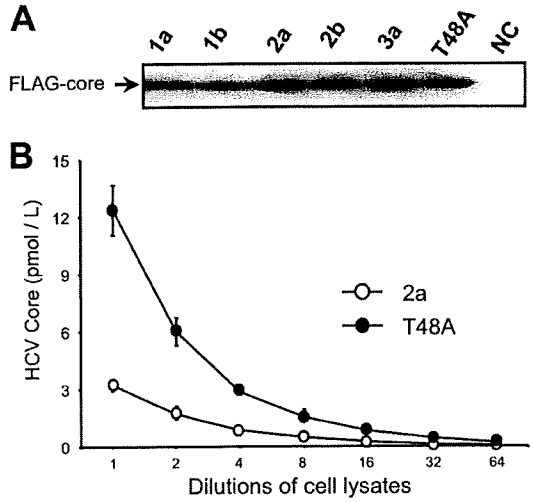

FIG. 2. Effect of T48A substitution in the core Ag of the JFH-1 isolate with regard to sensitivity of the CLEIA method. Samples of wild-type or mutated core Ag cell lysate were analyzed by immunoblotting (A) and CLEIA (B). The data shown in panel B represent the mean values and standard deviations (n = 3). NC, negative control.

TABLE 2. Comparison of the modified CLEIA with the original version for detection of the core Ags of genotypes 1a, 1b, 2a, 2b, and 3a[a]

Genotype	CLEIA	HCV core antigen concn (fmol/liter) in serially diluted cell lysates at indicated fold dilution							
		1	2	4	8	16	32	64	128
1a	Original	11,147	5,527	2,611	1,484	691	403	195	101
	Modified	10,511	5,700	2,676	1,420	716	444	200	111
1b	Original	11,612	5,618	3,081	1,551	779	409	223	113
	Modified	11,192	6,028	2,824	1,522	804	431	197	101
2a	Original	3,216	1,710	844	480	232	104	48	36
	Modified	12,101	6,255	3,153	1,676	805	422	212	106
2b	Original	10,559	5,635	2,811	1,286	762	387	194	94
	Modified	10,977	6,179	3,381	1,624	842	437	219	129
3a	Original	11,478	5,891	2,922	1,414	756	422	212	112
	Modified	11,208	6,225	3,126	1,555	791	445	215	100

[a] Data represent the mean values in triplicate measurements.

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the mutant JFH-1coreT48A) and measured the HCV core Ag concentration in cells expressing both mutated and wild-type JFH-1 core Ag. After confirming comparable levels of FLAG-tagged core Ag in the cell lysate samples by immunoblotting (Fig. 2A), HCV core Ag was quantified in the samples by serial dilution via the CLEIA method. As shown in Fig. 2B, the core Ag concentrations of JFH-1coreT48A were assessed to be 3.2- to 3.8-fold higher than those of the wild-type core Ag, suggesting that the sensitivity of HCV core Ag detection may have been affected by the 48th residue in the core Ag. Data for samples derived from genotypes 1a, 1b, 2b, and 3a were analogous to data for JFH-1coreT48A (data not shown). Although HCV isolates with threonine at the 48th position of the core Ag sequence comprise a relatively small proportion of the major genotype population, only 2.6% of the genotype 1a, 1b, 2a, 2b, and 3a isolates here (16 of 618 isolates; Table 1), attempts to overcome this problem would improve the overall sensitivity and usefulness of the assay. To achieve this aim, another monoclonal anticore Ab, whose epitope is comprised of amino acids 50 to 65, which are completely conserved among all the genotypes examined (Fig. 1C), was therefore used as a second Ab in a modified version of the CLEIA. We compared this modified assay with the original version by measurement of core Ag concentrations of the various genotypes (Fig. 2A) as illustrated in Table 2. The modified assay was able to quantify core Ag from genotypes 1a, 1b, 2a, 2b, and 3a with no significant differences observed between Ag levels in samples from different genotypes at each dilution.

It has been demonstrated that the HCV core Ag assay is a useful alternative to HCV RNA quantification for the diagnosis of hepatitis C and for monitoring the antiviral effects of treatment. Compared to various reverse transcription-PCR methods, HCV core assays are less expensive and easier to perform, without the requirement of sophisticated laboratory equipment and specially trained laboratory personnel. In addition, the core Ag assay can be used to measure a more diverse set of blood samples, such as sera stored for a long period of time, because the viral Ag is generally more stable than the RNA in sera or plasma. Despite the adequate performance of core Ag assays, we have shown that a single amino acid substitution at the 48th position of the core Ag changes the detection sensitivity. It is also noted that, although the original CLEIA should be improved, the ELISA used in this study may be substituted for it.

In conclusion, we have identified a distinct anticore Ab with a different epitope that might enable improved detection across all of the major HCV isolates. The findings of this study would provide useful information for the development of an improved assay with greater accuracy.

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Identification of bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication by tube-capture-RT-PCR

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ABSTRACT

We devised a screening method for hepatitis C virus (HCV) inhibitors by exploiting the JFH1 viral culture system. The viral RNA released in the medium was adsorbed onto PCR plates, and real-time RT-PCR was performed by directly adding the one-step RT-PCR reaction mixture to the wells. The "tube-capture-RT-PCR" method obviates the need for labor-intensive RNA isolation and should allow high-throughput screening of HCV inhibitors. To substantiate the validity of the assay for drug screening, a pilot screen of an inhibitor library composed of 95 compounds was performed. In addition to the known inhibitors of HCV replication included in the library, the assay identified the PKC inhibitor bisindolylmaleimide I (BIM I) as an HCV replication inhibitor. BIM I was also effective in reducing the viral protein level in genotype 1b and 2a subgenomic replicon cells, indicating inhibition of HCV replication. Further assays revealed that a broad range of bisindolylmaleimides and indolocarbazoles inhibit HCV, but no correlation was found between the PKC inhibition pattern and anti-HCV activity. These series of compounds represent new classes of inhibitors that may warrant further development.

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1. Introduction

Hepatitis C virus, a major cause of chronic liver disease, has infected over 170 million people. The current mainstream anti-HCV therapy is a combination of interferon (IFN) and ribavirin. However, the therapy is not effective in approximately half of HCV-infected patients and has considerable side effects in many patients; thus, there is an urgent need for novel HCV therapies.

Various assays for HCV drug screening have been reported, many of which rely on HCV replicon systems. Although HCV replicon-based systems have greatly facilitated HCV research and drug discovery, these systems do not completely reflect the entire HCV life cycle and are not capable of identifying inhibitors of several important steps such as viral attachment, entry, and release. The recently introduced HCV cell culture systems (Wakita et al., 2005) should overcome these limitations and enable identification of inhibitors that would not be recognized by the replicon-based screens.

Here we describe a simple screening method for discovering anti-HCV drugs using the JFH1 viral culture system. Antiviral activity was determined by RT-PCR measurement of viral RNA released in the medium of infected cells. To increase efficiency, we devised a method that avoids tedious RNA isolation.

As a proof of concept, the method was used to evaluate a compound library and successfully confirmed the anti-HCV activity of cyclosporin A. In addition, a potent and selective PKC inhibitor, BIM I, was also identified as an anti-HCV agent. We found that other bisindolylmaleimides and indolocarbazoles also inhibit HCV, whereas anti-HCV activity was not associated with PKC inhibition. HCV inhibition by bisindolylmaleimides or indolocarbazoles has not been reported, and we expect that our assay will facilitate the identification of previously unrecognized HCV inhibitors. The bisindolylmaleimides and indolocarbazoles are already in clinical trials and may merit attention as HCV drug candidates.

2. Materials and methods

2.1. Cells and virus

Plasmid pJFH1, containing full-length cDNA of the JFH1 isolate, was used to generate HCV production in cell culture, as described elsewhere (Wakita et al., 2005), and the supernatant was passaged in Huh 7.5.1 cells. To prepare virus stock for screening, naïve Huh 7.5.1 cells were infected with the passaged supernatant virus, and the medium was collected 7 days post-infection and stored at

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−80 °C until use. The infectious titers of the viruses were determined by immunofluorescence analysis of the infected Huh 7.5.1 cells using anti-core antibody (2H9). The infectious titers of the stocks were generally about 3×10^5–1×10^6 ffu/ml, corresponding to about 3×10^7–1×10^8 copies of JFH1 RNA/ml. A subgenomic replicon cell, clone 4-1, which harbors the genotype 2a HCV genome (Kato et al., 2003; Date et al., 2004) and clone 5-15, which harbors the genotype 1b HCV genome (Lohmann et al., 1999), were also cultured in Dulbecco's Modified Eagle's medium (DMEM) with fetal bovine serum (FBS).

2.2. Reagents

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. The PKC β isozyme selective inhibitor LY333531 (Ruboxistaurin) was from Alexis Corp. (Lausen, Switzerland). Other chemicals were purchased from Merck Calbiochem (Darmstadt, Germany). Interferon-α (IFN-α) was from PeproTech, Inc. (Princeton Business Park, Princeton, NJ).

2.3. Quantitative real-time RT-PCR

Huh 7.5.1. cells were seeded in 96-well plates at a density of 20,000 cells per well in a volume of 120 μl. The next day, 15 μl of test compounds was added and the cells were infected with 15 μl of virus stock of HCV-JFH1 at a multiplicity of infection (MOI) of 0.01. After 5 days of culture, 100 μl of medium was transferred to a PCR plate, incubated on ice for 30 min, centrifuged at 3500 rpm for 15 min, and then removed. Twenty microliters of One Step SYBR PrimeScript RT-PCR Kit reaction mixture (Takara-Bio Co., Otsu, Japan) was added into the PCR plate wells, and quantitative real-time PCR was performed using an ABI Prism 7000 sequence detector (PE Applied Biosystems, Foster City, CA). The primers used were 5′-GAGTGTCGTACAGCCTCCAG-3′ (nucleotides 97–116), and 5′-AGGCCTTTCGCAACCCA-3′ (nucleotides 280–264) from the noncoding region of HCV-JFH1, at a concentration of 200 nM. Media from the control wells without drug were serially diluted to create a standard curve, which was used to determine the relative amount of HCV RNA in the media of HCV-infected cells treated with the compounds. Cell growth was monitored by MTT assay, as described previously (Fukazawa et al., 1995).

For further analysis of the drug effect and determination of the copy number of HCV RNA in medium and cells, HCV RNA was extracted from 140 μl medium with the QIAamp Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany), and eluted with 60 μl of elution buffer. Eight microliters of the viral RNA eluate was subjected to quantitative real-time PCR using Taqman EZ RT-PCR Core reagents (PE Applied Biosystems). The primers were 5′-CGGGAGAGCCATAGTGG (nucleotides 129–145) and 5′-AGTACCACAAGGCCTTTCG (nucleotides 289–271) at a concentration of 200 nM, and the Taqman probe was FAM-5′-CTGCGGAACCGGTGAGTACAC-3′-TAMRA (nucleotides 147–167) at a concentration of 300 nM (Takeuchi et al., 1999). Standard JFH1 RNA for measurement of copy number was transcribed from plasmid pSRG-JFH1-Luci, which was derived from pSRG-JFH1 (Kato et al., 2003), using the AmpliScribe T7 High Yield Transcription Kit (Epicentre Biotechnologies, Madison, WI). The transcribed RNA was purified and diluted with ribonuclease-free water containing yeast tRNA and 0.2% DTT, as previously described (Suzuki et al., 2005).

2.4. Western blotting

Cells were lysed with Radio-ImmunoPrecipitation Assay (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 μg/ml of each of antipain, pepstatin, and leupeptin, and centrifuged. The amount of protein in the supernatant was then measured. Cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with antibodies against core (2H9), NS5A (Austral Biologicals, San Raman, CA), α-tubulin (Merck Calbiochem), and GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and specific proteins were visualized by chemiluminescence.

3. Results

3.1. Assay development

To establish an efficient RT-PCR-based screen for anti-HCV agents, we searched for methods that could be carried out without labor-intensive RNA isolation. We tested whether tube-trapping methods used to obtain plant viral RNAs for RT-PCR (Rowhani et al., 1995; James, 1999; Suehiro et al., 2005) could be applied to HCV. A JFH1 stock solution (3×10^5 ffu/ml, 3×10^7 copies/ml) was serially diluted fourfold, put into the wells of the PCR plate, incubated on ice for 30 min, and then centrifuged at 3500 rpm for 15 min. The supernatant was removed and quantitative RT-PCR was performed by direct addition of the one-step RT-PCR reaction mixture. We found that HCV, like plant viruses, are adsorbed onto the well wall during incubation. As shown in Fig. 1a, RNA adsorption appeared to be linear over a broad range of viral concentrations. HCV RNA could still be detected after seven fourfold dilutions, indicating that the "tube-capture" is a quantitative method that can detect less than 200 copies of HCV RNA. We compared the CT values from this "tube-capture method" with those from the conventional method of RNA extraction. The efficiency of RNA recovery by "tube-capture" was calculated to be about 9% of the conventional method. However, as shown in Fig. 1a, there was a close correlation between the CT values obtained from the two methods ($R = 0.988$), demonstrating the usefulness of this method.

3.2. Identification of BIM I as an inhibitor of HCV infection

To explore the possibility of tube-capture-RT-PCR as a simple screen for discovering anti-HCV compounds, we first tested whether the method would detect the antiviral activity of IFN-α. Huh7.5.1 cells were seeded in a 96-well plate and infected with HCV-JFH1 at an MOI of 0.01. After 5 days, HCV RNA released in the medium was assayed by the tube-capture method. Under these conditions, the CT from the control medium was usually about 18–20. As shown in Fig. 1b, a substantial reduction in the amount of HCV RNA was observed when the cells were infected in the presence of IFN-α.

For further validation of the ability of the assay to identify HCV inhibitors, we performed a pilot screen using an inhibitor kit provided by the Screening Committee of Anticancer Drugs (SCADS inhibitor kit I). This kit contains 95 inhibitors including cyclosporin A, a compound reported to inhibit HCV replication.

Cyclosporin A was identified (Fig. 1c), providing a proof of concept for screening for anti-HCV drug candidates. In addition, our assay also identified the PKC inhibitor bisindolylmaleimide I (BIM I) (Fig. 2a, black columns). The IC_{50} was about 0.1 μM, which is comparable to that of cyclosporin A and about 200-fold lower than the IC_{50} for cell growth (Fig. 2b). In addition, BIM I inhibited the cytopathic effect of HCV JFH1. Infection with HCV resulted in about a 20% reduction of cell growth. BIM I at 1 μM enhanced the growth of

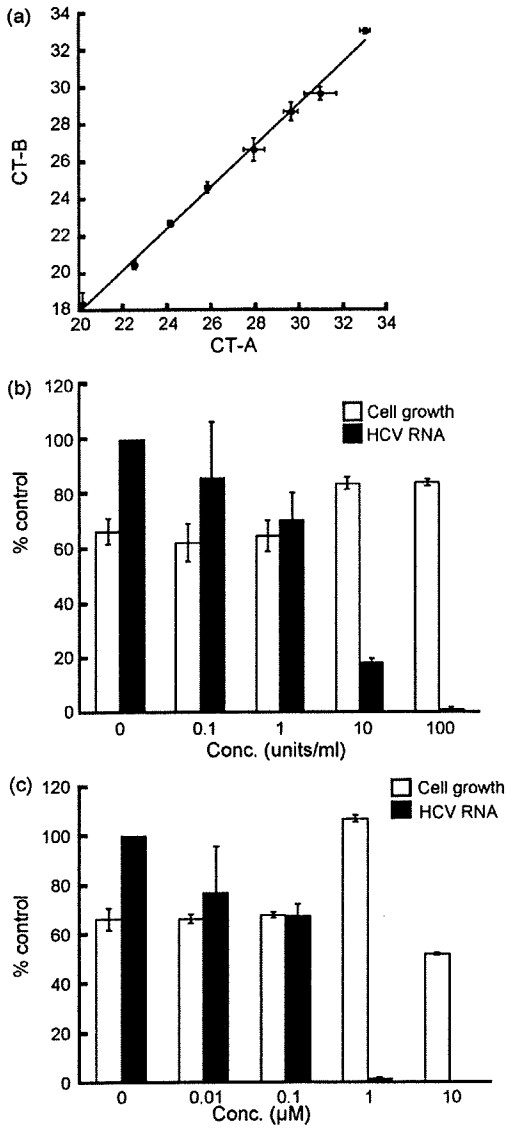

Fig. 1. RT-PCR-based screen for anti-HCV agents using the JFH1 viral culture system. (a) Correlation between CT values from "tube-capture-RT-PCR" (CT-A) and ordinary RNA extraction (CT-B). JFH1 stock solution (3×10^5 ffu/ml) was serially diluted four-fold and quantitative real-time PCR was performed as described under Section 2. CT-A was the average CT of three wells using tube-capture-RT-PCR and CT-B was the average CT of three HCV RNA eluates using a kit (QIAamp Viral RNA mini). (b) and (c) Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of IFN-α (b) or cyclosporin A (c). HCV RNA in the medium (closed (black) columns) was assessed by "tube-capture-RT-PCR" as described under Section 2. Open (white) columns represent percentage of cell growth compared with that of control cells without virus and compound. Columns, mean of triplicate wells; bars, SD.

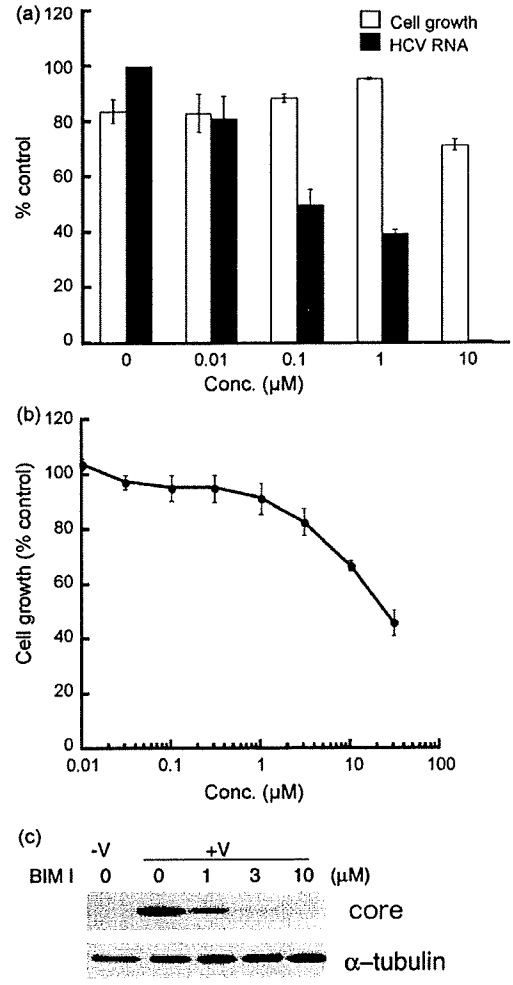

Fig. 2. BIM I inhibits HCV. (a) Effects of BIM I on HCV JFH1 RNA. Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of BIM I and assayed for HCV RNA and cell growth as in Fig. 1. Columns, mean of triplicate wells; bars, SD. (b) Effects of BIM I on growth of Huh 7.5.1 cells. (c) Effects of BIM I on HCV core protein in cells. Cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of BIM I and cultured for 2 days. Cells were lysed and subjected to western blotting as described under Section 2. "V" indicates infection with HCV.

infected cells, almost to the level of uninfected cells (Fig. 2a, white columns). Recovery of cell growth was also observed with INF-α or cyclosporin A treatment (Fig. 1b and c). BIM I reduced cell growth of uninfected cells only at concentrations of 1 μM or higher (Fig. 2b). BIM I also inhibited the production of the HCV core protein with marginal effects on host α-tubulin levels (Fig. 2c).

3.3. BIM I inhibits HCV replication

To our knowledge, the anti-HCV effects of BIM I or other PKC inhibitors have not been reported. Because the majority of current HCV drug screening relies on replicon-based models, we investigated the possibility that BIM I targets a step in the HCV life cycle that is not included in the replicon systems, such as attachment, entry or release. We treated two subgenomic replicon cells with BIM I and examined the amount of NS5A protein.

As shown in Fig. 3a and b, BIM I dose-dependently reduced NS5A in both 1b and 2a subgenomic replicon cells, but not the host GAPDH. The results indicate that BIM I inhibits a process involved in the replication of HCV subgenomic replicons. However, although the NS5A level appeared to be more vulnerable, cell growth was substantially suppressed by BIM I (Fig. 3c). Whereas a significant difference between the IC_{50} for HCV RNA and cell growth was observed in the HCV cell culture system, the reduction of NS5A in replicon cells overlapped with the effects on cell growth.

To further elucidate the stage of the HCV life cycle affected by BIM I, Huh 7.5.1 cells were inoculated with higher titers of JFH1 (MOI 2) and then treated with 3 μM BIM I, starting at different time points after infection. JFH1 appeared to complete the life cycle in about 48 h, judging from the expression profiles of viral RNA and proteins in cells (Fig. 4a). When BIM I was added at the time of infection,

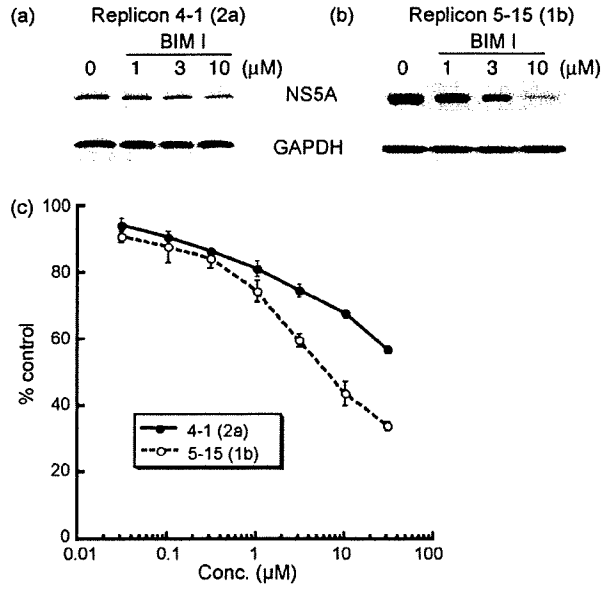

Fig. 3. Effect of BIM I on subgenomic replicon cells. (a) Subgenomic replicon cells harboring genotype 2a (4-1) (a) and genotype 1b (5-15) (b) were treated with BIM I for 2 or 4 days, respectively. Cells were lysed and analyzed by western blotting with anti-NS5A antibody or anti-GAPDH antibody. (c) Effect of BIM I on cell growth of the two replicon cells. Cells were incubated for 2 (4-1) or 4 days (5-15) with BIM I, and cell growth was measured by MTT assay.

the amount of viral RNA in the medium after 48 h decreased to less than 20% of control cells without inhibitor treatment. Addition of BIM I at 6 h post-infection still resulted in a reduction of viral RNA to 30% of control, but after 24 h the antiviral activity of BIM I was significantly diminished and only a modest decline to about 80% was observed (Fig. 4b). These results suggest that interference with RNA synthesis or translation of viral proteins accounts, at least in part, for the anti-HCV activity of BIM I.

3.4. HCV inhibition by bisindolylmaleimide and indolocarbazole compounds does not involve PKC

Since the discovery of staurosporine as a broad-spectrum protein kinase inhibitor, a variety of bisindolylmaleimide and indolocarbazole inhibitors with different potencies and selectivity have been developed. BIM I is one such compound that is highly specific for PKC and is broadly used to analyze PKC-mediated events. To gain insight into the relevance of the PKC inhibitory spectrum and antiviral activity, panels of different bisindolylmaleimide and indolocarbazole compounds were tested in the assays. Contrary to our expectation, no correlation was found between the ability to inhibit PKC and HCV.

Another bisindolylmaleimide PKC inhibitor without the N-dimethylaminopropyl chain, BIM IV, displayed similar significant anti-HCV activity (Fig. 5). Other structurally related pan- and isozyme-specific PKC inhibitors such as BIM II, Ro31-8220 (BIM IX), LY333,531 and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (PKCβ Inhibitor, Calbiochem) or an indolocarbazole compound K252c also inhibited HCV replication (not shown). However, as shown in Fig. 5, the non-PKC-inhibitory analog BIM V (Toullec et al., 1991) and arcyriaflavin A, an indolocarbazole compound with no reported effects on PKC (Zhu et al., 2003), were also effective in reducing HCV RNA. BIM V was actually more potent than BIM I. Whereas the effect of BIM I on HCV overlapped with cytotoxicity in this particular experiment, BIM V was virtually nontoxic at a dose (1 μM) that reduced

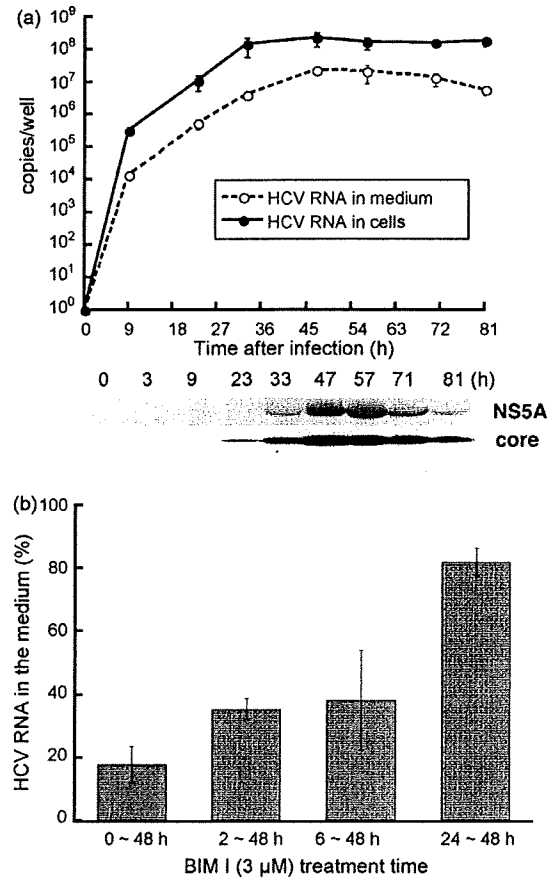

Fig. 4. (a) Expression profile of the HCV RNA and proteins. Huh 7.5.1 cells were infected with HCV as described above, but at an MOI of 2. The cells and medium were harvested at the indicated time and analyzed for HCV RNA and proteins. The core and NS5A protein were detected in the cell lysate. (b) Effect of time of addition. BIM I (3 μM) was added to Huh 7.5.1 cells just before and 2, 6, or 24 h after HCV addition at an MOI of 0.2. After 48 h of incubation following virus infection, the HCV RNA in the medium was extracted and measured with quantitative real-time RT-PCR. The indicated values represent the averages for two independent experiments.

HCV RNA to less than 20% of control. The results indicate that a broad range of bisindolylmaleimide and indolocarbazole compounds inhibit HCV replication, albeit in a PKC-independent manner.

4. Discussion

HCV replicon systems have made significant contributions in HCV research and drug development. Nevertheless, as drug screening tools, replicon systems have limitations because they are not capable of identifying inhibitors of several important events in the viral life cycle. The use of HCV cell culture systems should overcome the drawbacks of the replicon systems and facilitate the identification of inhibitors with novel mechanisms of action. Actually, it has recently been shown that use of an infectious HCV system identified inhibitors that a replicon-based screen did not recognize (Zhang et al., 2008).

We developed a simple screening method for HCV inhibitors that measures the viral RNA released from JFH1-infected cells. The assay does not require specially engineered viruses. The "tube-capture-RT-PCR" method obviates the need for labor-intensive RNA isolation and significantly increases the efficiency of screening. The validity of the assay was confirmed by successful identification of known

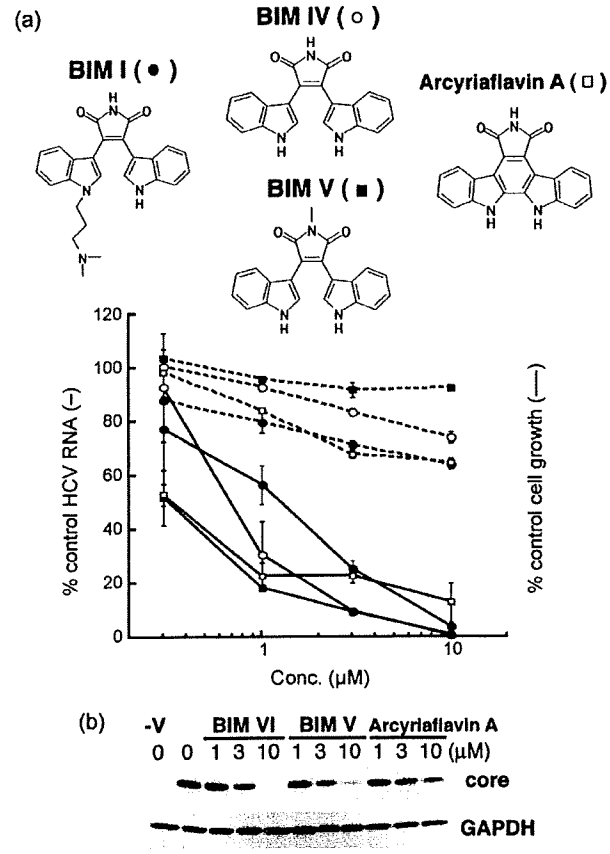

Fig. 5. Effects of bisindolylmaleimide or indolocarbazole compounds on HCV infection. (a) Effects of BIM I, BIM IV, BIM V, and arcyriaflavin A on HCV RNA in the medium. Huh 7.5.1 cells were seeded and infected with HCV at an MOI of 0.01 in the presence of drugs. After 4 days of incubation, the HCV RNA in the medium was extracted and quantified. The relative amounts of HCV RNA with BIM I (closed circle), BIM IV (open circle), BIM V (closed rectangle), and arcyriaflavin A (open rectangle) are represented by solid lines. Cell viability, represented by dotted lines, was determined by MTT assay of a parallel culture without HCV challenge. (b) Effects of BIM IV, BIM V and arcyriaflavin A on core proteins in cells. The cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of compounds and analyzed as described in Fig. 2c. "–V" indicates control without HCV infection.

HCV inhibitors in the pilot screen. In addition, the assay identified the PKC inhibitor BIM I.

BIM I is a widely used compound, and it was somewhat surprising to us that its anti-HCV activity had not been reported. HCV replication in the cell culture system appeared to be considerably more susceptible to BIM I than in the replicon systems, and this is probably why this compound had not been identified as an HCV inhibitor.

Because virus replication is closely linked to host cell growth, HCV inhibition could occur as a result of cell growth inhibition. However, as shown in Fig. 2a, BIM I reduced the cytopathic effect of HCV infection just like interferon-α and cyclosporin A. BIM I, at 1 μM, enhanced the growth of infected cells almost to the level of uninfected cells, presumably because HCV replication, but not cell growth, was inhibited, resulting in a reversal of the cytopathic effect.

Because the PKC inhibitory properties of bisindolylmaleimides and indolocarbazoles have been characterized extensively, we tested a panel of commercially available compounds in the assay to gain insight into the role of PKC in HCV replication. However, no correlation between PKC inhibition and antiviral activity could be found. Anti-HCV activity did not involve PKC inhibition, apparently because a non-PKC-inhibitory analog, BIM V, was also active. Furthermore, PKC inhibitors with different structures, such as calphostin C and H-7, did not show specific inhibition of HCV (data not shown).

Previous studies have indicated that bisindolylmaleimide PKC inhibitors have cellular targets other than PKC. It has been shown that BIM I and Ro31-8220 inhibit p70S6K and p90RSK (Alessi, 1997; Roberts et al., 2005) and that BIM V inhibits p70S6K (Marmy-Conus et al., 2002). Although we did not monitor the activities of these enzymes in our experiments, inhibition of p70S6K is unlikely to be responsible for the anti-HCV effect of PKC inhibitors, because Ishida et al. reported that silencing of p70S6K enhanced HCV RNA abundance (Ishida et al., 2007).

Bisindolylmaleimides and indolocarbazoles have also been reported to inhibit the ATP-binding cassette (ABC) transporters P-glycoprotein and multidrug resistance-associated protein 1 (MRP1), efflux pumps that play important roles in cancer drug resistance (Merritt et al., 1999; Gekeler et al., 1995). More recently, Robey et al. reported that BIMs I, II, III, IV, and V, K252c, and arcyriaflavin A inhibit ABCG2, an ABC half-transporter that confers resistance to various antitumor agents (Robey et al., 2007). Whether ABC transporters play any role in HCV infection awaits further study. We are currently examining the anti-HCV effects of other ABC transporter inhibitors.

In conclusion, we developed a simple infectious HCV system-based assay that can be used for high-throughput screening of HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles. These compounds might represent lead substances for the development of new HCV drugs. Further analysis of the mechanism of HCV-inhibition by these compounds might reveal a new mechanism of regulation of HCV infection.

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ORIGINAL ARTICLE

Double-stranded RNA-induced interferon-beta and inflammatory cytokine production modulated by hepatitis C virus serine proteases derived from patients with hepatic diseases

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Abstract We previously demonstrated that hepatitis C virus (HCV) serine protease NS3-4A was unable to cleave TRIF (adaptor protein of Toll-like receptor 3), resulting in a lack of suppression of the TRIF-mediated pathway, whereas NS3-4A cleaved Cardif (adaptor protein of retinoic acid-inducible gene I or melanoma differentiation-associated gene-5), resulting in an interruption of the Cardif-mediated pathway in non-neoplastic human hepatocyte PH5CH8 cells. To elucidate these observations, we examined the cleavage potential of NS3-4A for TRIF in PH5CH8 cells, genome-length HCV RNA-replicating O cells, and HCV-infected cells, and we demonstrated that NS3-4A lacked the ability to cleave endogenous TRIF, regardless of HCV strains derived from patients with different stages of hepatic disease. Furthermore, we demonstrated that inflammatory cytokine production by NF-κB activation via the TRIF-mediated pathway also remained unsuppressed by NS3-4A. These results suggest that the inhibitory effects of NS3-4A on antiviral signaling pathways are limited to the Cardif-mediated pathway in human hepatocytes.

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Introduction

Hepatitis C virus (HCV) infection causes a number of liver diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [5, 24, 32, 33]. The progression of liver disease from chronic hepatitis to hepatocellular carcinoma by persistent HCV infection is a serious health problem [37]. In order to elucidate the relationship between the mechanism of persistent HCV infection and liver disease progression, it will be necessary to examine the virus life cycle and develop more effective anti-HCV reagents based on these observations. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* [20, 36]. The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least ten proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and a virally encoded serine protease located in the amino-terminal domain of NS3. The serine protease activity of NS3 requires NS4A, a protein that consists of 54 aa residues, to form a stable complex with NS3 [11, 12, 19].

During infection by RNA viruses such as HCV, double-stranded RNA (dsRNA) is produced by viral RNA replication in virus-infected cells, and dsRNA is in turn recognized by Toll-like receptor (TLR) 3, which is expressed on the cell surface or in endosome vesicles [3, 13]. Additionally, dsRNA is recognized by retinoic-acid-inducible gene I (RIG-I) and/or melanoma differentiation-associated gene 5 (MDA5), which are both localized in the cytoplasm [18, 40, 41]. The stimulation of TLR3 by extracellular dsRNA leads to the activation of two signaling pathways that bifurcate at TRIF [17, 34], i.e., interferon

(IFN)-β production is induced via activation of the TRIF/TRAF3/TBK1/IRF-3 pathway, and inflammatory cytokines such as IL-6 or IL-8 are produced via activation of the TRIF/TRAF6/TAK1/NF-κB pathway (see Fig. 5a). On the other hand, the stimulation of RIG-I or MDA5 by intracellular dsRNA may induce both IFN-β and inflammatory cytokine production by similar signaling pathways that bifurcate at Cardif (i.e., the Cardif/TRAF3/TBK1/IRF-3 pathway and the Cardif/TRAF6/TAK1/NF-κB pathway) [39]. IFN-β and the inflammatory cytokines are upregulated to induce an antiviral state in virus-infected cells, and then these production levels return to a steady state in virus-eliminated cells.

Several groups, including ours, have previously reported that the HCV serine protease NS3-4A inhibited intracellular dsRNA-induced IFN-β production via the cleavage of Cardif [6, 29, 30]. The findings of these reports have indicated that Cardif is a key molecule for establishing persistent HCV infection. On the other hand, we also previously demonstrated that NS3-4A (1B-1 and O strains of genotype 1b) was not able to inhibit extracellular dsRNA-induced IFN-β production due to a lack of ability to cleave TRIF [6]; however, in another previous report, it was demonstrated that NS3-4A (N strain of genotype 1b) was able to inhibit IFN-β production via the cleavage of TRIF [27]. These latter results, taken together, suggest that among HCV strains, NS3-4A possesses a range of ability to cleave TRIF. In the present study, NS3-4As derived from patients with different stages of liver disease were used to compare the potential of NS3-4As to inhibit IFN-β production and NF-κB activation via intracellular or extracellular dsRNA.

Materials and methods

Cell culture

Non-neoplastic human hepatocyte PH5CH8 cells susceptible to HCV infection and supportive of HCV replication were cultured as reported previously [16]. Genome-length HCV RNA-replicating O cells [14], their cured Oc cells [14] and other HuH-7-derived RSc cells [25] were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carsbad, CA, USA) supplemented with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [1], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct various expression vectors. pCX4pur/myc-TRIF(P367D), (P367E), (P367D/S368C), or (P367E/S368D) mutants were constructed using PCR mutagenesis with primers containing base alterations. pCX4pur/myc-TRIF [6] was used as the template for PCR mutagenesis. The NS3-4A expression vectors used in this study were constructed using oligonucleotides (Supplementary Table S1 in Electronic Supplementary Material) as described below. RNA was extracted using an ISOGEN extraction kit (Nippon Gene, Toyama, Japan) and serum from 13 HCV-infected patients: three healthy carriers (1B-3, 1B-4, and 1B-5 strains [15]), a patient with acute hepatitis (AH1 strain [21]), and seven patients with chronic hepatitis (CH1, CH3, CH4, CH5, CH6, CH7, and CH8 strains). In addition, serum was obtained from two patients with hepatocellular carcinoma (HCC1 and HCC2 strains [2]). Informed consent was obtained from each patient before the study. The DNA fragments, including the NS3-4A region, were amplified by RT-nested PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) and oligonucleotides for cDNA synthesis, first-round PCR, and second-round PCR (Supplementary Table S1). The obtained DNA fragments were subcloned into the *Xba*I site of pBR322MC [22], and the nucleotide sequences of the NS3-4A regions were determined. The oligonucleotides for the construction of the NS3-4A expression vector were designed from the nucleotide sequences of the NS3-4A regions (Supplementary Table S1). The DNA fragments encoding NS3-4A were amplified by PCR using KOD-plus DNA polymerase and the specifically designed oligonucleotides, and the amplified fragments were cloned into the *Eco*RI and *Not*I sites of pCX4bsr. The nucleotide sequences of the constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular evolutionary analysis

Molecular evolutionary trees were constructed from the aa sequences of the NS3-4A regions using the UPGMA method and the program GENETYX-MAC (Software Development, Tokyo, Japan).

JFH-1 infection experiments

The infection of RSc cells with JFH-1 was performed as described previously [25]. Briefly, 1.0×10^5 RSc cells were seeded onto 6-well plates 24 h before infection. Then, an inoculum of JFH-1 was added to the cells at a multiplicity of infection of 0.1. After 96 h of JFH-1 infection, cell lysates were prepared as described below.

Immunoprecipitation and Western blot analysis

The preparation of cell lysates from PH5CH8 cells stably expressing NS3-4A and two mutants (S1165A and W1528A) [6] was performed as described previously [31].

Cell lysates were subjected to immunoprecipitation using anti-TRIF antibody (Exalpha Biologicals, Maynard, MA, USA) or anti-Cardif antibody (Bethyl Laboratories, Montgomery, TX, USA). Bound proteins were collected from cell lysates using Protein G Sepharose (Amersham) and were subjected to immunoblot analysis. Anti-NS3 antibody (polyclonal R212; a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), and anti-NS4A antibody (C14II3-3; also a generous gift from Dr. Kohara) were used to detect NS3 and NS4A proteins. Anti-myc antibody (PL14; Medical and Biological Laboratories, Nagoya, Japan), anti-EGFP antibody (JL-8; Clontech), and anti-β-actin antibody were used in this study as primary antibodies. Immunocomplexes were detected using a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β (−125)-Luc [4], containing the IFN-β gene promoter region (−125 to +19) and pNF-κB-Luc (Stratagene). The reporter assay was carried out as described previously [8]. Briefly, a total of 0.3×10^5 cells were seeded onto 24-well plates 24 h before transfection. Then, PH5CH8 cells were transfected with 0.1 μg pIFN-β (−125)-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA), used as an internal control reporter, for the measurement of IFN-β promoter activity. For the measurement of NF-κB promoter activity, PH5CH8 cells were transfected with 0.01 μg pNF-κB-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.02 ng pRL-CMV. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h, and then poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium for 6 h at 50 μg/ml (M-pIC) before the reporter assay. Three independent triplicate transfection experiments were conducted in order to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

RNA interference and real-time LightCycler PCR

siRNA duplexes targeting the coding regions of human TLR3 [31], TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 (Dharmacon), used as a control, were chemically synthesized. Using PH5CH8 cells with drastically decreased TLR3 or TRIF mRNA levels [6], NF-κB promoter activity was measured as described above, and dsRNA-induced inflammatory cytokine production levels were examined by using a primer set for IL-6 or IL-8 [38]. Total cellular RNA extraction and real-time LightCycler PCR were performed as described previously [6, 7].

Results

NS3-4A lacks the ability to cleave endogenous TRIF

We recently reported that NS3-4A serine protease (1B-1 strain of genotype 1b) was unable to cleave TRIF expressed in human PH5CH8 hepatocyte cells [6]. To account for this lack of cleavage ability, we examined the ability of NS3-4A to cleave TRIF mutants converted to a sequence similar to the consensus sequence required for cleavage ability by NS3-4A (Fig. 1a). The results obtained with

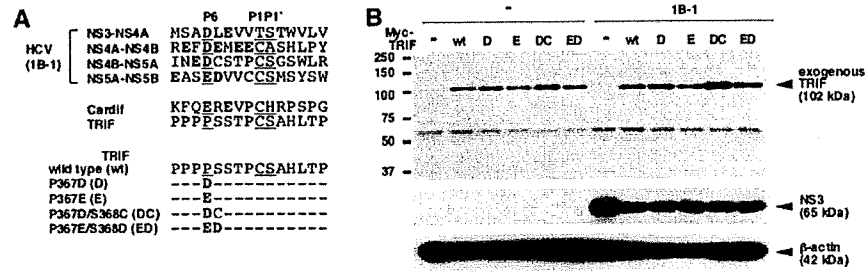

Fig. 1 NS3-4A does not cleave exogenous TRIF or its mutants at the P6 position. **a** The alignment of amino acid sequences surrounding the site cleaved *in trans* or *in cis* by NS3-4A. The consensus sequences required for cleavage by NS3-4A are underlined (P6, P1, and P1′ positions). The amino acids with a negative charge are indicated in boldface type. **b** TRIF mutants with a negative charge at the P6 position also remain uncleaved by NS3-4A. Wild-type TRIF and TRIF mutants (P367D, P367E, P367D/S368C, and P367E/S368D) are indicated as wt, D, E, DC, and ED, respectively. PH5CH8 cells stably expressing NS3-4A (1B-1) were transfected with the pCX4pur vector (as a control, –) or myc-TRIF expression vectors (wild-type strain or mutants). Production of myc-TRIF and NS3 in the cells was analyzed by immunoblot analysis using anti-myc and anti-NS3 antibody, respectively. PH5CH8 cells infected with retrovirus pCX4bsr were used as a control (–). β-actin was used as a control for the amount of protein loaded per lane