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Limited suppression of the interferon-β production by hepatitis C virus serine protease in cultured human hepatocytes

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Keywords
antiviral response; hepatitis C virus; innate immune response; interferon-β; serine protease

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Toll-like receptors and RNA helicase family members [retinoic acid-inducible gene I (RIG-I) and melanoma differentiation associated gene-5 (MDA5)] play important roles in the induction of interferon-β as a major event in innate immune responses after virus infection. TRIF (adaptor protein of Toll-like receptor 3)-mediated and Cardif (adaptor protein of RIG-I or MDA5)-mediated signaling pathways contribute rapid induction of interferon-β through the activation of interferon regulatory factor-3 (IRF-3). Previously, it has been reported that the hepatitis C virus NS3-4A serine protease blocks virus-induced activation of IRF-3 in the human hepatoma cell line HuH-7, and that NS3-4A cleaves TRIF and Cardif molecules, resulting in the interruption of antiviral signaling pathways. On the other hand, it has recently been reported that non-neoplastic human hepatocyte PH5CH8 cells retain robust TRIF- and Cardif-mediated pathways, unlike HuH-7 cells, which lack a TRIF-mediated pathway. In the present study, we further investigated the effect of NS3-4A on antiviral signaling pathways. Although we confirmed that PH5CH8 cells were much more effective than HuH-7 cells for the induction of interferon-β, we obtained the unexpected result that NS3-4A could not suppress the interferon-β production induced by the TRIF-mediated pathway, although it suppressed the Cardif-mediated pathway by cleaving Cardif at the Cys508 residue. Using PH5CH8, HeLa, and HuH-7-derived cells, we further showed that NS3-4A could not cleave TRIF, in disagreement with a previous report describing the cleavage of TRIF by NS3-4A. Taken together, our findings suggest that the blocking of the interferon production by NS3-4A is not sufficient in HCV-infected hepatocyte cells.

Persistent infection by hepatitis C virus (HCV) frequently causes chronic hepatitis [1,2], which progresses to liver cirrhosis and hepatocellular carcinoma [3,4]. This is a serious health problem because approximately 170 million people are currently infected with HCV worldwide [5]. To resolve the mechanism of persistent HCV infection, it will be necessary to better understand the virus life cycle and then to develop more effective anti-HCV reagents. HCV is an enveloped positive ssRNA (9.6 kb) virus belonging to the *Flaviviridae* family [6,7]. The HCV genome encodes a large polyprotein precursor of approximately 3000 amino acid residues, which is cleaved co- and post-translationally into at least ten proteins in the order: core, envelope 1

Abbreviations
CARD, caspase recruitment domain; E1, envelope 1; EGFP, enhanced green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HEK293, human embryonic kidney 293; IFN, interferon; IRF-3, interferon regulatory factor 3; IKK-ε, inhibitor of κB kinase ε; MDA5, melanoma differentiation associated gene-5; MyD88, myeloid differentiation factor 88; NS2, nonstructural protein 2; RIG-I, retinoic acid-inducible gene I; siRNA, small interfering RNA; TBK, Tank-binding kinase 1; TLR, Toll-like receptor; TRIF, Toll-IL1 receptor domain-containing adaptor inducing IFN-β.

(E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and virally encoded serine protease located in the amino-terminal domain of NS3. Serine protease activity of NS3 requires NS4A, a protein consisting of 54 amino acid residues, to form a stable complex with the NS3 [8–10].

Virus-infected cells trigger the innate immune response by recognizing viral components, including DNA, ssRNA, dsRNA and glycoproteins. This response initiates signaling pathways leading to the induction of protective cellular genes, including type-I interferons [initially interferon (IFN)-β, and then IFN-α] and proinflammatory cytokines that directly limit viral replication. Within these signaling pathways, Toll-like receptors (TLRs) and RNA helicase family members play very important roles in the recognition of the viral components [11,12].

IFN-β is induced by dsRNA, a common intermediate in many RNA virus infections, including HCV. The viral dsRNA as well as the synthetic dsRNA analogue poly(I-C) are recognized by TLR3, which is expressed on the cell surface or in endosome vesicles [13,14]. On the other hand, it has been shown that retinoic acid-induced gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) also recognize dsRNA molecules [15–17]. A recent study showed that MDA5 and RIG-I recognize different types of dsRNA: MDA5 recognizes poly(I-C), and RIG-I recognizes *in vitro* transcribed dsRNA [18]. Very recently, it was discovered that viral 5′-triphosphate RNA is the ligand for RIG-I [19,20]. Both MDA5 and RIG-I contain DexD/H-box helicase domains that serve as intracellular cytoplasmic dsRNA and 5′-triphosphate RNA receptors, respectively [15–20]. After dsRNA is recognized, the cytoplasmic domain of TLR3 recruits TIR-domain-containing adaptor inducing IFN-β (TRIF) through a myeloid differentiation factor 88 (MyD88)-independent pathway (TRIF-mediated pathway) [21–23]. In contrast, the caspase recruitment domains (CARDs) of MDA5 or RIG-I recruit the CARD adaptor inducing IFN-β, Cardif (also known as IPS-1, MAVS, or VISA), which was recently identified as an adaptor protein located in the outer membrane of mitochondria (this recruitment is known as the Cardif-mediated pathway) [24–27].

The TRIF- and Cardif-mediated signaling pathways rapidly induce IFN-β through the phosphorylation of multiple cellular factors, including IFN regulatory factor-3 (IRF-3) and kinases, including the Tank-binding kinase 1 (TBK-1) and inhibitor of κB kinase ε (IKK-ε) [28–31]. Although IRF-3 is located in the cytoplasm in an inactive state [28,29], phosphorylation (Ser385, 386, 396, 398, 402, 405, and Thr404) of IRF-3 by TBK-1 and IKK-ε induces dimerization and nuclear translocation of IRF-3, leading to transcriptional activation of IFN-β [28–31].

Recent studies have found that several RNA virus proteins could inhibit the early signaling activation (TRIF- and Cardif-mediated pathways) leading to IFN-β production [32,33]. Regarding HCV, Foy *et al.* [33] found that NS3-4A serine protease blocked HCV-induced activation of IRF-3 in the human hepatoma cell line HuH-7. Additional studies regarding this finding have shown that NS3-4A blocks the Cardif-mediated signaling pathway by cleaving the Cardif molecule and blocking downstream IFN-β activation [24,34,35], and that TBK-1, IKK-ε, and TRIF may also be targeted for cleaving by NS3-4A [36–38]. With respect to TRIF, NS3-4A was reported to cleave this molecule in both an *in vitro* experiment using a reticulocyte lysate system and an *in vivo* experiment using human embryonic kidney 293 (HEK293) and UNS3-4A-24 osteosarcoma cells [36]. These studies suggest that NS3-4A has the ability to inhibit both TRIF- and Cardif-mediated signaling pathways.

On the other hand, we recently demonstrated that HCV proteins exhibited conflicting effects on the IFN-β production in non-neoplastic human hepatocyte PH5CH8 cells [39,40]: Core and NS5B synergistically enhanced IFN-β expression and this enhancement was dependent on the RNA-dependent RNA polymerase activity of NS5B, but NS3-4A significantly inhibited the production of IFN-β induced by the combination of Core and NS5B. Furthermore, Li *et al.* [41] recently reported that PH5CH8 cells retained robust and functionally active TRIF- and Cardif-mediated signaling pathways, unlike HuH-7 cells, which lacked the TRIF-mediated pathway [41,42]. Therefore, using poly(I-C) as an inducer of IFN-β, we investigated the effects of NS3-4A on antiviral signaling pathways in PH5CH8 cells. Our results showed that the extracellular TLR3/TRIF signaling pathway was not blocked by NS3-4A because NS3-4A did not cleave TRIF, unlike in the previous study [36].

Results

Human hepatocyte PH5CH8 cells more readily activate IFN-β transcription in response to dsRNA compared to HuH-7 cells and their sublines

Recently, Li *et al.* [41] reported that PH5CH8 cells showed a better response to dsRNA, including IFN-β induction, than other human hepatoma cell lines (HuH-7, HepG2, and Hep3B). Therefore, using a dual

luciferase reporter assay, we first confirmed that PH5CH8 cells were much more effective at inducing IFN-β than HuH-7 cells and HuH-7-derived cell sublines (O [43], Oc [43], and OR6c [44]) that can support HCV RNA replication.

When the dsRNA analog, poly(I-C), was transfected into cells using a liposome-mediated procedure (intracellular dsRNA, T-pIC), PH5CH8 cells showed a more potent (> 25-fold) activation of the IFN-β gene promoter than HuH-7 and HuH-7-derived cell lines (Fig. 1A). Furthermore, when poly(I-C) was added to the culture medium (extracellular dsRNA; M-pIC), a significant elevation (12-fold) of the IFN-β gene promoter was observed in PH5CH8 cells only (Fig. 1B). These results were confirmed by quantitative RT-PCR analysis of endogenous IFN-β mRNA induction in cells treated with poly(I-C) (T-pIC, Fig. 1C; M-pIC, Fig. 1D). In both T-pIC and M-pIC treatments, the induction level of IFN-β mRNA was markedly higher in PH5CH8 cells than in O, Oc, OR6c, and HuH-7 cells (Fig. 1C,D). Next, we carried out quantitative RT-PCR analysis of TLR3, TRIF, RIG-I, MDA5, Cardif, and IRF-3 mRNAs to clarify their expression levels in the steady state and the effects of poly(I-C)

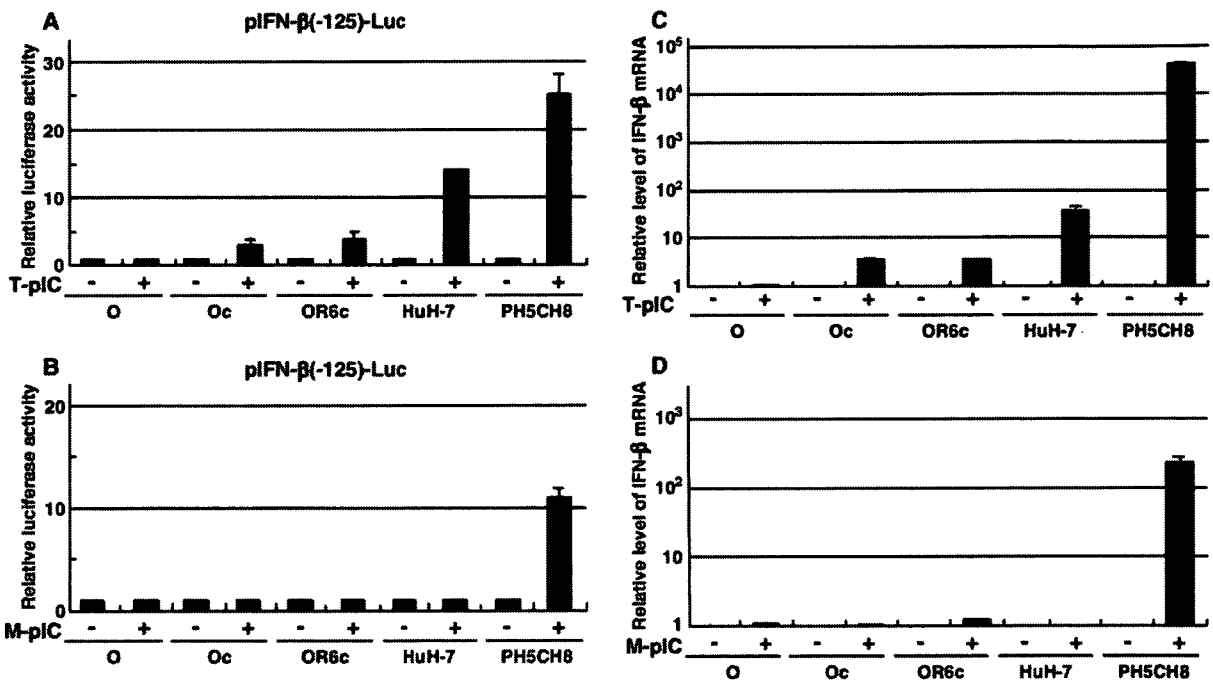

Fig. 1. PH5CH8 cells show high-level IFN-β production in response to dsRNA. (A) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with T-pIC. The following HuH-7-derived cell sublines were used: O, cloned cells [43] replicating genome-length HCV RNA; Oc, cured cells which were created by eliminating genome-length HCV RNA from the O cells by IFN treatment; and OR6c, cured cells which were created by eliminating genome-length HCV RNA from the cloned OR6 cells [44] by IFN treatment. Cells grown in 24-well plates were cotransfected with pIFN-β-(−125)-Luc and pRL-CMV (internal control reporter) and cultured for 42 h, and then poly(I-C) (1 μg) was transfected into the cells for 6 h before the reporter assay as described in the Experimental procedures. The relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). The lysate of cells without poly(I-C) treatment was used as a control. Data are the means ± SD from three independent experiments, each performed in triplicate. (B) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with M-pIC. The dual luciferase reporter assay was performed as described in (A) except that poly(I-C) was added to the medium (50 $\mu g \cdot mL^{-1}$) for 6 h before the reporter assay. (C) Quantitative RT-PCR analysis of IFN-β mRNA in various cells treated with T-pIC. Poly(I-C) (1 μg) was transfected into the cells for 6 h before the sampling for RNA preparation. Total RNA extracted from the cells was subjected to real-time LightCycler PCR analysis using the primer set of IFN-β (202 bp). Data are the means ± SD from three independent experiments. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of IFN-β mRNA concentration to that of GAPDH. The IFN-β mRNA levels were calculated relative to the level in the O cells treated with T-pIC, which was set at 1.0. (D) Quantitative RT-PCR analysis of IFN-β mRNA in various cells treated with M-pIC. Poly(I-C) was added to the medium (50 $\mu g \cdot mL^{-1}$) for 6 h before the sampling for RNA preparation. Quantitative RT-PCR analysis for IFN-β mRNA was performed as described in (C). The IFN-β mRNA level was calculated relative to the level in the O cells treated with M-pIC, which was set at 1.0.

treatment (T-pIC and M-pIC). In T-pIC treatment, RIG-I and MDA5 mRNAs were clearly induced in PH5CH8 and HuH-7 cells, and TLR3 mRNA was induced only in PH5CH8 cells. Moreover, there was no such induction in the other cell lines examined (supplementary Table S1). In M-pIC treatment, TLR3, RIG-I, and MDA5 were induced only in PH5CH8 cells (supplementary Table S1). The fact that these mRNAs were induced at substantial levels only in PH5CH8 cells treated with T-pIC or M-pIC suggests that the elevation of these mRNAs is mediated by the IFN-β induced by poly(I-C) treatment. In summary, these results revealed that PH5CH8 cells retain both the Cardif- and TRIF-mediated pathways for IFN-β production, whereas HuH-7 cells retain only the Cardif-mediated pathway, and that the HuH-7-derived cells lines used are lacking in both pathways for IFN-β production.

Parental PH5CH and PH5CH clones other than PH5CH8 also exhibit IFN-β response toward poly(I-C) treatment

PH5CH8 is one of eight cell lines that were previously cloned from parental PH5CH cells to examine HCV susceptibility *in vitro* [45]. Therefore, we used a dual luciferase assay to examine the effects of poly(I-C) treatment on the IFN-β gene promoter in PH5CH cells and these cloned cell lines. When T-pIC treatment was employed, the parental cells and all the cloned cell lines exhibited good IFN-β response, and the activation level in PH5CH2 and PH5CH6 cells was higher than that in PH5CH8 cells (Fig. 2A). However, when M-pIC treatment was used, the IFN-β response in the cloned cells and the parental cells was less than 50% of that in PH5CH8 cells (Fig. 2B). From these results, we concluded that PH5CH8 is the best cell line for the study of the dsRNA-induced antiviral signaling pathways.

M-pIC treatment activates IRF-3 through the TLR3/TRIF signaling pathway

To confirm that the TRIF-mediated pathway is activated in M-pIC treatment, and to determine if its activation is mediated by the TLR3 but not the TLR4 signaling pathway, we examined whether or not activation of IRF-3 by M-pIC treatment is specifically mediated by the TLR3 signaling pathway using TLR3-, TLR4-, and TRIF-specific small interfering RNA (siRNAs) [46,47]. Quantitative RT-PCR analysis revealed that the TLR3, TLR4, and TRIF mRNAs were drastically decreased (more than 70% reduction) in the

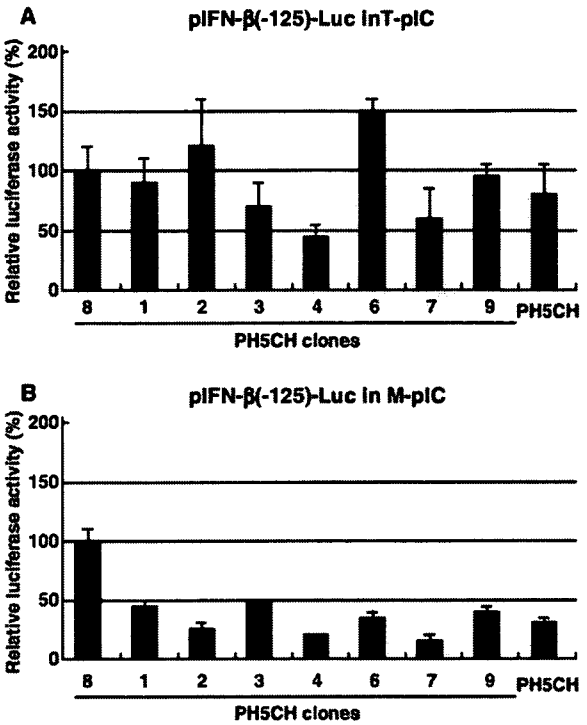

Fig. 2. IFN-β responses of parental PH5CH and PH5CH cloned cells by dsRNA treatment. (A) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with T-pIC. The T-pIC treatment and the dual luciferase reporter assay were performed as described in Fig. 1A. The IFN-β gene promoter activity level was calculated relative to the level in the PH5CH8 cells, which was set at 100. (B) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with M-pIC. The M-pIC treatment and the dual luciferase reporter assay were performed as described in Fig. 1B. The relative level of the IFN-β gene promoter activity was calculated as described in (A).

PH5CH8 cells transfected with TLR3, TLR4, and TRIF siRNAs, respectively, but not in the PH5CH8 cells transfected with the GL2 siRNA used as a control (Fig. 3A). We also confirmed that IRF-3 mRNA was not decreased in PH5CH8 cells transfected with any of these siRNAs (Fig. 3A). Under this condition, we performed a luciferase reporter assay using an IFN-β gene promoter in PH5CH8 cells treated with M-pIC. The activation of the IFN-β gene promoter was greatly suppressed (by more than 80%) in PH5CH8 cells transfected with TLR3 or TRIF siRNA, but not in the PH5CH8 cells transfected with GL2 or TLR4 siRNA (Fig. 3B). This result suggests that the activation of IRF-3 by M-pIC treatment is mediated by the TLR3/TRIF signaling pathway. We obtained further evidence by examining the status of the phosphorylation and dimerization of IRF-3. The results

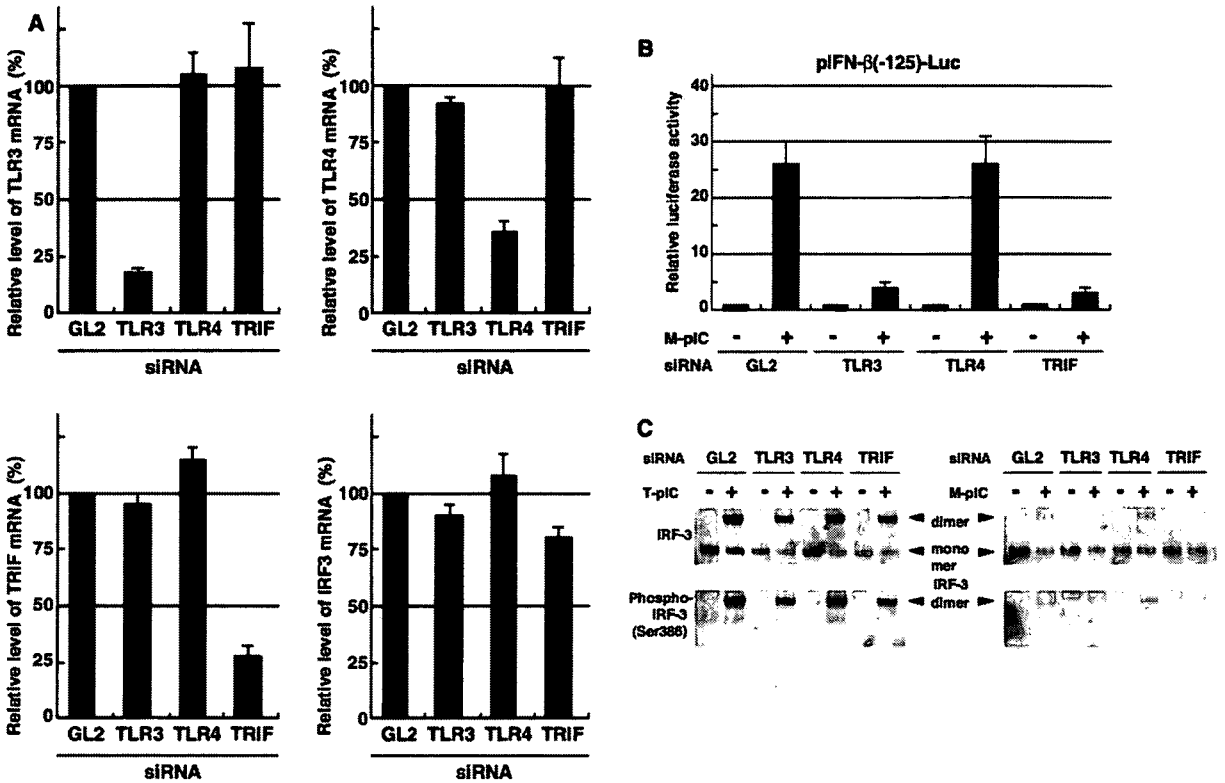

Fig. 3. Extracellular dsRNA treatment activates IRF-3 through the TLR3/TRIF signaling pathway in PH5CH8 cells. (A) Down-regulation of TLR3, TLR4, and TRIF mRNAs by transfection of TLR3, TLR4, and TRIF siRNAs, respectively. PH5CH8 cells were transfected with dsRNA duplexes targeting TLR3, TLR4, TRIF or luciferase GL2. After 3 days, the expression levels of TLR3, TLR4, TRIF, and IRF-3 mRNAs were determined by the quantitative RT-PCR as described previously [67]. (B) Dual luciferase reporter assay of the IFN-β gene promoter using siRNA-transfected PH5CH8 cells treated with M-pIC. The poly(I-C) treatment and the dual luciferase reporter assay were performed as described in Fig. 1. (C) Phosphorylation and dimerization analyses of IRF-3 in the siRNA-transfected PH5CH8 cells treated with poly(I-C). The poly(I-C) treatment was performed as described in Fig. 1. The lysate of cells transfected with GL2, TLR3, TLR4, or TRIF siRNA was prepared, and subjected to Native-PAGE as described in the Experimental procedures. The phosphorylation and dimerization of IRF-3 were analyzed by immunoblotting using anti-phospho-IRF-3 (Ser386) serum and anti-IRF-3 serum, respectively.

obtained by M-pIC treatment revealed that both the phosphorylation and dimerization of IRF-3 were almost completely abrogated in the cells transfected with TLR3 or TRIF siRNA, but not in those transfected with the GL2 and TLR4 siRNAs (Fig. 3C, right panel). Such a suppression of IRF-3 activation was not observed by T-pIC treatment (Fig. 3C, left panel), suggesting that the activation of IRF-3 by T-pIC treatment is mainly mediated by the Cardif-mediated signaling pathway [16].

HCV NS3-4A blocks the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway

Several studies [24,33,36,48–50] have demonstrated that NS3-4A blocks IFN-β induction by inhibiting the nuclear translocation of IRF-3 in HuH-7 cells harboring HCV replicons and HCV (JFH1 strain of genotype 2a)-infected HuH-7 cells. However, it has also been reported that HuH-7 cells possess weak or defective dsRNA-induced antiviral signaling pathways [41,42] (Fig. 1). Therefore, we examined whether or not NS3-4A can block the induction of IFN-β by poly(I-C) in PH5CH8 cells that retain dsRNA-induced signaling pathways. The results were quite different between T-pIC treatment and M-pIC treatment. First, in T-pIC treatment, the results showed that NS3-4As (the 1B-1 and HCV-O strains of genotype 1b) could drastically inhibit the enhancement of the IFN-β gene promoter activity, and that this suppressive effect of NS3-4A was dependent on its serine protease activity, because the NS3-4A/S1165A mutant lacking the serine protease activity did not exhibit the suppressive effect,

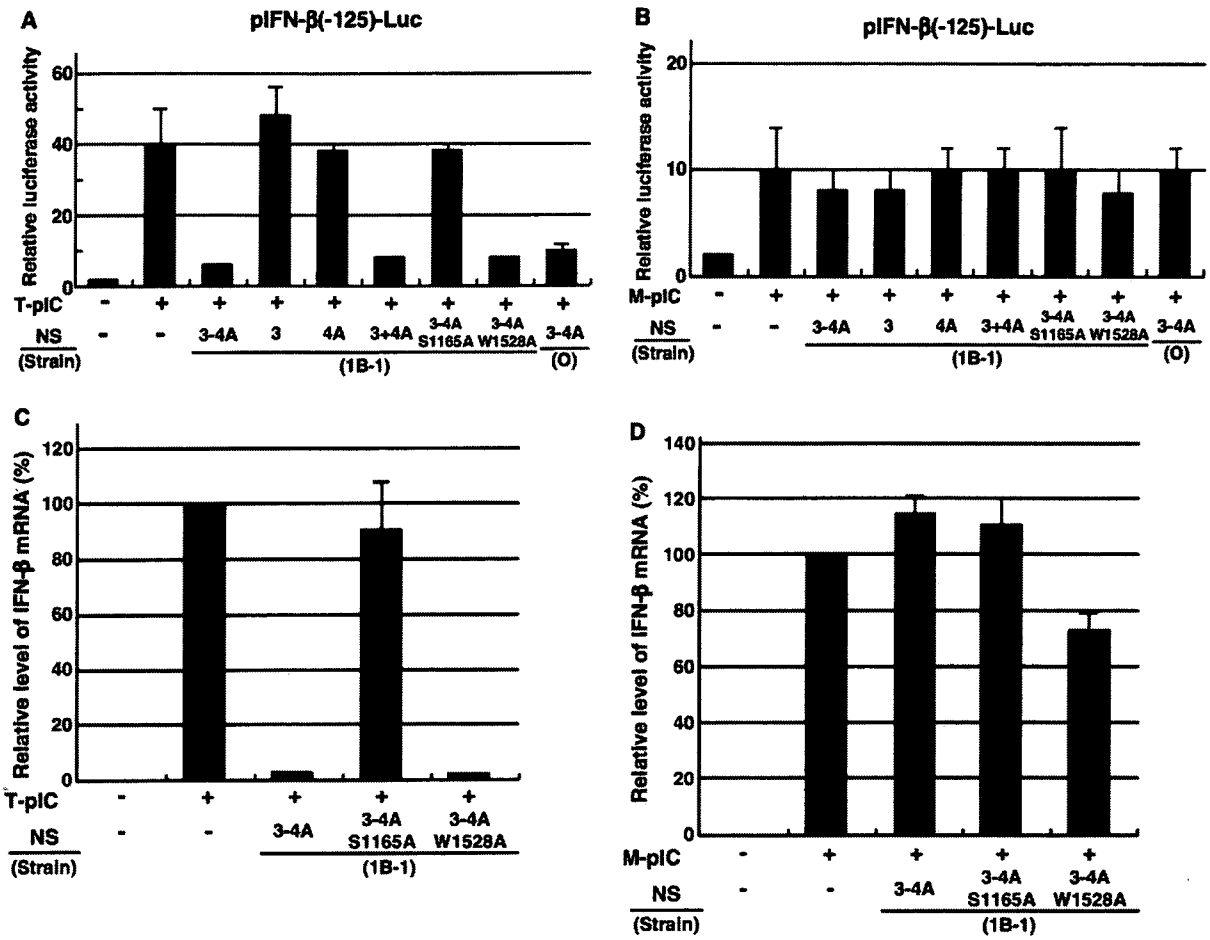

Fig. 4. NS3-4A blocked the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway. The poly(I-C) treatment, dual luciferase reporter assay, and quantitative RT-PCR analysis were performed as described in Fig. 1. The pCX4bsr expression vectors encoding NS3-4A, NS3, or NS4A from the 1B-1 strain and NS3-4A from the HCV-O strain were used for the transfection. The pCX4bsr expression vector encoding the NS3-4A/S1165A mutant (1B-1 strain) lacking serine protease activity or the NS3-4A/W1528A mutant (1B-1 strain) lacking RNA helicase activity was also used for the transfection. The lysate of PH5CH8 cells transfected with the pCX4bsr vector was used as a control (NS–). (A) Effect of NS3-4A on the IFN-β gene promoter activated by T-plC treatment. (B) Effect of NS3-4A on the IFN-β gene promoter activated by M-plC treatment. (C) Effect of NS3-4A on the IFN-β mRNA induction by T-plC treatment. PH5CH8 cells stably expressing the NS3-4A or NS3-4A mutant (S1165A or W1528A) from the 1B-1 strain were subjected to T-plC treatment. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (NS–). The IFN-β mRNA level was calculated relative to the level in the control PH5CH8 cells treated with T-plC, which was set at 100. (D) Effect of NS3-4A on the IFN-β mRNA induction by M-plC treatment. PH5CH8 cells that were the same as in (C) were subjected to M-plC treatment. The IFN-β mRNA level was calculated relative to the level in the control PH5CH8 cells treated with M-plC, which was set at 100.

although the NS3-4A/W1528A mutant lacking RNA helicase activity did (Fig. 4A). In addition, we confirmed that NS3 alone or NS4A alone did not exhibit the suppressive effect, but coexpression of NS3 and NS4A did, suggesting that the NS3/4A complex in *trans* [51] also can block IFN-β induction. In M-plC treatment, however, we found that NS3-4As (strains 1B-1 and O) could not suppress the induction of the IFN-β gene promoter (Fig. 4B). Similar results were also obtained in the other cloned cell lines, PH5CH3 and PH5CH6 (data not shown), and in HeLa cells (supplementary Fig. S1). The results of the reporter assay were confirmed by quantitative RT-PCR analysis of endogenous IFN-β mRNA induced by T-plC or M-plC treatment in PH5CH8 cells. We found that the NS3-4A and NS3-4A/W1528A mutants, but not the NS3-4A/S1165A mutant, could suppress the induction of IFN-β mRNA following

T-pIC treatment (Fig. 4C), but none of these NS3-4As could suppress the induction of IFN-β mRNA following M-pIC treatment (Fig. 4D).

We next examined the effects of NS3-4A on the phosphorylation and dimerization of IRF-3 in PH5CH8 cells. We observed that both T-pIC and M-pIC treatments induced the phosphorylation at Ser386 and Ser396 of IRF-3, and formed the dimerization of IRF-3 (Fig. 5A,B, lanes 1 and 2), and that NS3-4A remarkably inhibited the phosphorylation and dimerization of IRF-3 in the cells treated with T-pIC, depending on its protease activity (Fig. 5A). However, the phosphorylation and dimerization of IRF-3 induced by M-pIC treatment was not inhibited by NS3-4A (Fig. 5B). From these results, we concluded that, in PH5CH8 cells, NS3-4A could not block the TRIF-mediated signaling pathway, although it could block the Cardif-mediated signaling pathway.

NS3-4A blocks the Cardif-mediated pathway by cleaving Cardif

NS3-4A is able to cleave the Cardif [24,34,35] and TRIF [36] molecules, resulting in the blocking of dsRNA-induced antiviral signaling pathways. However, our finding that IFN-β production was not suppressed by NS3-4A in cells treated with M-pIC seemed to contradict the finding of a previous study [36] in which NS3-4A-mediated cleavage of TRIF inhibited dsRNA-activated signaling through the TLR3 pathway. Therefore, we evaluated whether or not NS3-4A could impair the functional ability of

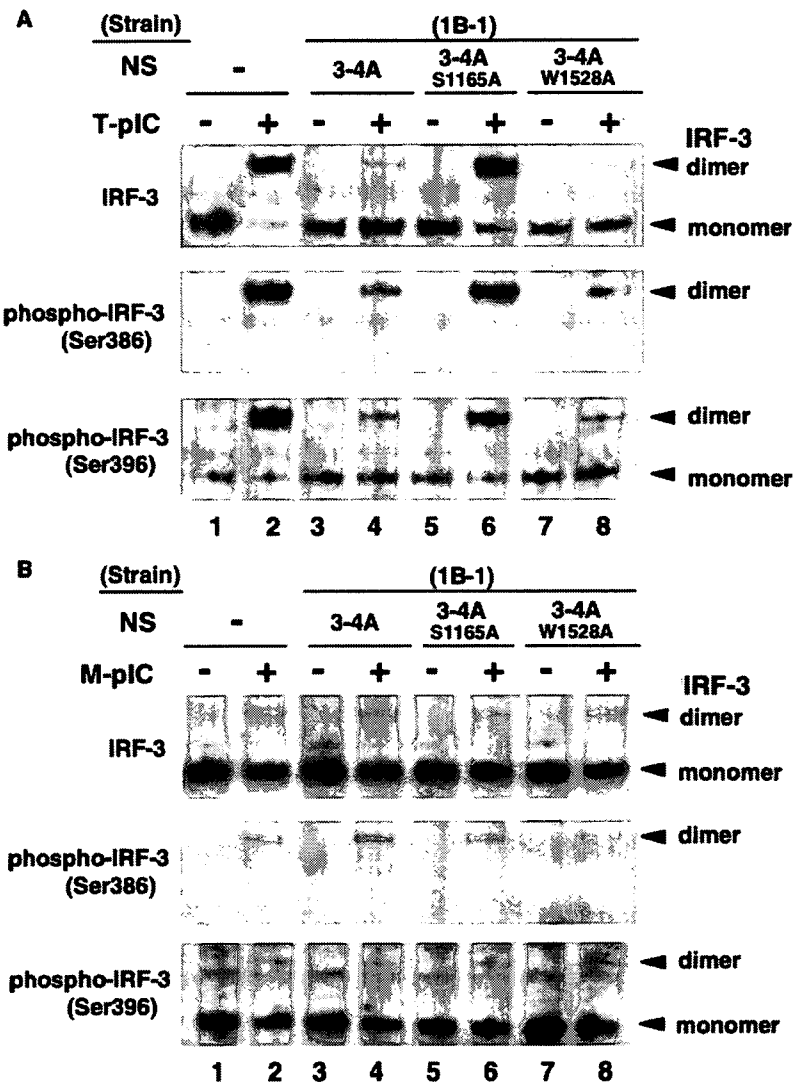

Fig. 5. Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in PH5CH8 cells treated with intracellular or extracellular dsRNA. PH5CH8 cells that were the same as in Fig. 4C were used. The poly(I-C) treatment was performed as described in Fig. 1. (A) Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in the PH5CH8 cells treated with T-pIC. The phosphorylation and dimerization analyses of IRF-3 were performed as described in Fig. 3C. Anti-phospho-IRF-3 (Ser396) serum was also used for the analysis. (B) Effects of NS3-4A on phosphorylation and dimerization of IRF-3 in the PH5CH8 cells treated with M-pIC. The phosphorylation and dimerization analyses of IRF-3 were performed as described in (A).

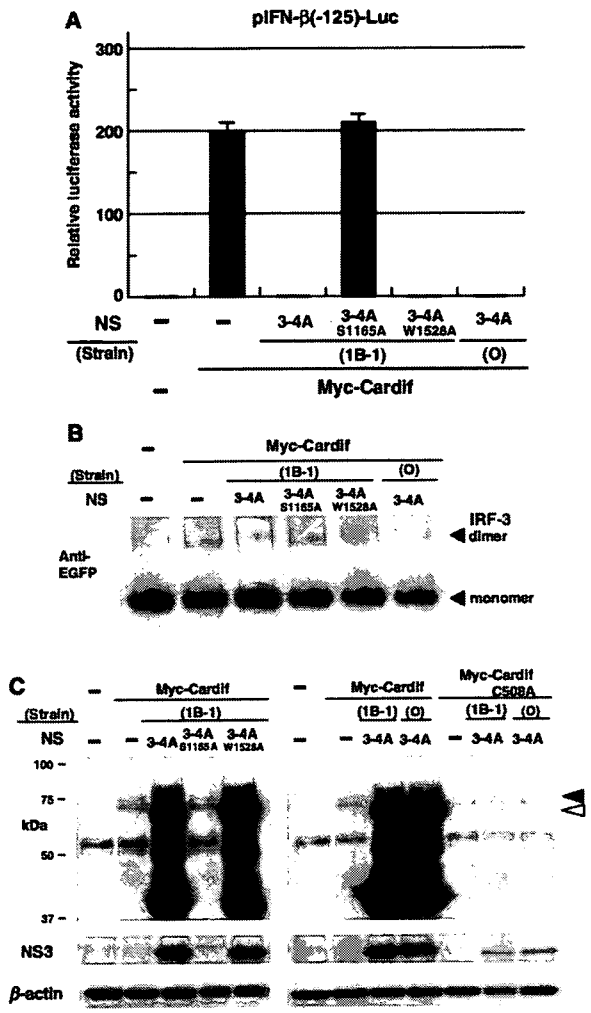

Fig. 6. NS3-4A blocks Cardif-mediated pathways by cleaving Cardif. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of Cardif in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, as described in Fig. 4, and the pCX4pur expression vector encoding myc-Cardif. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dual luciferase reporter assay was performed as described in Fig. 1A. (B) Effect of NS3-4A on IRF-3 dimerization induced by the ectopic expression of Cardif in PH5CH8 cells. The enhanced green fluorescent protein (EGFP)-IRF3 expression vector was used for the cotransfection in PH5CH8 cells with the myc-Cardif and NS3-4A (wild-type or its mutant S1165A or W1528A) expression vectors. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dimerization analysis of IRF-3 was preformed as described in Fig. 3C using anti-EGFP serum. (C) Cardif is cleaved by NS3-4A in PH5CH8 cells. PH5CH8 cells were cotransfected with the myc-Cardif (wild-type or its mutant C508A) and NS3-4A expression vectors (wild-type or its mutant S1165A or W1528A). Production of the myc-Cardif and NS3 in these cells was analyzed by immunoblotting using anti-myc and anti-NS3 sera, respectively. The PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors were used as a control (NS–). β-actin was used as a control for the amount of protein loaded per lane. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively.

TRIF as well as Cardif in PH5CH8 cells. First, we confirmed the effect of NS3-4A on the activation of the IFN-β gene promoter by the Cardif exogenously expressed in PH5CH8 cells. The results of the luciferase reporter assay revealed that NS3-4As (strains 1B-1 and HCV-O) completely suppressed the activation (200-fold induction) of the IFN-β gene promoter by Cardif, and that this suppression was dependent on the serine protease activity of NS3-4A (Fig. 6A). This result was supported by the results of the dimerization analysis of IRF-3 (Fig. 6B). Next, we confirmed that wild-type Cardif, but not the Cardif mutant (C508A located in the C-terminal region), was cleaved by the NS3-4As (strains 1B-1 and HCV-O), and that this cleavage was dependent on its serine protease activity (Fig. 6C). These results are in agreement with previous studies in which NS3-4A blocked the intracellular dsRNA signaling pathways through cleavage at the Cys508 residue of Cardif [24,34,35].

NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF

Because we demonstrated that NS3-4A blocked the intracellular dsRNA signaling pathways through cleavage of Cardif in PH5CH8 cells, we performed the same analysis regarding TRIF exogenously expressed in PH5CH8 cells. The results of the luciferase reporter assay using the IFN-β gene promoter revealed that NS3-4As (strains 1B-1 and HCV-O) could not suppress the activation (1000-fold induction) of the IFN-β gene promoter by TRIF (Fig. 7A). This result was also supported by the results of the dimerization analysis of IRF-3 (Fig. 7B). Furthermore, we demonstrated that the exogenously expressed TRIF was not cleaved by NS3-4As (strains 1B-1 and HCV-O) (Fig. 7C). These results indicate that NS3-4A could not block the TRIF-mediated signaling pathway, and suggest that NS3-4A did not suppress the M-pIC-induced production of IFN-β because NS3-4A did not have the ability to cleave TRIF.

To confirm the results obtained in PH5CH8 cells, we examined the status of Cardif and TRIF molecules expressed exogenously in the O cells replicating genome-length HCV-O RNA efficiently and their cured Oc cells. The results revealed that Cardif was cleaved in the O cells but not in the Oc cells (Fig. 8A,B), and that the cleavage of Cardif occurred

when NS3-4As (strains 1B-1 and HCV-O) were expressed in the Oc cells (Fig. 8B). From these results, we confirmed that NS3-4A could cleave Cardif in the O and Oc cells. In contrast, TRIF was not cleaved in either O or Oc cells (Fig. 8C). We further confirmed that TRIF was not cleaved in the O cells transfected with TLR3 siRNA, indicating that the resistance of TRIF to NS3-4A is not related to the presence of TLR3 (Fig. 8C). We also performed the same analysis using HeLa cells, and obtained results (supplementary Fig. S2) similar to those obtained in PH5CH8 cells (Figs 6C, 7C and 8). In addition, we observed that, like TRIF, exogenously expressed MDA5 and RIG-I were not cleaved by NS3-4A in PH5CH8 cells (data not shown). Taken together, the above results indicate that NS3-4A cleaves the Cardif molecule, resulting in interruption of the Cardif-mediated pathway, but NS3-4A is not able to cleave the TRIF molecule, and thus the TRIF-mediated pathway is not suppressed by NS3-4A.

Discussion

In the present study, we demonstrated that parental PH5CH cells and their clones retained both TRIF- and Cardif-mediated pathways as antiviral dsRNA signaling pathways, and confirmed that the PH5CH8 cell line was far more useful for the study of antiviral pathways than HuH-7 or the cell lines cloned from it. From the results of the present study and a previous study [41], we considered the possibility that immortalized hepatocyte cells possess the functional TRIF- and Cardif-mediated signaling pathways. Based on this assumption, we examined IFN-β production in three other immortalized human hepatocyte cell lines, NKNT-3 [52], IHH10.3 [53], and IHH12 [53], after treatment with poly(I-C). However, the results revealed that none of these immortalized cell lines responded to both M-pIC and T-pIC treatments. Therefore, we suggest that PH5CH and the cell lines cloned from it are uniquely suitable for the comprehensive study of antiviral TRIF- and Cardif-mediated signaling pathways.

We failed to obtain evidence that NS3-4A was able to cleave TRIF as reported by Li *et al.* [36]. In our study (Fig. 7C), there was no evidence of the cleavage of the TRIF molecule in NS3-4A-expressed PH5CH8

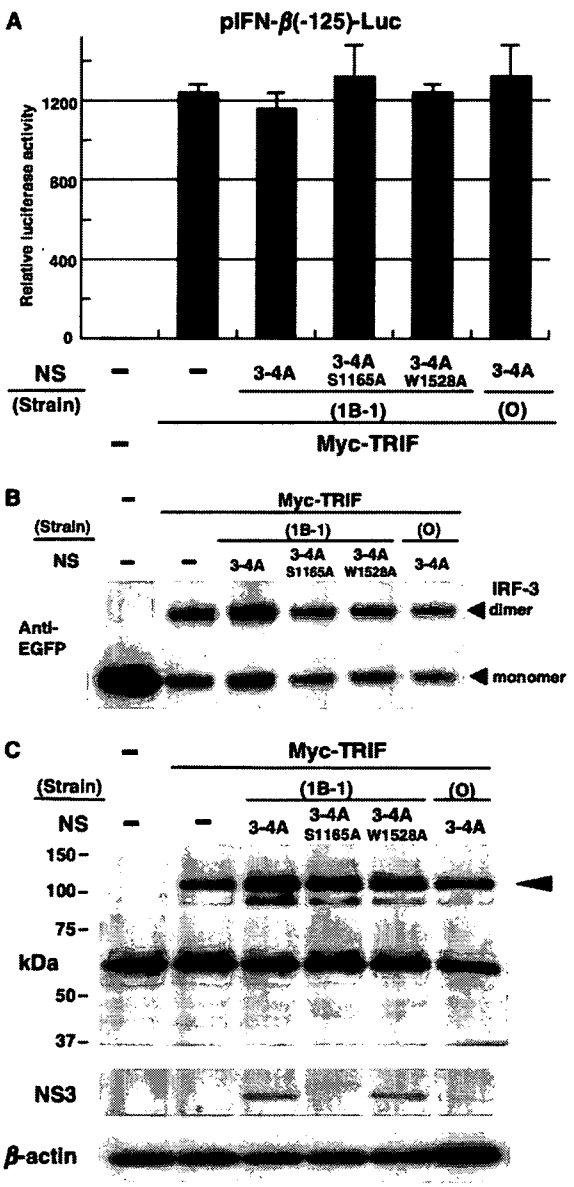

Fig. 7. NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of TRIF in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, and the pCX4pur expression vector encoding myc-TRIF. The lysate of PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors was used as a control (NS–). The dual luciferase reporter assay was performed as described in Fig. 1A. (B) Effect of NS3-4A on IRF-3 dimerization induced by the ectopic expression of TRIF in PH5CH8 cells. The dimerization analysis of IRF-3 was performed as described in Fig. 6B except that the myc-TRIF expression vector was used in place of the myc-Cardif expression vector. (C) TRIF is not cleaved by NS3-4A in PH5CH8 cells. PH5CH8 cells were cotransfected with the myc-TRIF and NS3-4A (wild-type or its mutant S1165A or W1528A) expression vectors. Production of myc-TRIF and NS3 in these cells was analyzed by immunoblotting using anti-myc and anti-NS3 sera, respectively, as described in Fig. 6C. The black arrowhead indicates the noncleaved TRIF.

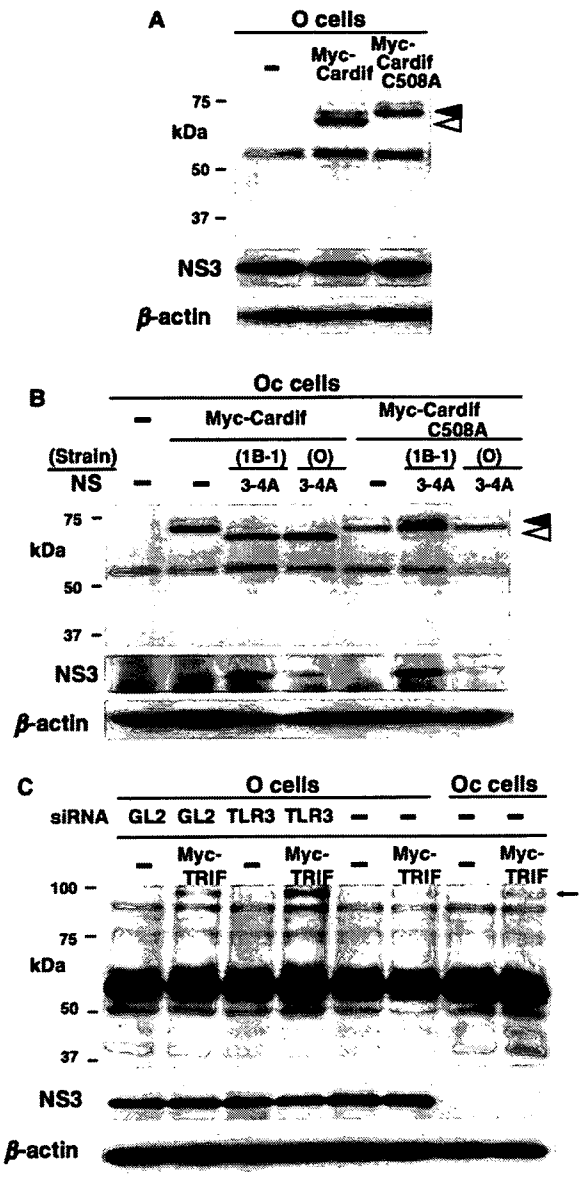

Fig. 8. TRIF is not cleaved in genome-length HCV RNA replicating cells. (A) Cardif is cleaved in the O cells replicating genome-length HCV-O RNA efficiently. The O cells were transfected with the myc-Cardif (wild-type or its mutant C508A) expression vector. Production of the myc-Cardif and NS3 in the O cells was analyzed by immunoblotting as described in Fig. 6C. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively. (B) Cardif is cleaved by NS3-4A in the cured Oc cells. The Oc cells were cotransfected with the myc-Cardif (wild-type or mutant C508A) and NS3-4A expression vectors. The production of the myc-Cardif and NS3 in these cells was analyzed by immunoblotting as described in Fig. 6C. The black and white arrowheads indicate Cardif and the cleaved Cardif, respectively. (C) TRIF is not cleaved in the O cells. The O and Oc cells were transfected with the myc-TRIF expression vector. The O cells transfected with GL2 or TLR3 siRNA were also used for the analysis. Production of myc-TRIF in these cells was analyzed by immunoblotting as described in Fig. 6C. The black arrowhead indicates the noncleaved TRIF.

cells. Nor did we observe any cleavage of TRIF by the NS3-4A expressed in the Oc cells, which exhibited almost no response to the T-pIC and M-pIC treatments (Figs 1 and 8C), or the HeLa cells, which exhibited a good response to the T-pIC and M-pIC treatments (supplementary Figs S1 and S2). We further observed that TRIF was not cleaved in the O cells, in which the HCV NS protein precursor was efficiently processed by NS3-4A (Fig. 8C). Regarding the cellular localization of NS3-4A, it has recently been reported that the localization of NS3-4A expressed transiently in HuH-7 cells was the same as that in genome-length HCV RNA replicating cells, and that NS3-4A was localized not only on the endoplasmic reticulum, but also on mitochondria [54]. From these findings, we suggest that NS3-4A is unable to cleave TRIF in cultured human cells.

Although amino acid sequences (PSSTPC/SAHLT, cleavage at Cys372; the P6 residue is underlined) surrounding the NS3-4A *trans*-cleavage site in TRIF [36] resemble those (DLEVVT/STWVL for NS3-4A; DEMEEC/ASHLP for NS4A/4B; DCSTPC/SGSWL for NS4B/5A; EDVVCC/SMSYS for NS5A/5B; the P6 residue is underlined) in the NS proteins from the 1B-1 and HCV-O strains and that (EREVPC/HRPSP, cleavage at Cys508; the P6 residue is underlined) in Cardif, only the TRIF site lacks the acidic P6 residue that is conserved and important in viral cleavage sites [55]. Accordingly, we examined whether or not a TRIF mutant (P to E at the P6 residue) is cleaved by NS3-4A in PH5CH8 cells. However, no cleavage of the TRIF mutant was observed (unpublished data). To clarify why TRIF is not cleaved by NS3-4A, further analysis will be necessary.

Although the results obtained in the present study suggest that the suppression of IFN-β production by NS3-4A is limited in human hepatocyte cells, it has recently been reported [56] that HCV can block the dsRNA-induced signaling pathway via the NS3-4A-independent pathway in addition to the NS3-4A-dependent pathway. However, because HuH-7 cells infected with the HCV genotype 2a clone (JFH1) were used in that study, it is not clear whether or not the TRIF-mediated pathway is also inhibited by the NS3-4A-independent pathway. To clarify this point, it will be necessary to study an HCV infection system using human hepatocyte cells in which both the TRIF- and

Cardif-mediated pathways are functional, such as PH5CH8 cells.

We clearly demonstrated that Cardif was cleaved by NS3-4As of 1B-1 and HCV-O strains obtained from healthy HCV carriers [57]. Although we observed that this cleavage was dependent on the protease activity of NS3-4A (Fig. 6), the correlation between the inhibitory effect of NS3-4A on the Cardif-mediated signaling pathway and the protease activity of NS3-4A remains unclear. Furthermore, we have no evidence that all NS3-4As derived from patients with HCV are able to cleave the Cardif molecule. To clarify these issues, further comparative analysis among HCV strains obtained from patients with different hepatic disease conditions will be needed. In addition, in the present study, we observed that the bands corresponding to the cleaved Myc-Cardif became extremely intense in PH5CH8 cells (Fig. 6C). This phenomenon has been observed in previous studies [24,34,49]. Although these previous studies did not explain what caused this phenomenon, we speculate that the cleaved Myc-Cardif is transferred to the cytosolic (soluble) fraction, although noncleaved Myc-Cardif remains in the membrane (insoluble) fraction. To clarify the reason for this phenomenon, several experiments may be needed.

In summary, we show that NS3-4A could not cleave TRIF, but could cleave Cardif, in PH5CH8 cells possessing functional TRIF- and Cardif-mediated antiviral signaling pathways, and suggest that the disruption of the IFN-β production system by NS3-4A is not sufficient in HCV-infected hepatocyte cells. This information will be useful for understanding the roles of NS3-4A in persistent HCV infection.

Experimental procedures

Cell culture

Non-neoplastic human hepatocyte PH5CH-derived cloned cells, including PH5CH8 cells, which are susceptible to HCV infection and supportive of HCV replication [45], were maintained as described previously [58]. HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum. The O cells replicating genome-length HCV RNA were cultured in DMEM with 10% fetal bovine serum and G418 (300 $\mu g \cdot mL^{-1}$; Geneticin, Invitrogen) as described previously [43]. The Oc and OR6c cured cells, which were created by eliminating genome-length HCV RNA from O cells [43] and OR6 cells [44] by IFN treatment, respectively, were also cultured in DMEM with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [59], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct the various expression vectors. pCX4bsr/NS3-4A(1B-1), pCX4bsr/NS3(1B-1) and pCX4bsr/NS4A(1B-1) were constructed according to the previously described method [60]. The DNA fragments encoding NS3-4A, NS3, and NS4A derived from the HCV 1B-1 strain belonging to genotype 1b (accession no. AB0802999) [61] were subcloned into the *Eco*RI and *Not*I sites of pCX4bsr. To construct pCX4bsr/NS3-4A(O), the DNA fragment encoding NS3-4A derived from the HCV-O strain belonging to genotype 1b [43] were also subcloned into the *Eco*RI and *Not*I sites of pCX4bsr. pCX4bsr/NS3-4A (1B-1)/S1165A and pCX4bsr/NS3-4A(1B-1)/W1528A were constructed by PCR mutagenesis with primers containing base alterations according to the previously described method [62]. To construct pCX4pur/myc-Cardif, the DNA fragment encoding Cardif (IPS-1/MAVS/VISA, accession no. DQ181928) was amplified from cDNAs obtained from PH5CH8 cells by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The primer sequences containing the *Sph*I (forward) and *Not*I (reverse) recognition sites for Cardif were designed to enable expression of the Cardif ORF. The obtained DNA fragment was subcloned into the *Sph*I and *Not*I sites of pCX4pur/myc, which can express myc-tagged protein, according to the previously described method [39]. To construct pCX4pur/myc-TRIF, the *Eco*RI-*Not*I fragment of pCXpur/myc-TRIF encoding myc-TRIF ORF [39] was subcloned into the *Eco*RI and *Not*I sites of pCX4pur. To construct pEGFP-C1/IRF-3, the DNA fragment encoding IRF-3 (accession no. NM_001571) was amplified by PCR as described above. The primer sequences containing the *Xho*I (forward) and *Hin*dIII (reverse) recognition sites for IRF-3 were designed to enable expression of the IRF-3 ORF. The obtained DNA fragment was subcloned into the *Xho*I and *Hin*dIII sites of pEGFP-C1 (Clontech, Mountain View, CA, USA), and the obtained pEGFP-C1/IRF-3 was used for IRF-3 dimerization analysis. The nucleotide sequences of these constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Poly(I-C) treatment

Poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium at 50 $\mu g \cdot mL^{-1}$ (M-pIC), or 1 μg of poly(I-C) was complexed with Lipofectamine™ 2000 (Invitrogen) for transfection (T-pIC). Cells were assayed for poly(I-C)-induced responses 6 h after exposure by either route.

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β-(−125)-Luc [63], containing the IFN-β gene promoter region (−125 to +19). The reporter assay was carried out as previously described [40]. Briefly, a total of 0.3×10^5 cells were seeded in a 24-well plate, 24 h before transfection. Then, 0.1 μg firefly luciferase reporter vector, 0.2–0.4 μg HCV protein expression plasmid (pCX4bsr series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA) as an internal control reporter were transfected into the various cell lines. To maintain the efficiency of transfection, up to 0.4 μg of pCX4bsr was added instead of HCV protein expression vectors. In some cases, 20 ng of pCX4pur/myc-Cardif or pCX4pur/myc-TRIF were added as the effector plasmid. 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) was added to the medium or transfected into the cells for 6 h before the reporter assay. Three independent triplicate transfection experiments were conduced 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.

Western blot analysis

Preparation of cell lysates, SDS/PAGE, and immunoblotting were performed as described previously [64]. Anti-NS3 (Novocastra Laboratories, Newcastle, UK), anti-myc (PL14; Medical and Biological Laboratories, Nagoya, Japan) or anti-β-actin serum (AC-15; Sigma, St Louis, MO, USA) was used in this study as a primary antibody. Immunocomplexes were detected by a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

IRF-3 dimerization analysis

Preparation of cell lysates and native-polyacrylamide gel electrophoresis were performed as described previously [65]. After the separation of proteins, immunoblotting was performed as described above. Anti-IRF3 serum (FL-425; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for the detection of the endogenous IRF-3 dimerization. Anti-phospho-IRF-3 (Ser386) serum (IBL, Gunma, Japan) and anti-phospho-IRF-3 (Ser396) serum (Upstate Biotechnology, Lake Placid, NY, USA) were used for detection of the phosphorylated IRF-3. The dimerization of exogenous IRF-3 was detected by anti-EGFP monoclonal serum (JL-8; Clontech).

Preparation of PH5CH8 cells stably expressing HCV proteins

PH5CH8 cells were infected with retrovirus pCX4bsr encoding various HCV proteins, as described previously [64]. pCX4bsr/NS3-4A(1B-1), pCX4bsr/NS3-4A(1B-1)/S1165A, and pCX4bsr/NS3-4A(1B-1)/W1528A were used to obtain the PH5CH8 cells stably expressing NS3-4A(1B-1), the NS3-4A(1B-1)/S1165A mutant lacking the serine protease activity [51], and the NS3-4A(1B-1)/W1528A mutant lacking the helicase activity [66], respectively. At 2 days postinfection, PH5CH8 cells were changed with fresh medium containing blasticidin (20 $\mu g \cdot mL^{-1}$), and the culture was continued for 7 days to select the cells expressing HCV proteins.

Real-time LightCycler PCR

Total cellular RNA was extracted using an Isogen extraction kit (Nippon Gene, Toyama, Japan). Before reverse transcription, the RNA was treated with RNase-free DNase I (TaKaRa Bio, Ohtsu, Japan) to completely remove the genomic DNA as described previously [40]. Real-time LightCycler PCR was performed according to a method described previously [67]. The sequences of sense and antisense primers for TRIF (accession no. AB093555) were 5′-AAGCCATGATGAGCAACCTC-3′ and 5′-GTGTCC TGTTCCTTCCTCCAC-3′. The sequences of sense and antisense primers for RIG-I (accession no. NM_014314) were 5′-AATGAAAGATGCTCTGGATTACTTG-3′ and 5′-TTGTCTCTGGGTTTAAGTGGTACTC-3′. The sequences of sense and antisense primers for MDA5 (accession no. NM_022168) were 5′-AAGTCATTAGTAAA TTTCGCACTGG-3′ and 5′-TCATCTTCTCTCGGAAAT CATTAAC-3′. In addition, we used primer sets for IFN-β [40], TLR3 [39], TLR4 [39], Cardif [24] and GAPDH [40].

RNA interference

siRNA duplexes targeting the coding regions of human TLR3 [39], TLR4 (Dharmacon, Lafayette, CO, USA; catalog no. M-008088-00), TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 [68] (Dharmacon) as a control were chemically synthesized. PH5CH8 cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen). Total RNA was extracted at 3 days after transfection, and real-time LightCycler PCR was performed to examine RNA-mediated interference efficiency as described above.

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Supplementary material

The following supplementary material is available online:

Fig. S1. NS3-4A blocked the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway in HeLa cells.

Fig. S2. NS3-4A is capable of cleaving Cardif, but not TRIF in HeLa cells.

Table S1. Quantitative RT-PCR analysis of mRNA expression of several factors involving in innate immune response in the various cell lines.

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Modulation of host metabolism as a target of new antivirals ☆

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Abstract

The therapy for chronic hepatitis C (CH–C) started with interferon (IFN) monotherapy in the early 1990s and this therapy was considered effective in about 10% of cases. The present standard therapy of pegylated IFN with ribavirin achieves a sustained virologic response in about 50% of patients. However, about half of the CH–C patients are still at risk of fatal liver cirrhosis and hepatocellular carcinoma. The other significant event in hepatitis C virus (HCV) research has been the development of a cell culture system. The subgenomic replicon system enables robust HCV RNA replication in hepatoma cells. And recently, the complete life cycle of HCV has been achieved using a genotype 2a strain, JFH1. These hallmarks have provided much information about the mechanisms of HCV replication, including information on the host molecules required for the replication. Anti-HCV reagents targeting HCV proteins have been developed, and some of them are now in clinical trials. However, the RNA-dependent RNA polymerase frequently causes mutations in the HCV genome, which lead to the emergence of drug-resistant HCV mutants. Some of the cellular proteins essential for HCV RNA replication have already been discovered using the HCV cell culture system. These host molecules are also candidate targets for antivirals. Here, we describe the recent progress regarding the anti-HCV reagents targeting host metabolism.
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Keywords: Hepatitis C virus; Replicon; Antiviral; Interferon; Host metabolism; Statin

Contents

Abbreviations: HCV, hepatitis C virus; CH, chronic hepatitis; HCC, hepatocellular carcinoma; IFN, interferon; SVR, sustained virological response; PEG-IFN, pegylated-IFN; GBV-B, GB virus B; uPA-SCID, urokinase plasminogen activator-severe combined immunodeficiency; NS, nonstructural; RdRp, RNA dependent RNA polymerase; CyPB, cyclophilin B; CsA, cyclosporine A; HSP90, heat shock protein 90; La, La auto antigen; PTB, polypyrimidine tract-binding protein; ALT, alanine aminotransferase; Neo, neomycin phosphotransferase; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; ORF, open reading frame; FKBP8, FK-506-binding protein 8; GGPP, geranylgeranyl pyrophosphate; FPP, farnesyl pyrophosphate; FTase, farnesyltransferase; GGTase-I, geranylgeranyl-transferase type I; GGTI, GGTase-I inhibitor; HMG–CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LOV, lovastatin; ATV, atorvastatin; FLV, fluvastatin; PRV, pravastatin; SMV, simvastatin; EC_{50}, 50%; effective concentration to inhibit HCV RNA replication; PTV, pitavastatin; RSV, respiratory syncytial virus; CMV, cytomegarovirus; HIV, human immunodeficiency virus; ICAM-1, integrin intercellular adhesion molecule 1; LFA-1, lymphocyte function associated antigen-1; DRM, detergent resistant membrane; SPT, serine palmitoyltransferase; ER, endoplasmic reticulum; GSL, glycosphingolipid; SBD, sphingolipid-binding domain; IMPDH, inosine monophosphate dehydrogenase; XMP, xanthosine 5′ ; monophosphate; MPA, mycophenolic acid; RMP, ribavirin monophosphate; RDP, ribavirin diphosphate; RTP, ribavirin triphosphate; GTP, guanosine triphosphate; SARS, severe acute respiratory syndrome; HBV, hepatitis B virus; VLP, virus-like particle; PIAS1, protein inhibitor of activated STAT1; PRMT1, protein arginine methyltransferase 1; PP2Ac, catalytic subunit of protein phosphatase 2A; AdoMet, S-adenosyl-L-methionine; PUFAs, polyunsaturated fatty acids; AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid.

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1. Introduction

Hepatitis C virus (HCV) was discovered in 1989 [1] as the causative agent of chronic hepatitis C (CH–C), liver cirrhosis and hepatocellular carcinoma (HCC) [2]. It is estimated that 170 million people worldwide are infected with HCV [3]. The ultimate goal of both clinical and basic HCV studies is the suppression of liver-related death caused by HCV infection. With respect to clinical studies, interferon (IFN) has played a major role in the treatment of patients with CH–C. IFN therapy started with IFN monotherapy in the early 1990s, and a sustained virologic response (SVR) was obtained in about 10% of patients [4]. IFN therapy was developed by the hepatologists, and the current therapy of pegylated IFN (PEG-IFN) with ribavirin has improved the SVR to about 50% [4]. Therefore, the next stage of the therapy for CH–C is to develop new anti-HCV reagents to improve the SVR.

During the development of IFN therapy, the most striking discovery in the basic research was the development of a cell culture system for robust HCV RNA replication. In 1999, Lohmann et al. [5] achieved subgenomic HCV RNA replication in a human hepatoma cell line, HuH-7. The advantages of this novel system (known as the replicon system) were that it provided not only a way to screen for anti-HCV reagents but also information about the mechanism of HCV RNA replication. This cell culture system has been further improved, and recently the complete life cycle of HCV was achieved using a genotype 2a HCV strain, JFH1 [6–8]. This newest system has extended the targets of the anti-HCV therapy to the virus infection and release.

The effects of anti-HCV reagents selected from the cell culture-based screening should be evaluated using an animal model system for HCV infection before they can be released to clinical trial. Chimpanzees were the only animal model in the early HCV studies [9]. However, the use of chimpanzees is limited for ethical and financial reasons. In addition to chimpanzees, a study using tree shrews (*Tupaia belangeri chinensis*) has been reported [10]. A different approach to the study of HCV using animal models was achieved using the related GB virus B (GBV-B). GBV-B belongs to the *Flaviviridae* family and can be transmitted to tamarins and marmosets [11,12]. These animal models may be valuable surrogate models for HCV study. Another approach was demonstrated in a study using urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mice transplanted with human hepatocytes [13]. This chimeric mouse model can support chronic HCV viremia under the circumstance without immune system. Mass screening for anti-HCV reagents using cell culture systems will become a more powerful tool when combined with small animal model systems to evaluate the antiviral effects of selected reagents before clinical trial.

In considering a new strategy for CH–C to be used in place of or in combination with IFN, the main targets are HCV proteins and HCV RNA. With respect to the HCV proteins, two of these, nonstructural (NS) 3-4A and NS5B, have been well-characterized as protease and RNA-dependent RNA polymerase (RdRp), respectively [14,15]. Several reagents have been reported to be inhibitors of NS3-4A serine protease, including SCH6 [16,17], SCH503034 [18], VX-950 [19,20], and BILN-2061 [21]. Valopicitabine (NM283) was reported to inhibit NS5B RdRp [22]. HCV RNA itself is also a target of antivirals, and recent RNA interference technologies using siRNA or shRNA have targeted HCV RNA [23–25]. As RdRp lacks proofreading activity, the high mutation rate of RdRp allows the virus to escape from the reagents targeting HCV proteins and HCV RNA. These anti-HCV reagent-targeting viral proteins and genome will be reviewed in another section.

Other targets are the cellular proteins essential for HCV RNA replication and infection. The expression of HCV proteins is thought to affect the host cells' gene expression profiles and vise versa [26]. The interaction of the specific cellular proteins with HCV proteins is essential for HCV replication (Table 1). Cyclosporine A (CsA) is one of the best characterized inhibitors targeting the cellular proteins required for HCV replication [27–36]. The interaction of cyclophilin B (CyPB) with NS5B is required for HCV RNA replication [28]. CsA inhibits HCV RNA replication by interrupting the interaction between NS5B and CyPB. Heat shock protein 90 (HSP90) has also been reported to be an essential cellular protein for HCV RNA replication [37–39]. Knockdown or inhibition of HSP90 has been shown to result in the anti-HCV activity in cell culture and in uPA-SCID mouse systems [37].

Table 1
Host molecules as targets of anti-HCV

Target molecules	Reagents	References
HMG–CoA reductase	Statin	[68–71]
Serine palmitoyltransferase	NA255	[81]
	Myriocin	[82]
IMP dehydrogenase	VX-497	[98]
	Ribavirin	[74,86]
	Mizoribin	[74]
	MPA	[97]
Protein arginin methyltransferase	AdoMet, Betaine	[102]
α-Glucosidase	Deoxynojirimycin	[101]
Cyclophilins	CsA	[27–36]
	NIM811	[27,33]
	DEBIO-025	[34]
HSP90	Geldanamycin, Radicicol	[37–39]
FKBP8		[38]
Unknown	PUFAs	[70,103,108]

FKBP8, a member of the FK506-binding protein family, specifically interacts with NS5A and forms a complex with HSP90 [38]. The La autoantigen (La) and polypyrimidine tract-binding protein (PTB) are also candidate cellular proteins for the inhibition of HCV RNA replication [40], although no inhibitors for these proteins have been reported to date. Thus, inhibition of the metabolism has recently been reported as a target of the new antivirals. Here, we survey the recent progress on enzyme inhibitors of the cholesterol, sphingolipid, and guanosine triphosphate (GTP) synthesis pathways, as well as other metabolic pathways.

2. Current standard therapy for chronic hepatitis C

HCV was discovered to be the causative agent of non-A, non-B hepatitis by the Chiron Corporation in 1989 [1]. However, a treatment for patients with non-A, non-B hepatitis was established before the discovery of HCV. In 1986, Hoofnagle et al. reported that IFN-α treatment normalized the serum alanine aminotransferase (ALT) levels in patients with non-A, non-B hepatitis [41]. Since the initial discovery of its anti-HCV activity, IFN-α has become the major reagent for CH–C treatment [4]. The replication of HCV RNA itself seems to stimulate IFN production signaling, and our recent results have suggested that core and/or NS5B induce IFN-stimulated genes [42–44]. However, viral NS3-4A protease inhibits the IFN production, although it does not completely shut it off. Therefore, exogenous IFN administration is needed for patients with CH–C. The SVR is affected by multiple factors, such as genotype, viral load and duration of therapy. IFN-α monotherapy was begun in the early 1990s, but an SVR was achieved in only about 10% of patients. In the early 2000s, IFN-α and ribavirin combination therapy was developed and the SVR was improved to about 30-40%. Furthermore, IFN itself has been modified by the attachment of PEG, thereby enhancing its stability in the blood. The SVR of the current standard therapy by PEG-IFN and ribavirin is as high as 50% [4]. In the current PEG-IFN and ribavirin combination therapy, the genotype of HCV is one of the major determinants of the SVR. HCV genotypes are classified into 6 groups, and genotype 1 is currently considered a problem due to its IFN resistance [45]. For example, in genotype 1 HCV, 12 months of treatment resulted in an SVR in 50% of patients, while in genotype 2, 6 months of treatment achieved an SVR of 80-90% [46]. The precise mechanisms of the IFN resistance remain unclear. However, the recently developed IFN-resistant HCV replicon-harboring cells will be useful for studies examining ways to improve the SVR [47–49]. Therefore, the focus in the treatment of patients with CH–C has shifted to increasing the SVR in genotype 1 HCV.

3. Cell culture-based HCV RNA-replication system

Before the development of an HCV replicon system, screening of anti-HCV reagents was rather difficult. The HCV replicon system developed by Lohmann et al. [5] was the first milestone in HCV study using a cell culture system. The replicon system has provided a wealth of information concerning the replication machinery of HCV. We can make strategies for the Achilles' heel of HCV based on the information regarding HCV RNA replication. The HCV replicon has been improved to be a suitable system for the screening of anti-HCV reagents by the introduction of reporter genes such as luciferase [50]. However, this system does not contain a structural region. Therefore, selectable genome-length HCV RNA-replicating cell culture systems have been developed [51–54]. The second milestone was the infectious virus production system established by the three groups using a genotype 2a HCV strain, JFH1 [6–8]. This system has extended the range of the HCV study to the viral entry and release. Therefore, the life cycle of HCV in the cells has been reconstructed *in vitro*. Since the development of the HCV replicon and infectious HCV production systems, many cellular proteins have been identified as essential host molecules for HCV RNA replication.

3.1. From HCV replicon to infectious HCV production

The HCV replicon reported by Lohmann et al. contained neomycin phosphotransferase (Neo) and encephalomyocarditis virus (EMCV) internal ribosome entry sites (IRES) instead of the HCV structural regions (Fig. 1) [5]. This HCV replicon consists of 2 cistrons. In the first cistron, Neo is translated by HCV-IRES and in the second cistron NS3-NS5B is translated by EMCV-IRES introduced in the region upstream of the NS region (Fig. 1). After the development of the HCV replicon system [52,53,55–58], genome-length HCV RNA replication systems using different HCV strains (H, N, Con1, and O) were developed by several groups [51–54]. In these genome-length HCV RNA replication systems, a complete open reading frame (ORF) of HCV was introduced into the second cistron instead of the NS region (Fig. 1).

For the mass screening for anti-HCV reagents, evaluation of the levels of HCV RNA or HCV proteins requires time and complicated procedures. To facilitate the monitoring of the replication level of HCV RNA, the reporter gene (Renilla luciferase) was fused to the Neo gene. In this system, anti-HCV activity was evaluated by the value of the reporter instead of the