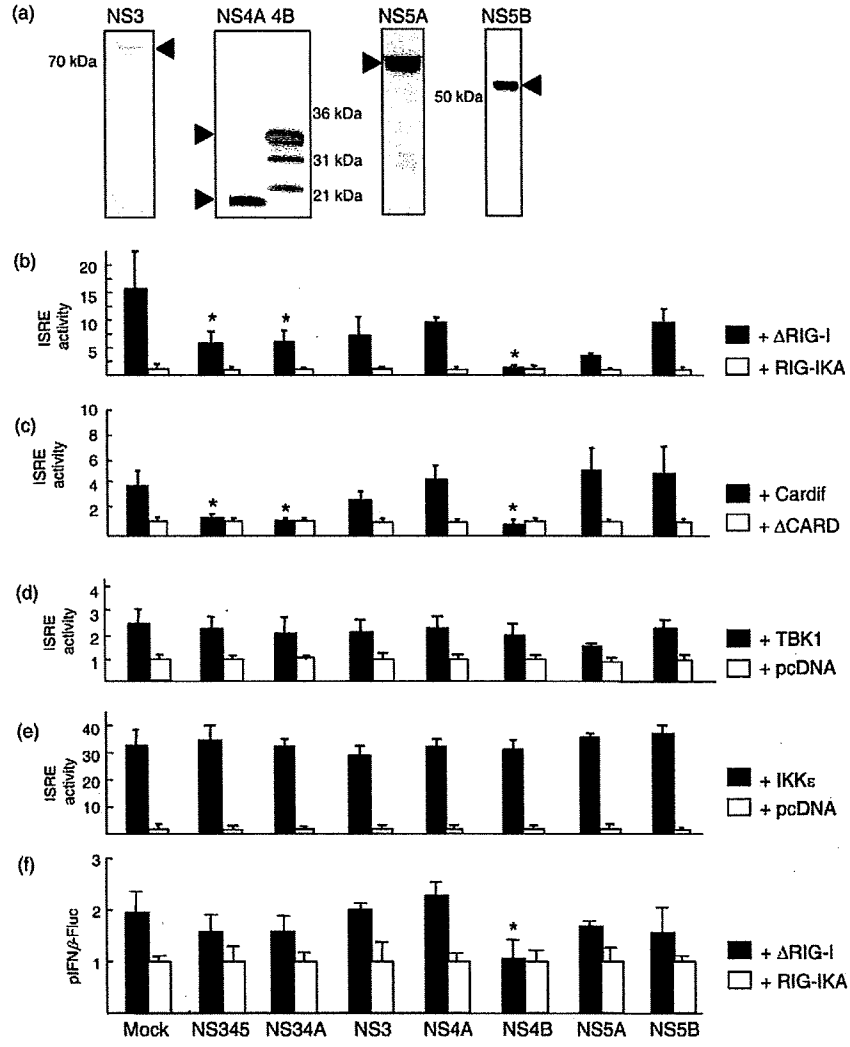

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, the indicated plasmids expressing ΔRIG-I (b), Cardif (c), TBK1 (d) and IKKε (e), and the indicated plasmids expressing individual HCV non-structural proteins. Plasmids RIG-IKA, ΔCARD or pcDNA were used as negative controls as indicated. Twenty-four hours after transfection, luciferase activities were measured. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (f) pIFN-β and pRL-CMV were co-transfected into Huh7 cells, with plasmids expressing individual HCV non-structural proteins and plasmid expressing ΔRIG-I. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. Plasmid RIG-IKA was used as a negative control.

To investigate strain-specific differences in the characteristics of NS4B proteins, we performed co-transfection assays using NS4B expression constructs from HCV N (Beard *et al.*, 1999) and JFH1 (Wakita *et al.*, 2005) strains, as well as HCV strain J4 (Fig. 4a and b). All NS4B constructs suppressed ΔRIG-I- or Cardif-mediated ISRE activation. These results suggested that the above-described effects of NS4B were independent of HCV strain.

NS4B has been reported to induce an unfolded protein response or endoplasmic reticulum (ER) stress through ATF6 or IRE1-X box protein (XBP1) pathways (Zheng *et al.*, 2005). The ER stress induces production of IL-8, which has been reported to interfere with the IFN system (Polyak *et al.*, 2001). Therefore, we detected expression of IL-8 using RT-PCR in cells with and without overexpression of NS4B. As shown in Fig. 4(c), no significant difference was observed in IL-8 mRNA levels among mock-, NS34A- and NS4B-transfected cells. These results showed that NS4B overexpression in the present study did not induce expression of IL-8 and that the IFN-antagonizing effects of NS4B were independent of IL-8.

It has been reported that NS34A suppresses the TLR3-mediated IFN response (Breiman *et al.*, 2005; Ferreon *et al.*, 2005). However, overexpression of HCV non-structural proteins did not suppress ISRE activation that was induced by overexpression of TLR-3 or TRIF (Fig. 5a and b), nor did NS34A from two different HCV strains, J4 and N, show significant suppression of TRIF-mediated ISRE activation (Fig. 5c). Although strain-specific differences might be involved, these data suggest that neither NS34A nor NS4B affect the TLR3-triggered, TRIF-mediated IFN expression signalling pathway.

The NS4B N terminus is involved in inhibition of the RIG-I-mediated pathway

Given the result that NS4B suppressed the RIG-I-mediated IFN expression pathway, we next investigated which domain of NS4B was responsible. We constructed plasmids that expressed truncated NS4B in which the protein-coding frame was truncated at four positions corresponding to the five transmembrane domains (Lundin *et al.*, 2003) (Fig. 6a).

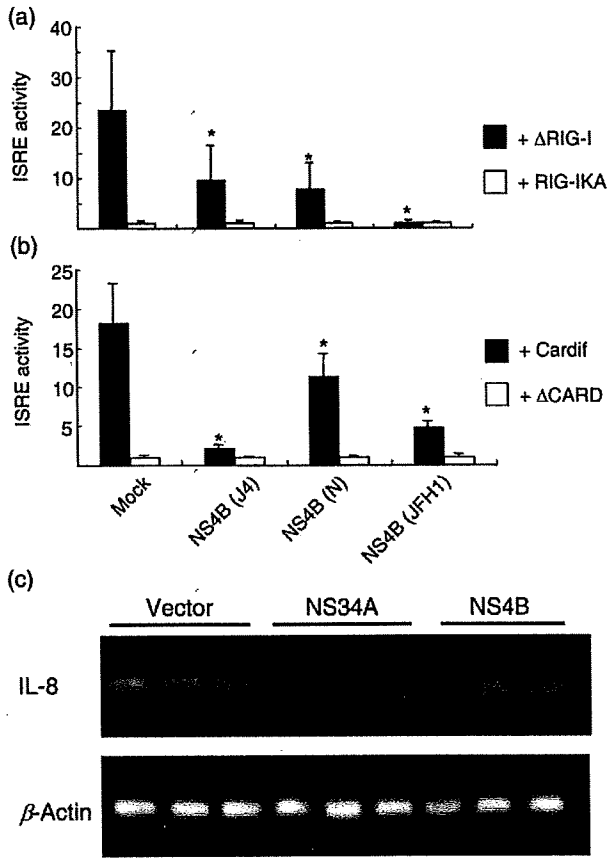

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. *, $P<0.05$. (c) Semi-quantitative detection of IL-8 mRNA by RT-PCR. cDNA was prepared from Huh7 cells transfected with empty vector or with NS34A or NS4B expression plasmid.

Expression and subcellular localization of NS4B truncated proteins were visualized by indirect immunofluorescence assays (Fig. 7). Each of the NS4B truncated proteins was localized predominantly to the perinuclear rim as dense spots. Some of the spots were similar to the staining of the ER-resident host protein PDI, consistent with previous reports (Lindström *et al.*, 2006; Lundin *et al.*, 2006). These truncated expression plasmids were co-transfected with Cardif expression plasmids into Huh7 cells. As shown in Fig. 6(b), Cardif-mediated ISRE activation was significantly suppressed by co-transfection of NS4Bt1–156 and NS4Bt1–186, as well as full-length NS4B, whilst transfection of NS4Bt90–260 and NS4Bt110–260 did not significantly suppress Cardif-mediated ISRE activation. The shortest construct, NS4Bt131–260, partially retained the ability to reduce ISRE activity. These results suggested that the N-terminal domain of NS4B, which includes aa 1–110, might function directly to suppress RIG-I-mediated IFN expression responses.

DISCUSSION

The recent discovery of cytoplasmic dsRNA sensor molecules has resulted in rapid expansion of knowledge about the IFN-mediated virus defence pathway (Yoneyama *et al.*, 2004). Several reports suggest that viruses target the IFN system to establish replication in the host cells (Kato *et al.*, 2006). We have confirmed that the dsRNA-triggered, IRF-3-mediated IFN activation pathway was blocked in several replicon-supporting cell lines (Fig. 1). Similarly, the dsRNA responses were substantially suppressed in HCV JFH-1 cell culture compared with parental Huh7 cells (Fig. 2b and c). Overexpression analyses showed that RIG-I- and Cardif-mediated ISRE activation was significantly suppressed in HCV replicon-expressing cells, which recovered after elimination of the replicon by IFN treatment (Fig. 2a). In contrast, TBK1- or IKKε-mediated ISRE activation was not suppressed in replicon-expressing cells. Overexpression of individual HCV non-structural proteins revealed that not only NS34A but also NS4B inhibited the ISRE activation signal (Figs 3, 4 and 5). These results suggested that HCV non-structural proteins suppress the IFN induction pathway and that the target host molecule could be Cardif or an unknown adaptor molecule acting between Cardif and TBK1/IKKε.

NS4B protein is a 27 kDa hydrophobic integral membrane protein that is localized in the ER with other non-structural proteins. Studies on other flaviviruses such as Kunjin virus and BVDV support the notion that NS4B may indeed be an essential part of the replication mechanism (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000; Li & McNally, 2001; Qu *et al.*, 2001). These systems have demonstrated that intact NS4B is necessary in a *cis* configuration in the polyprotein for maintaining viral replication (Grassmann *et al.*, 2001; Khromykh *et al.*, 2000). Furthermore, single mutations in NS4B alter the cytopathic effects of BVDV and even mediate changes in the cellular tropism of Dengue virus (Hanley *et al.*, 2003; Qu *et al.*, 2001). In HCV, the search for cell-culture-adaptive mutations in HCV subgenomic replicons has led to the generation of mutations in the NS4B region that confer higher replication levels and resistance to IFNs, as well as broadening the tropism for different cell lines (Lohmann *et al.*, 2003; Sumpter *et al.*, 2004; Zhu *et al.*, 2003). These pieces of evidence may imply that NS4B is not only part of the replication machinery but may also have other functions that enable establishment of viral replication.

NS4B truncation assays showed that RIG-I/Cardif-mediated ISRE activation was significantly suppressed by expression of N terminus-containing constructs (Fig. 6). These results imply that the N-terminal domain of NS4B, which is located between positions 1 and 110, may be essential for suppressing IFN expression responses in host

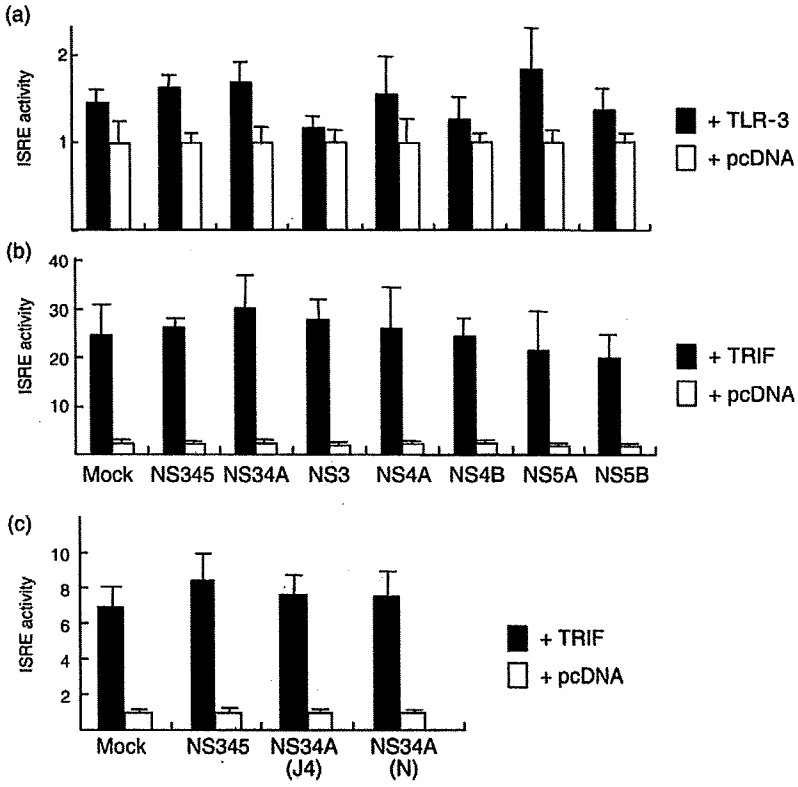

Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD. (c) NS34A (J4) and NS34A (N) denote plasmids expressing HCV NS34A derived from HCV strains J4 and N, respectively. The NS4B plasmids indicated were co-transfected with pISRE-TA-Luc, pRL-CMV and plasmids expressing TRIF or pcDNA. Luciferase activities were measured 24 h after transfection. The *y*-axis shows relative values. Assays were carried out in triplicate and results are given as means ± SD.

cells. Lindström *et al.*, (2006) investigated single point mutations in NS4B that negatively affected expression efficiency of the HCV replicon and reported that most of the active mutations were located around the N-terminal domain. A distinctive feature of NS4B is that it requires membrane rearrangement to form its native structure

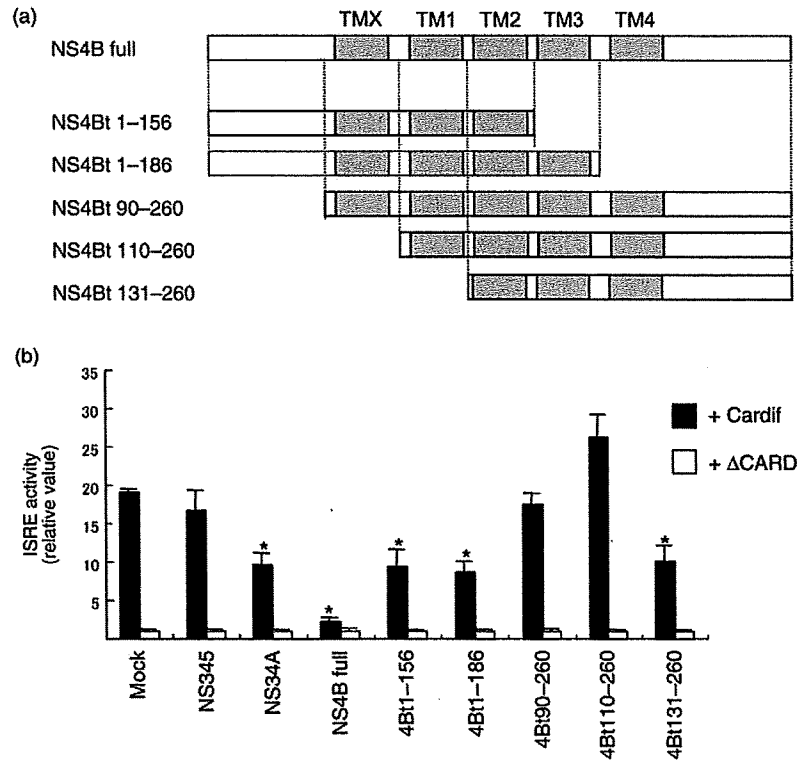

Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pISRE-TA-Luc and the Cardif- or ΔCARD-expressing plasmids indicated were co-transfected into Huh7 cells. Luciferase activities were measured 24 h after transfection. Results are given as means ± SD.

(Lindström *et al.*, 2006; Lundin *et al.*, 2006). The HCV polyprotein is translated from a single reading frame and subjected to proteolytic cleavage by the host signal peptidase and two viral proteases (Grakoui *et al.*, 1993). The mature form of NS4B is localized in the ER and constitutes a subcellular structure called the membrane-associated focus (MAF) (Gretton *et al.*, 2005). Once the NS4B is cleaved, the N-terminal peptide of NS4B is translocated from the cytoplasmic to the luminal side, giving it a fifth transmembrane region (Lundin *et al.*, 2006). The N-terminal amphipathic helix (AH) 1 of NS4B is necessary for this translocation and for MAF formation; NS4B molecules that were truncated at the AH1 lacked the ability to create the MAF, to translocate and to replicate (Elazar *et al.*, 2004; Lindström *et al.*, 2006).

In our assay, NS4Bt131–260 regained the ability to reduce ISRE activity. As we confirmed that all mutants co-localized with the ER, there may be some effect of the N-terminal localization of this mutant. The precise mechanism of NS4B suppression is still not clear and further experiments are needed.

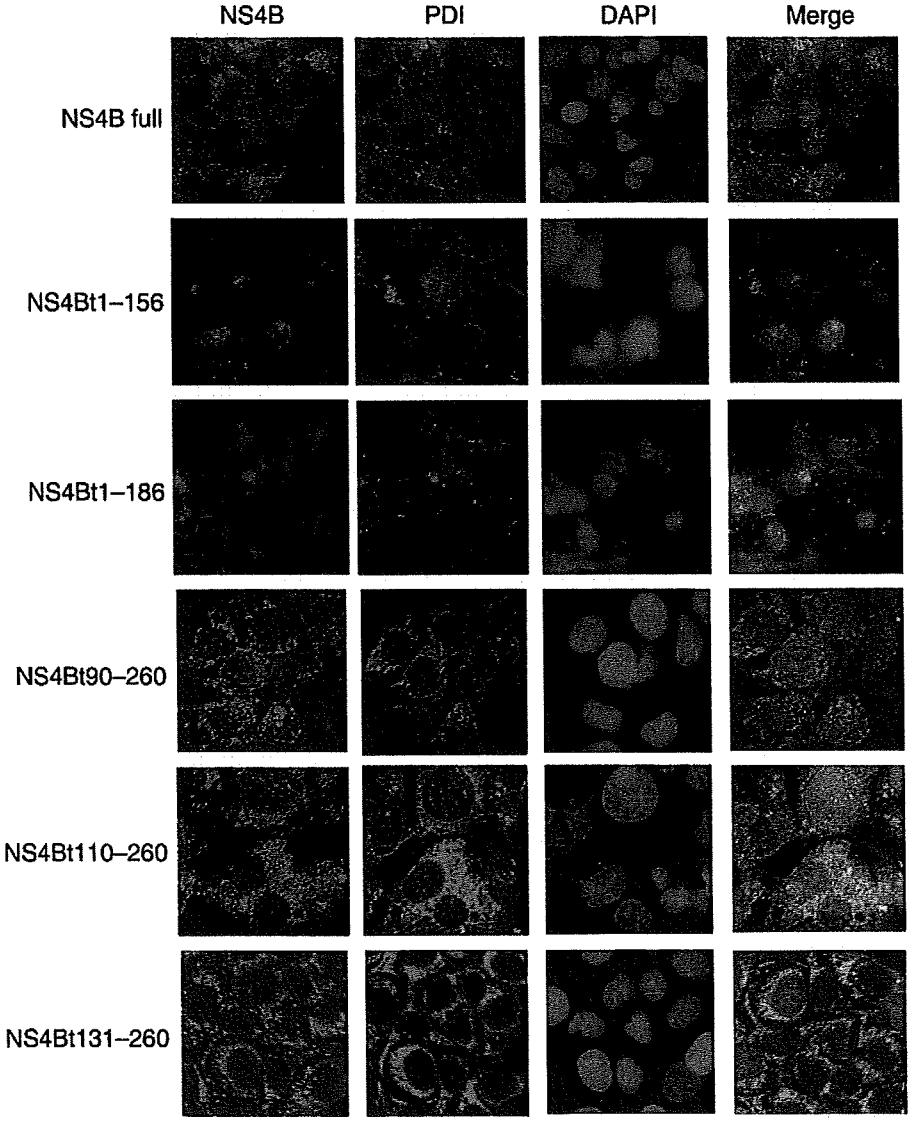

Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG, whilst PDI was stained with Alexa Fluor 568-labelled goat anti-rabbit IgG. DAPI staining revealed the nuclear chromatin. Representative immunofluorescence images derived from a number of experiments are shown as four images of a single focal plane of Huh7 cells, showing NS4B proteins (green), PDI (red), DAPI staining (blue) and the superimposed images (merge).

NS4B has been reported to induce an unfolded protein response or ER stress (Zheng *et al.*, 2005). Accumulation of unfolded or misfolded proteins in the ER is detected by three ER sensor proteins, ATF6, IRE-1 and PKR-like ER kinase (PERK), and triggers the unfolded protein response as a stress response and induces expression of molecular chaperon proteins, global shut-off of protein translation and apoptotic cell death (Mori, 2000). Therefore, it may be possible that transgenic overexpression of NS4B induces ER stress and suppresses overall protein synthesis, including that of IFNs. In our experiments, however, NS4B suppressed the ISRE-mediated IFN gene activation but did not suppress non-specific protein expression, as demonstrated by *Renilla* luciferase activity in the control plasmid driven by the herpes simplex thymidine kinase promoter. In addition, the growth and viability of cells that overexpressed NS4B did not differ from untransfected cells or from those transfected with the other HCV proteins. IL-8 overproduction induced by ER stress was not observed in our NS4B-overexpressing cells (Fig. 4c). These findings indicated that the inhibitory effect of NS4B is specific to the IFN induction pathway and is not a non-specific effect through ER stress.

In conclusion, we have shown that dsRNA-induced IFN expression was suppressed by NS4B. These virus–host interactions probably contribute to HCV persistence and to the pathogenesis of HCV-associated liver disease.

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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)

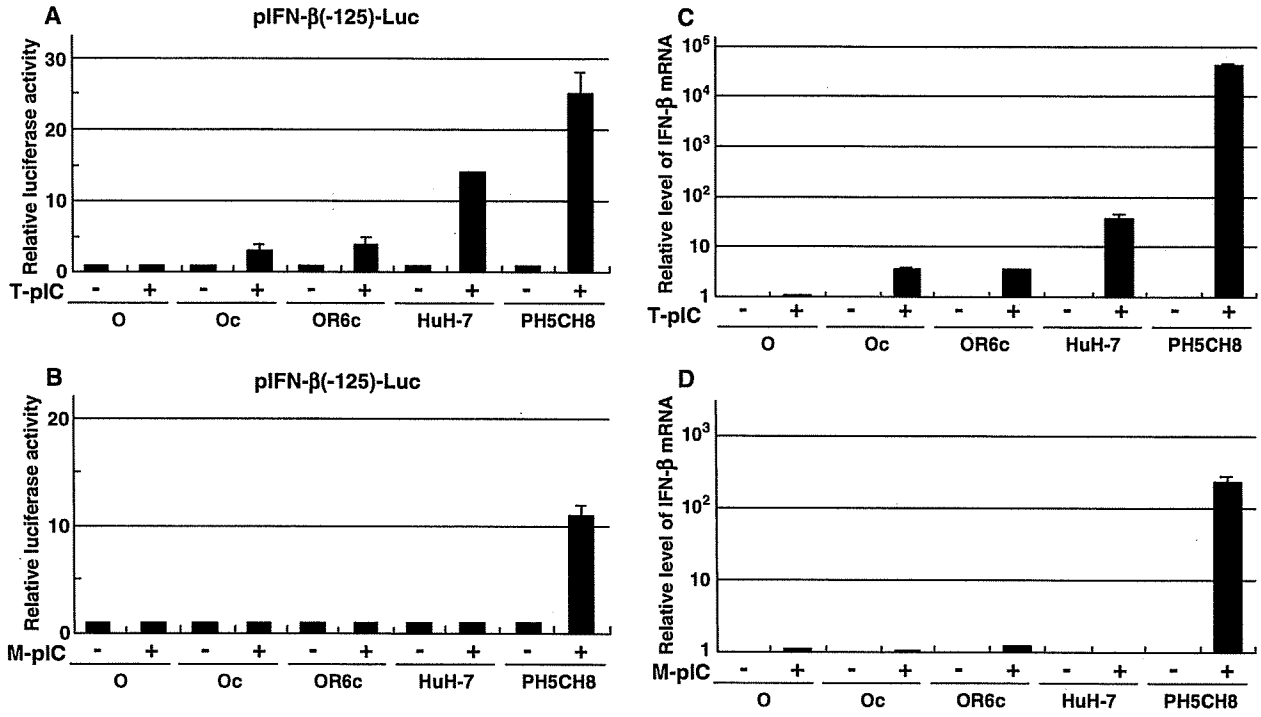

Fig. 1. PH5CH8 cells show high-level IFN-β production in response to dsRNA. (A) Dual luciferase reporter assay of the IFN-β gene promoter using the various cells treated with T-pIC. The following HuH-7-derived cell sublines were used: O, cloned cells [43] replicating genome-length HCV RNA; Oc, cured 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

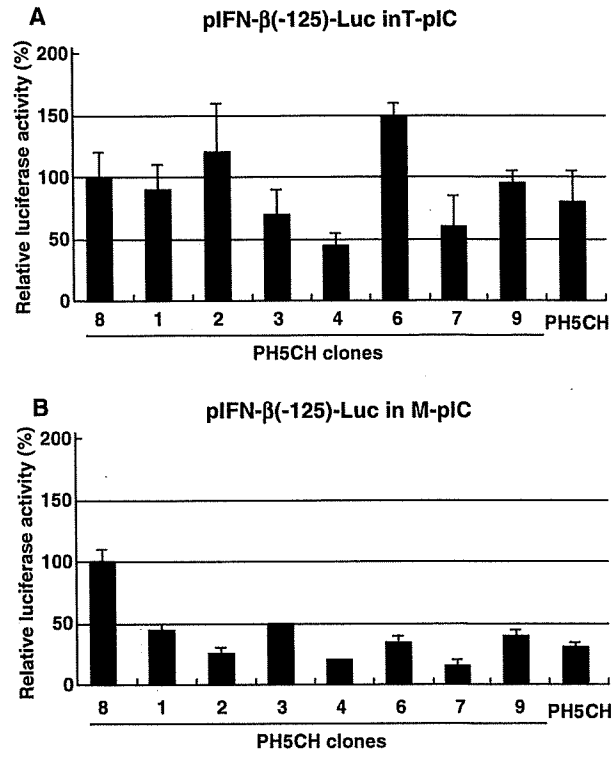

Fig. 2. IFN-β responses of parental PH5CH and PH5CH cloned cells by dsRNA treatment. (A) Dual luciferase reporter assay of the IFN-β gene promoter using parental PH5CH and PH5CH cloned cells treated with T-pIC. The T-pIC treatment and the dual luciferase reporter assay were performed as described in 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

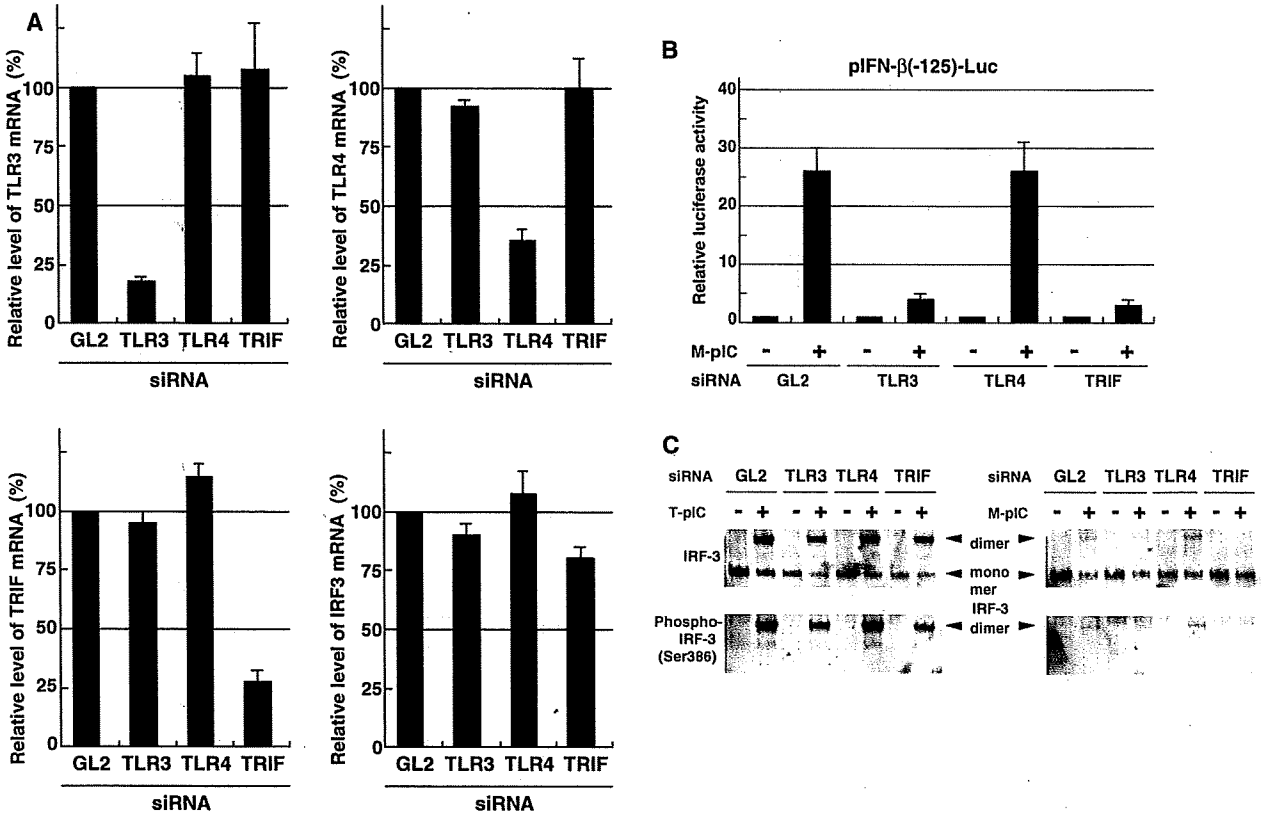

Fig. 3. Extracellular dsRNA treatment activates IRF-3 through the TLR3/TRIF signaling pathway in PH5CH8 cells. (A) Down-regulation of TLR3, TLR4, and TRIF mRNAs by transfection of TLR3, TLR4, and TRIF siRNAs, respectively. PH5CH8 cells were transfected with dsRNA duplexes targeting TLR3, TLR4, TRIF or 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,

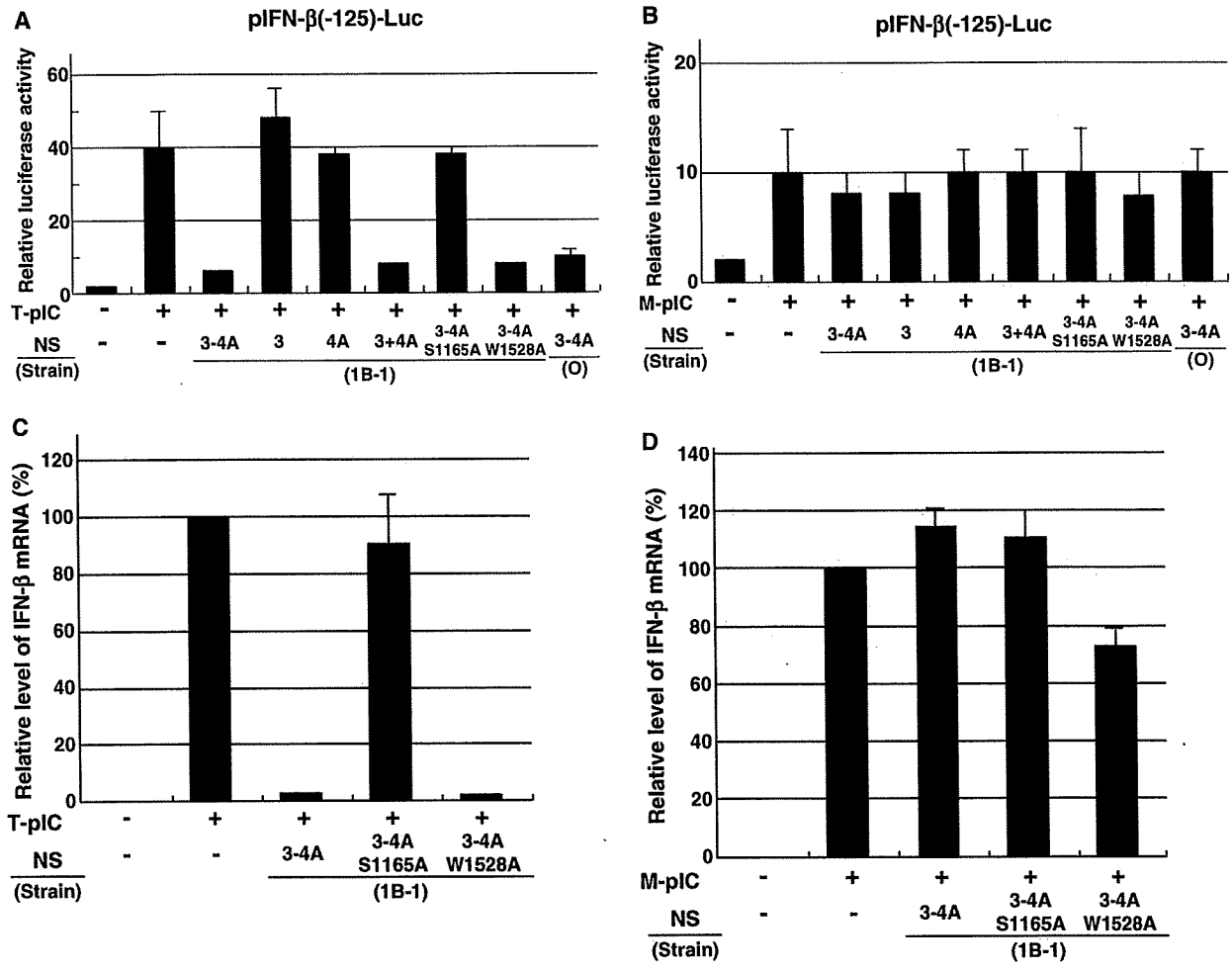

Fig. 4. NS3-4A blocked the Cardif-mediated signaling pathway, but not the TRIF-mediated signaling pathway. The poly(I-C) treatment, dual luciferase reporter assay, and quantitative RT-PCR analysis were performed as described in Fig. 1. The pCX4bsr expression vectors encoding NS3-4A, NS3, or NS4A from 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-pIC treatment. (B) Effect of NS3-4A on the IFN-β gene promoter activated by M-pIC treatment. (C) Effect of NS3-4A on the IFN-β mRNA induction by T-pIC treatment. PH5CH8 cells stably expressing the NS3-4A or NS3-4A mutant (S1165A or W1528A) from the 1B-1 strain were subjected to T-pIC 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-pIC, which was set at 100. (D) Effect of NS3-4A on the IFN-β mRNA induction by M-pIC treatment. PH5CH8 cells that were the same as in (C) were subjected to M-pIC treatment. The IFN-β mRNA level was calculated relative to the level in the control PH5CH8 cells treated with M-pIC, 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-pIC 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-pIC or M-pIC 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

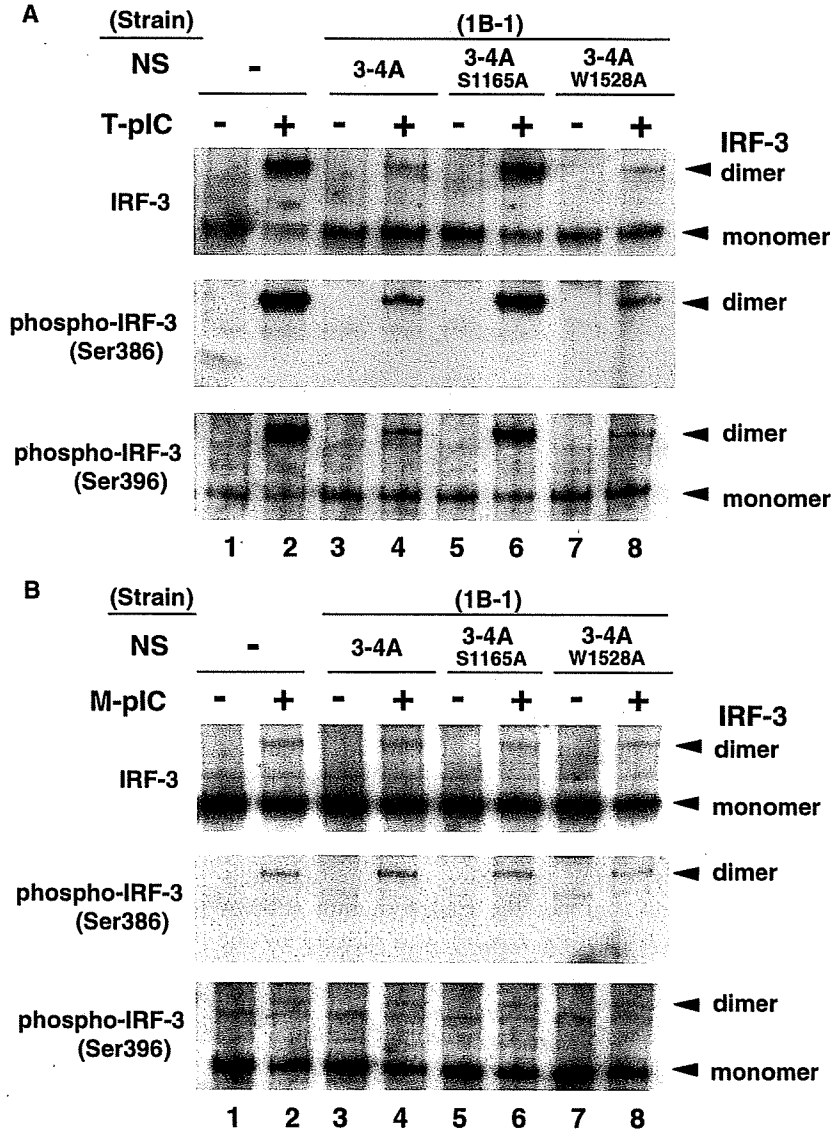

Fig. 5. Effect of NS3-4A on phosphorylation and dimerization of IRF-3 in PH5CH8 cells treated with intracellular or extracellular dsRNA. PH5CH8 cells that were the same as in Fig. 4C were used. The poly(I-C) treatment was performed as described in Fig. 1. (A) Effect of NS3-4A on phosphorylation and dimerization 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).

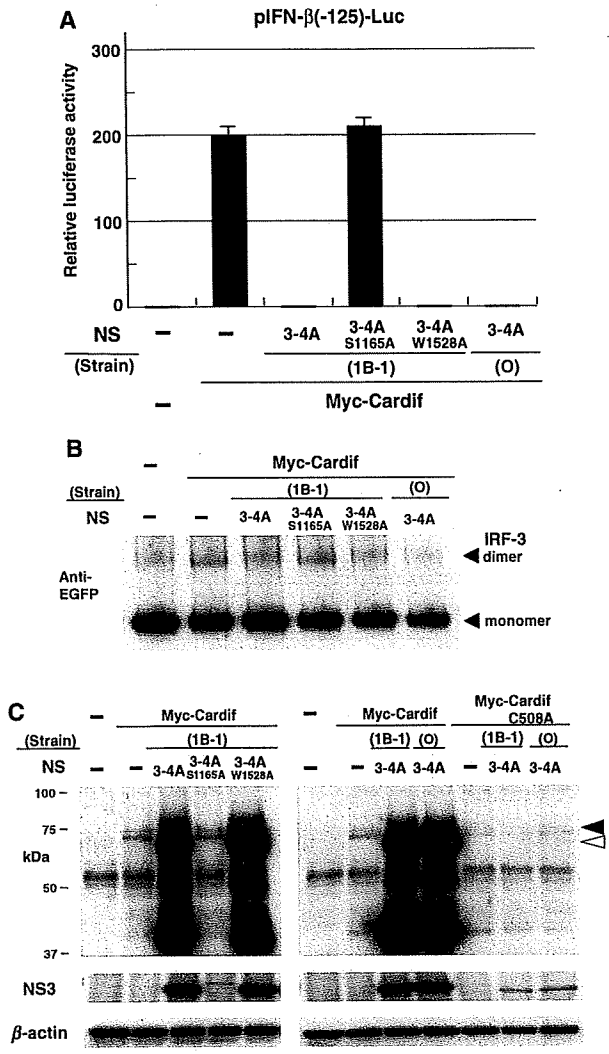

Fig. 6. NS3-4A blocks Cardif-mediated pathways by cleaving Cardif. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of Cardif in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant S1165A or W1528A, as described 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

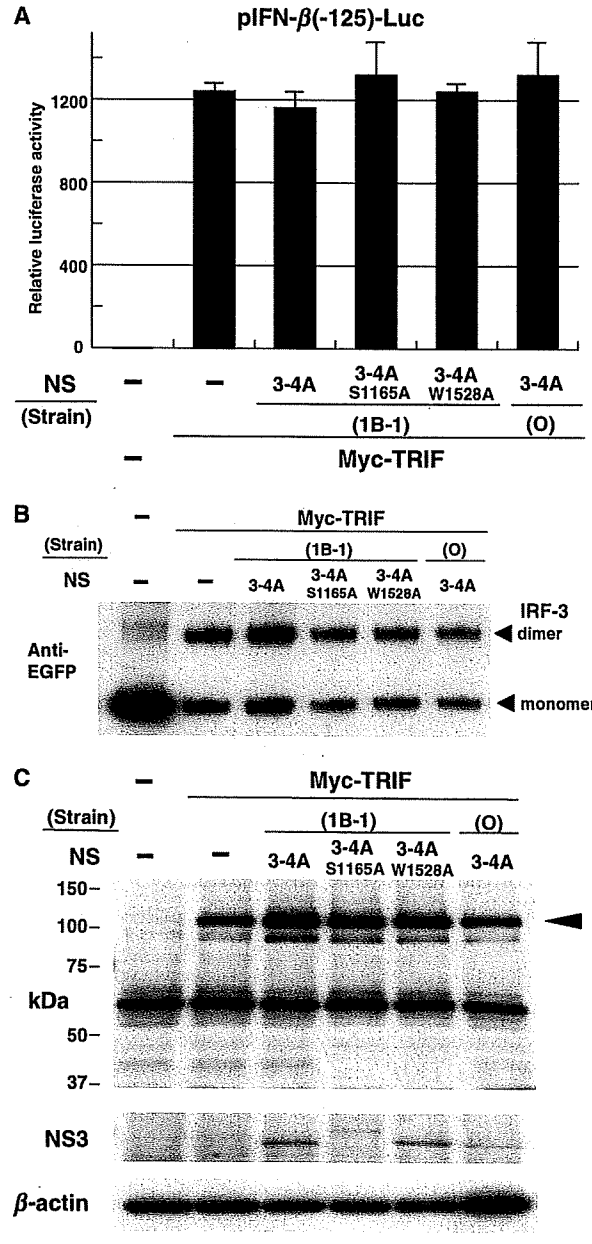

Fig. 7. NS3-4A does not block the TRIF-mediated pathway because it lacks the ability to cleave TRIF. (A) Effect of NS3-4A on the IFN-β gene promoter activated by the ectopic expression of TRIF in PH5CH8 cells. PH5CH8 cells were cotransfected with the pCX4bsr expression vector encoding NS3-4A or its mutant 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.

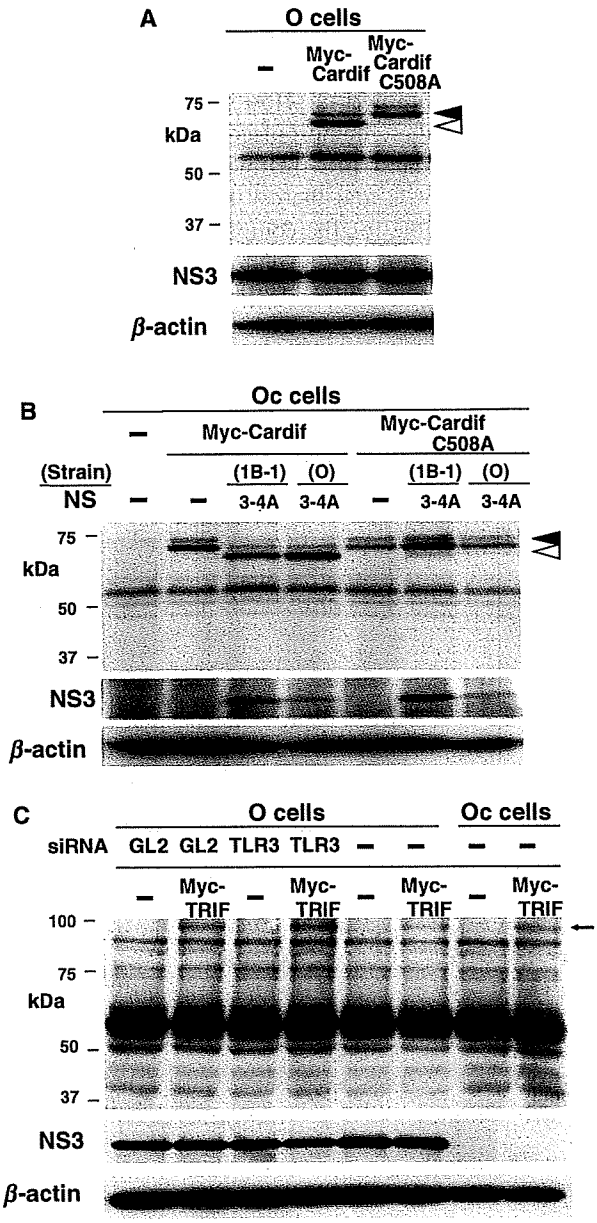

Fig. 8. TRIF is not cleaved in genome-length HCV RNA replicating cells. (A) Cardif is cleaved in the O cells replicating genome-length HCV-O RNA efficiently. The O cells were transfected with the myc-Cardif (wild-type or its mutant C508A) expression vector. Production of the myc-Cardif and NS3 in the 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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