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# Distinct Poly(I-C) and Virus-activated Signaling Pathways Leading to Interferon-β Production in Hepatocytes*

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**Innate cellular antiviral defenses are likely to influence the outcome of infections by many human viruses, including hepatitis B and C viruses, agents that frequently establish persistent infection leading to chronic hepatitis, cirrhosis, and liver cancer. However, little is known of the pathways by which hepatocytes, the cell type within which these hepatitis agents replicate, sense infection, and initiate protective responses. We show that cultured hepatoma cells, including Huh7 cells, do not activate the interferon (IFN)-β promoter in response to extracellular poly(I-C). In contrast, the addition of poly(I-C) to culture media activates the IFN-β promoter and results in robust expression of IFN-stimulated genes (ISG) in PH5CH8 cells, which are derived from non-neoplastic hepatocytes transformed with large T antigen. Small interfering RNA knockdown of TLR3 or its adaptor, Toll-interleukin-1 receptor domain-containing adaptor inducing IFN-β (TRIF), blocked extracellular poly(I-C) signaling in PH5CH8 cells, whereas poly(I-C) responsiveness could be conferred on Huh7 hepatoma cells by ectopic expression of Toll-like receptor 3 (TLR3). In contrast to poly(I-C), both cell types signal the presence of Sendai virus infection through a TLR3-independent intracellular pathway requiring expression of retinoic acid-inducible gene I (RIG-I), a putative cellular RNA helicase. Silencing of RIG-I expression impaired only the response to Sendai virus and not extracellular poly(I-C). We conclude that hepatocytes contain two distinct antiviral signaling pathways leading to expression of type I IFNs, one dependent upon TLR3 and the other dependent on RIG-I, with little cross-talk between these pathways.**

The innate immune system represents the first line of defense against viral infections in mammalian cells. It senses invading viral pathogens and initiates signaling pathways leading to the induction of protective cellular genes, including type I interferons (IFN-α and IFN-β)[1] and proinflammatory cytokines that directly limit viral replication and also help to shape subsequent adaptive immune responses (1, 2). Recognition of conserved molecular structures that are expressed by large groups of pathogens (pathogen-associated molecular patterns, PAMPs) is carried out by specific pattern recognition receptor (PRR) molecules (3, 4). The Toll-like receptors (TLRs) are a class of PRRs that have been shown to detect infection by many types of pathogens, including viruses (5). TLR3 is engaged specifically by double-stranded (ds) RNA that is present either in viral genomes or generated during viral replication, and is involved in the cellular recognition of RNA viruses and induction of type I IFN responses (6). However, several recent studies indicate that viral infection can also activate host responses through TLR3-independent pathways (7–9). Mouse TLR7 and human TLR8, which are expressed within endosomal membranes, detect GU-rich viral single-stranded RNA (7, 10, 11). Whereas TLR3 signaling requires the adaptor protein, Toll-IL1 receptor (TIR) domain-containing adaptor inducing IFN-β (TRIF/TICAM-1) (12–15), TLR7/8 engagement utilizes the MyD88 adaptor protein and requires endosomal acidification for activation of IFN responses (7, 10). Finally, virally encoded proteins may engage TLR2 or TLR4, activating MyD88-dependent pathways leading to expression of inflammatory cytokines and contributing to both viral clearance as well as pathogenesis (16–18).

The TLRs are not the only class of PAMP receptors that contribute to the recognition of virus infection. Yoneyama and colleagues (19) have demonstrated recently that IFN-β production is induced in response to Newcastle disease virus infection through a pathway that is independent of TLR3 but requires the retinoic acid-inducible gene I (RIG-I, or DDX58), a cellular RNA helicase with homology to caspase-recruitment domain (CARD) proteins.

The ability of these pathways to induce the expression of type I IFNs and subsequently a wide array of IFN-stimulated genes (ISGs) is likely to influence the outcome of infection by many human viruses. Such responses may have direct antiviral effects within the infected cell (2). Type 1 IFNs also exert

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[1] The abbreviations used are: IFN, interferon; dsRNA, double-stranded RNA; HCV, hepatitis C virus; IRF-3, interferon regulatory factor 3; ISG, interferon-stimulated gene; MDA5, melanoma differentiation associated gene-5; MyD88, myeloid differentiation factor 88; RIG-I, retinoic acid-inducible gene I; SenV, Sendai virus; siRNA, small interfering RNA; TLR, Toll-like receptor; TRIF, Toll-IL1 receptor domain-containing adaptor inducing IFN-β; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular patterns; CARD, caspase-recruitment domain; HBV, hepatitis B virus; pAb, polyclonal antibody; RT, reverse transcription; HAU, hemagglutinin unit(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

critically important immunoregulatory effects, including stimulation of natural killer cell cytotoxicity, activation of γδ T cells, and stimulation of immunoglobulin synthesis. Cytokines and chemokines induced by the activation of these signaling pathways also contribute to the maturation of dendritic cells and influence the priming of $T_H1$ and cytotoxic T cells (20). These responses thus may play critical roles in shaping subsequent adaptive T cell responses that are required for the ultimate elimination of viruses (21, 22).

Although the liver is a particularly important site of persistent viral infections in humans, very little is known about how these signaling pathways function specifically in hepatocytes. Despite this, there is strong, albeit indirect evidence, that type I IFN responses are important in the pathogenesis of chronic viral hepatitis. Both hepatitis B virus (HBV) and hepatitis C virus (HCV) cause persistent infections involving the hepatocyte, and both have evolved mechanisms to disrupt the induction of type I IFNs. Although not well understood, the core protein of HBV inhibits the transcription of IFN-β (23, 24). Similarly, the NS3/4A protease of HCV blocks Sendai virus (SenV)-induced activation of IFN regulatory factor 3 (IRF-3), a cellular transcription factor that plays a critical role in the expression of IFN-β (25). IRF-3 blockade is dependent upon the protease activity of NS3/4A, suggesting that NS3/4A proteolytically targets one or more cellular proteins residing within the signaling pathways leading to IRF-3 activation and type I IFN production (25). These and other data (26, 27) suggest that a more detailed understanding of the mechanisms by which virus infections trigger IRF-3 activation and IFN production in hepatocytes would be helpful in unraveling the pathogenesis of persistent HBV and HCV infections, and might possibly lead to the design of novel therapeutic interventions. Here, we describe efforts to better define the antiviral signaling pathways that are active in cultured hepatocyte-derived cell lines and that thus may be triggered by HBV or HCV infection *in vivo*.

## MATERIALS AND METHODS

*Cell Culture and Reagents*—Murine macrophage RAW264.7, human hepatoma Huh7, Huh7.5 (kindly provided by C. M. Rice via Apath), HepG2, Hep3B cells, and PH5CH8, a simian virus 40 (SV40) large T antigen-immortalized non-neoplastic human hepatocyte cell line (28), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G and streptomycin in a humidified 37 °C, 5% $CO_2$ incubator. Poly(I-C) was purchased from Sigma. Bafilomycin A1 was from Calbiochem. Recombinant human interleukin-1β was from Raybiotech. R-848 was kindly provided by K. A. Fitzgerald.

*Plasmids*—pIFN-β-luc, pCMV1FlagTLR3, pCMV1FlagTLR3DN, and pEF-Bos Flag-RIG-I were generous gifts of Rongtuan Lin, Ruslan Medizhitov, and Takashi Fujita, respectively. The ISG54 ISRE-Luc and pNFκB-Luc plasmids were from Stratagene. Sequence encoding a dominant negative form of MyD88, which lacks the amino-terminal death domain (29), was amplified from cDNA transcribed from PH5CH8 cell RNA, and cloned into pCDNA3.1 V5-His TOPO (Invitrogen). pPRDII-luc has been described previously (30). pCMV-β-galactosidase (Clontech) was used to normalize transfection efficiencies. Cells were transfected with plasmid DNAs using TransIT-LT1 (Mirus) according to the manufacturer's instructions.

*Sendai Virus Infection*—Cells were infected with 100 hemagglutinin units/ml of SenV (Charles River Laboratory) and harvested 16 h later for luciferase/β-galactosidase reporter assays or immunoblot analysis as previously described (25).

*Poly(I-C) Treatment*—Poly(I-C) was added directly to the medium at 50 μg/ml (M-pIC), or complexed with Lipofectin for transfection (T-pIC). Cells were assayed for poly(I-C) induced responses 6 h after exposure by either route.

*Reporter Gene Assay*—Cells ($5 \times 10^4$ cells per well in 24-well plates) were transfected with reporter plasmids (100 ng), pCMV-β-galactosidase (100 ng), and the indicated amounts of an expression vector. Twenty-four hours later, cells were mock treated or treated with 20 ng/ml interleukin-1β, or challenged with poly(I-C) or Sendai virus, then subsequently lysed and assayed for luciferase and β-galactosidase activities as indicated. For comparisons, luciferase activity was normalized to β-galactosidase activity. Data are expressed as mean relative luciferase activity ± S.D. for one representative experiment carried out in triplicate, typically from a minimum of three separate experiments. The –fold induction of promoter activity was calculated by dividing the relative luciferase activity of stimulated cells with that of mock-treated cells.

TABLE I
*Gene-specific primers for semiquantitative RT-PCR*

| Gene name | | Primer sequence[a] | Product size |
|---|---|---|---|
| | | | *bp* |
| IFN-β | Forward | gattcatctagcactggctgg | 186 |
| | Reverse | cttcaggtaatgcagaatcc | |
| TLR3 | Forward | tcacttgctcattctccctt | 157 |
| | Reverse | gacctctccattcctggc | |
| TRIF | Forward | ccagatgcaacctccactgg | 339 |
| | Reverse | ctgttccgatgatgattcc | |
| ISG56 | Forward | tagccaacatgtcctcacagac | 396 |
| | Reverse | tcttctaccactggtttcatgc | |
| RIG-I | Forward | cagtatattcaggctgag | 389 |
| | Reverse | ggccagttttccttgtc | |
| MDA5 | Forward | agtttggcagaaggaagtgtc | 480 |
| | Reverse | ggagttttcaaggatttgagc | |

[a] GAPDH primers were purchased from Clontech.

*Immunoblot Analysis*—Cellular extracts were subjected to immunoblot analysis as described (25). Briefly, protein samples were separated by SDS-PAGE, and transferred to Hybond enhanced chemiluminescent (ECL) nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with 3% nonfat milk in phosphate-buffered saline and processed for immunodetection using the following monoclonal or polyclonal (pAb) antibodies: anti-FLAG M2 and anti-actin monoclonal antibodies (Sigma), anti-TLR3 monoclonal antibody (Imgenex), rabbit anti-MyD88 pAb (Santa Cruz), rabbit anti-IRF-3 pAb (kindly provided by Michael David), rabbit anti-ISG15 pAb (kindly provided by Arthur Haas), rabbit anti-ISG56 pAb (kindly provided by Ganes Sen), rabbit anti-MxA and anti-Sendai virus pAbs (kindly provided by Ilkka Julkunen), peroxidase-conjugated secondary donkey anti-rabbit (Jackson ImmunoResearch), and sheep anti-mouse (Amersham Biosciences) pAbs. Protein bands were visualized using ECL Plus Western blotting detection reagents (Amersham Biosciences), followed by exposure to Kodak Bio-Max film.

*RNA Interference*—Transfections of siRNAs targeting TLR3, TRIF, or RIG-I were carried out using Oligofectamine (Invitrogen) with a final concentration of siRNA of 80 nM according to the manufacturer's instructions. The target sequences of siRNA used in this study were: TLR3, GGTATAGCCAGCTAACTAG; TRIF (13), GACCAGACGCCACTCCAAC; RIG-I, GGAAGAGGTGCAGTATATT; and MDA5, GGTGAAGGAGCAGATTCAG. siRNAs were purchased from Dharmacon and Ambion. The scrambled negative control siRNA was from Ambion.

*RT-PCR*—Total cellular RNA was extracted with TRIzol Reagent (Invitrogen), treated with DNase I to remove genomic DNA contamination, and reverse-transcribed using Advantage RT-4PCR kit (Clontech). The resulting cDNA was subjected to PCR using primers (Table I) specific for IFN-β, TLR3, TRIF, ISG56, RIG-I, MDA5, β-actin, and GAPDH, respectively. The quantity of the cDNA template included in these reactions and the number of amplification cycles were optimized to ensure that reactions were stopped during the linear phase of product amplification, permitting semiquantitative comparisons of mRNA abundance between different RNA preparations. To exclude the possibility of contaminating DNA, control reactions were performed in parallel in the absence of reverse transcriptase. PCR products were visualized by agarose gel electrophoresis.

## RESULTS

*Cultured Hepatocyte Cell Lines Differ in Their Ability to Activate IFN-β Transcription in Response to dsRNA or Virus Infection*—The induction of type I IFNs represents an early protective response to many viral infections in mammalian cells. IFN-β induction represents the immediate response of cells to viral infection, and precedes the transcription of most IFN-α species, the induction of which depends on autocrine/paracrine feedback of IFN-β and activation of IRF-7 (31). We thus focused on characterizing the IFN-β response. The molecular basis for induction of IFN-β expression has been exten-

sively studied and shown to be induced by dsRNA or other products of virus infection through coordinate activation of transcription factors IRF-3, NF-κB, and ATF-2/c-Jun (2). Most of these studies were conducted in human embryonic kidney 293 or epithelial cells, and relatively little is known about these events in hepatocytes, which constitute ~80% of the liver cell population (32), and are the primary cells within which both HBV and HCV replicate. Because primary differentiated hepatocytes are difficult to maintain in culture, we studied 3 different continuous cell lines derived from human hepatocellular carcinomas: Huh7 cells, which appear to be particularly permissive for HCV RNA replication (33, 34); HepG2 cells and Hep3B cells, the latter of which contains integrated HBV DNA and express the HBV envelope protein, HBsAg (35). Huh7 cells are not available from any standard repository, but are carried in many different laboratories and different laboratory variants may show significant variation in morphology and perhaps other characteristics. We thus studied 3 different Huh7 sublines: Huh7 SL, cells carried in this laboratory for many years; Huh7 2–3c, cells derived from Huh7 SL that were selected for their ability to support replication of genome-length HCV RNA and subsequently "cured" of the replicating RNA with IFN-α2b treatment (36), and Huh7 MG cells, Huh7 cells carried in the Gale laboratory at the University of Texas Southwestern Medical Center. We also studied the PH5CH8 cell line, which is a clonal variant of a non-neoplastic hepatocyte cell line, PH5CH, immortalized with the simian virus 40 large T antigen (28, 37, 38).

We characterized virus-induced activation of the IFN-β promoter in these various hepatocyte-derived cell lines after transient transfection with a reporter plasmid expressing luciferase under control of the IFN-β promoter. Although the PH5CH8 cell line has been reported to support replication of HCV (28), no cultured cell has been reported to be fully for HCV replication. Thus, to challenge these cells, we exposed them to the dsRNA analog, poly(I-C), or infected them with SenV. Interestingly, when poly(I-C) was added to the culture medium, there was no induction of IFN-β promoter activity in the Huh7, HepG2, or Hep3B hepatoma cells (M-pIC, Fig. 1*A*, *upper panel*). In contrast, the PH5CH8 cells demonstrated a 6-fold up-regulation of IFN-β promoter activity upon exposure to poly(I-C) (Fig. 1*A*, *upper panel*). Consistent with the reporter data, M-pIC treatment significantly up-regulated the expression of ISG15 and ISG56, which are responsive to either IRF-3 or IFN, in PH5CH8 cells, but not in hepatoma cell lines Huh7, HepG2, or Hep3B (Fig. 1*B*). To mimic intracellular dsRNA generated during viral replication, we also transfected poly(I-C) into cells using a liposome-mediated procedure. This resulted in much more potent stimulation of the IFN-β promoter in PH5CH8 cells, leading to a 17-fold increase in activity over basal promoter levels (T-pIC, Fig. 1*A*, *middle panel*). It also resulted in significant activation of the IFN-β promoter in HepG2 (8-fold induction) and Hep3B (4-fold induction) cells. However, under the same conditions, there was little if any activation of the promoter in any of the Huh7 cell sublines. These promoter assay results were confirmed by RT-PCR analysis of endogenous IFN-β mRNA synthesis in cells transfected with increasing concentrations of poly(I-C) (Fig. 1*C*). Whereas transfection of as little as 1 μg/ml poly(I-C) induced IFN-β transcription in HepG2, Hep3B, and PH5CH8 cells, there was no detectable IFN-β mRNA in Huh7 SL cells transfected with up to 100 μg/ml poly(I-C).

A similar pattern of IFN-β induction was observed following infection of these different cell lines with SenV. The greatest induction of IFN-β promoter activity occurred in PH5CH8 cells

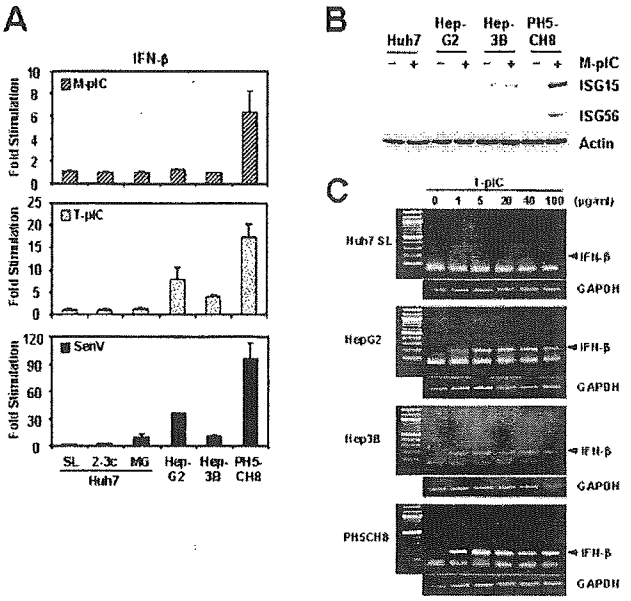


FIG. 1. **Activation of IFN-β transcription in various hepatocyte cell lines by dsRNA and virus.** *A*, cells grown in 24-well plates were cotransfected with IFN-β Luc and pCMV-β-galactosidase for 24 h before stimulation with 50 μg/ml poly(I-C) directly added to culture medium for 6 h (*M-pIC, upper panel*), 5 μg of poly(I-C) transfected with Lipofectin for 6 h (*T-pIC, middle panel*), or SenV 100 HAU/ml for 16 h (*lower panel*). -Fold induction of IFN-β promoter was calculated by dividing the relative luciferase activity of stimulated cells with that of mock-treated cells. *B*, immunoblot analysis of ISG15 and ISG56 expression in various hepatocyte cell lines either mock-treated or treated with 50 μg/ml M-pIC for 12 h. Actin was included as a loading control. *C*, cells were mock-treated (Lipofectin) or transfected with differing concentrations of poly(I-C) for 6 h before total RNA isolation. IFN-β mRNA was detected by semiquantitative RT-PCR.

(96-fold increase over basal promoter activity), followed by HepG2 (35-fold), and Hep3B (10-fold) (Fig. 1*A*, *lower panel*). However, there were significant differences in the magnitude of the IFN-β promoter activation induced by SenV infection in the different Huh7 cell lines. SenV infection induced IFN-β promoter activity by ~10-fold in the Huh7 MG cells, but only 1.5-fold in Huh7 SL cells and 3-fold in the cured Huh7 2–3c cells (Fig. 1*A*, *lower panel*).

We conclude from these results that cultured hepatoma cells generally have impaired poly(I-C)- and virus-activated IFN responses, compared with the PH5CH8 cells that were established from non-neoplastic hepatocytes. These latter cells retain robust IFN-β responses to extracellular and intracellular poly(I-C) as well as SenV infection. They are likely to more closely resemble normal hepatocytes *in vivo*, and thus may represent a superior cell model for investigation of antiviral responses in hepatocytes. The considerable variation we observed in the IFN response in different Huh7 cell lines mandates caution in comparing studies carried out with these cells in different laboratories, an important point because Huh7 cells are widely used for cell culture studies with HCV.

*TLR3 and TRIF Are Not Required for SenV Activation of the IFN-β Promoter in PH5CH8 Cells*—TLR3 is the only known TLR that recognizes viral dsRNA and it is expressed both on the cell surface and within intracellular vesicles (39, 40). We were thus interested in determining whether the virus-induced IFN response involves TLR3 in PH5CH8 cells. To investigate this, we transfected the cells with a synthetic siRNA duplex targeting human TLR3, or a scrambled negative-control siRNA, then challenged the cells by adding poly(I-C) to the media or infecting them with SenV prior to measuring IFN-β

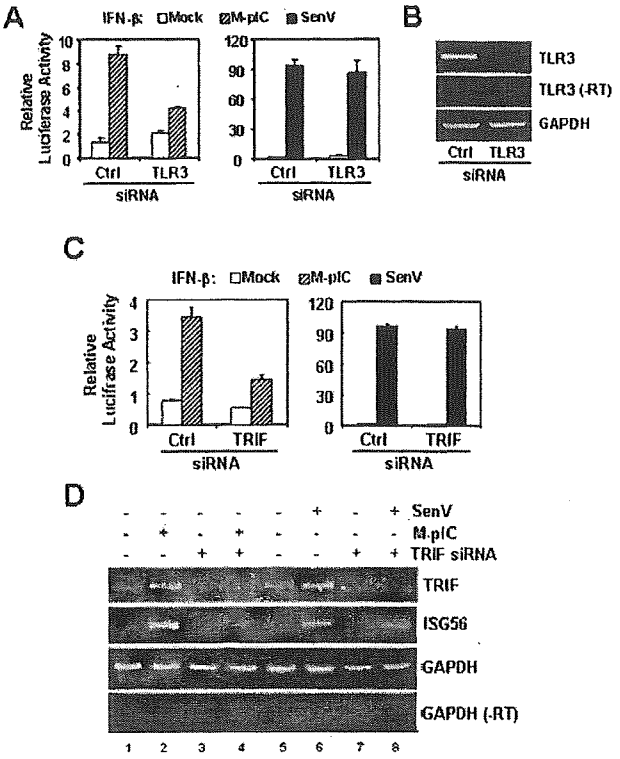


FIG. 2. **Extracellular dsRNA, but not SenV, activates IFN response via TLR3 and TRIF in PH5CH8 hepatocytes.** *A*, TLR3 expression is required for activation of IFN-β promoter by M-pIC but not by SenV. PH5CH8 cells grown in 24-well plates were transfected with control (*Ctrl*) or TLR3 siRNA, and pIFN-β Luc plus pCMV-β-galactosidase. 48 h later, cells were mock-treated or stimulated with 50 μg/ml M-pIC for 6 h or infected with SenV (100 HAU/ml) for 16 h. *B*, semiquantitative RT-PCR detection of TLR3 and GAPDH mRNAs in control/TLR3 siRNA-transfected cells. *C*, TRIF expression is required for activation of IFN-β promoter by M-pIC but not by SenV. PH5CH8 cells were transfected with Ctrl or TRIF siRNA and reporter plasmids and treated similarly as in *A*. *D*, semiquantitative RT-PCR detection of TRIF, ISG56, and GAPDH mRNAs in cells under conditions as described for *panel C*.

promoter activity (Fig. 2). PH5CH8 cells express a readily detectable abundance of TLR3 mRNA, which was reduced to almost undetectable levels upon transfection of TLR3-specific siRNA (Fig. 2*B*). The induction of IFN-β promoter activity by extracellular poly(I-C) was substantially reduced by this siRNA knockdown of TLR3 expression (Fig. 2*A*, *left panel*). In contrast, there was no appreciable effect on SenV-induced IFN-β promoter activation (Fig. 2*A*, *right panel*).

TRIF is an essential adaptor protein that links TLR3 to downstream kinases responsible for IRF-3 activation and IFN-β production (13–15). It may well play additional roles in intracellular signaling events, given its relatively large size (712 amino acids) compared with other TLR adaptors. Mice that are deficient for TRIF function have an impaired response to murine cytomegalovirus infection (12). The liver contains a higher abundance of TRIF than any other organ (14), suggesting that it may play a particularly important role in intrahepatic signaling. To determine whether TRIF is required to mount a type I IFN response against SenV in hepatocytes, we carried out a TRIF knockdown experiment in PH5CH8 cells. As shown in Fig. 2*D*, semiquantitative RT-PCR demonstrated that transfection of the TRIF-specific siRNA efficiently reduced TRIF mRNA abundance. As expected from the TLR3 knockdown (Fig. 2*A*), TRIF knockdown also significantly inhibited the induction of IFN-β promoter activity in response to extracellular poly(I-C) (Fig. 2*C*, *left panel*). It also significantly reduced extracellular poly(I-C) induction of ISG56 mRNA transcription (Fig. 2*D*, compare *lanes 2 versus 4*). In contrast, there was no effect on either IFN-β promoter activity (Fig. 2*C*, *right panel*) or ISG56 mRNA transcription (Fig. 2*D*, compare *lanes 6 versus 8*) triggered by SenV infection. Similar results were obtained in an ISG54 ISRE promoter assay (data not shown). We conclude from these data that exposure to extracellular poly(I-C) triggers activation of the IFN-β promoter in PH5CH8 cells through a TLR3-TRIF dependent pathway, whereas SenV induction of IFN-β transcription is TLR3- and TRIF-independent.

*SenV Induction of IFN-β in Hepatocytes Does Not Utilize MyD88 nor Require Endosomal Acidification*—In addition to TLR3, recent data indicate that TLR7, TLR8, and TLR9 may sense viral components and lead to type I IFN production. Murine TLR7 and human TLR8 recognize viral ssRNA, whereas TLR9 senses unmethylated CpG DNA present in the murine cytomegalovirus genome (41). A common feature of these three TLRs is that they all localize within endosomes and signal through MyD88 and IRF-7 (42, 43). In addition, the responses induced through these TLRs require intact endocytic pathways and are thus sensitive to endosomal acidification inhibitors, such as chloroquine and bafilomycin A1 (7, 10). Because SenV induction of IFN-β occurs via a TLR3-TRIF independent pathway in PH5CH8 cells, we considered the possibility that TLR7/8 may initiate this response. To determine whether MyD88 is required for SenV activation in the PH5CH8 cells, as would be the case were it mediated by TLR7, TLR8, or TLR9, we measured promoter activation in PH5CH8 cells transfected with a vector expressing a dominant-negative MyD88 mutant that lacks the amino-terminal death domain that is required for interaction with IRF-7 (42) (MyD88DN). Expression of MyD88DN significantly inhibited the activation of the NF-κB-dependent PRDII promoter (44) by interleukin-1β, which is known to signal through MyD88 (29). However, we found that the activity of both PRDII and IFN-β promoters was induced to similar levels by SenV with or without MyD88DN co-expression (Fig. 3*A*). Consistent with these reporter data, SenV-induced hyperphosphorylation of IRF-3 was not affected by overexpression of MyD88DN (Fig. 3*B*). This was also true for SenV-induced expression of ISG56, which is responsive to either IRF-3 or IFN (45), and MxA, which responds only to IFN (46).

To determine whether SenV activation of the IFN-β promoter requires endosomal acidification, PH5CH8 cells were pretreated with the endosomal inhibitor bafilomycin A1 prior to infection with SenV. Although bafilomycin A1 treatment completely ablated NF-κB activation in RAW264.7 cells induced by a TLR7/8 ligand, R-848 (Fig. 3*C*), SenV-stimulated IFN-β and PRDII promoter activity was minimally reduced in cells treated with the compound (Fig. 3*D*). Moreover, immunoblot analyses indicated that there were no differences in SenV-induced hyperphosphorylation of IRF-3, or ISG15 and MxA expression in these cells in the presence or absence of bafilomycin A1 (Fig. 3*E*). Bafilomycin A1 did not alter the expression of SenV proteins.

These data suggest that SenV induction of IFN responses is MyD88-independent in PH5CH8 cells, and does not require active endocytic pathways. Both lines of evidence argue strongly against the involvement of TLR7/8. In addition, because MyD88 is thought to be used by all TLRs other than TLR3 (and TLR4 for signaling to IRF-3), these data, taken in the context of the absence of any inhibition of the SenV response by TRIF knockdown (Fig. 2, *C* and *D*), suggest that the recognition of SenV by PH5CH8 cells is not dependent upon any known TLR.

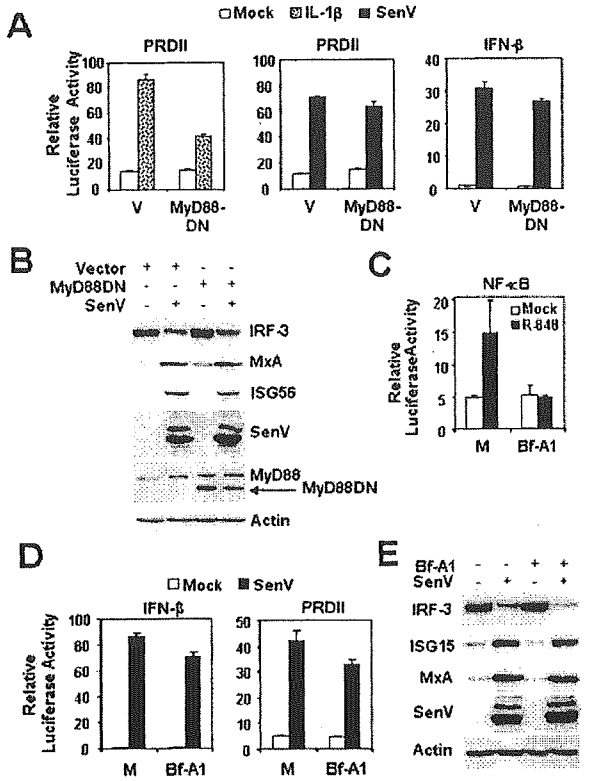


FIG. 3. **SenV activation of type I IFN response in hepatocytes is MyD88-independent and does not require endosomal acidification.** *A*, PH5CH8 cells in 24-well plates were cotransfected with the indicated reporter plasmids (100 ng), pCMV-β-galactosidase (100 ng), and 600 ng of a dominant-negative form of MyD88 (*MyD88DN*) or empty vector (*V*) for 24 h before mock-infected or infected with SenV (100 HAU/ml) for 16 h (*middle* and *right panels*) or mock/treated with 20 ng/ml interleukin-1β for 8 h (*left panel*). *B*, immunoblot detection of IRF-3, MxA, ISG56, SenV, and MyD88 in PH5CH8 cells using the conditions as described for the *middle* and *right panels* of *A*. *C*, NF-κB promoter activity in RAW264.7 cells mock-treated or treated with bafilomycin A1 and then mock-treated with R-848. Cells transfected with pNF-κB Luc and pCMV-β-galactosidase for 24 h were pretreated with medium alone (*M*), or 100 nM bafilomycin A1 (*Bf-A1*) for 1 h and then mock-treated with 1 μM R-848 for 6 h in the presence of M/Bf-A1. *D*, SenV-induced IFN-β and PRDII promoter activity in PH5CH8 cells treated with medium alone or Bf-A1. *E*, immunoblot detection of IRF-3, ISG15, MxA, and SenV in PH5CH8 cells as described for the conditions of *panel D*.

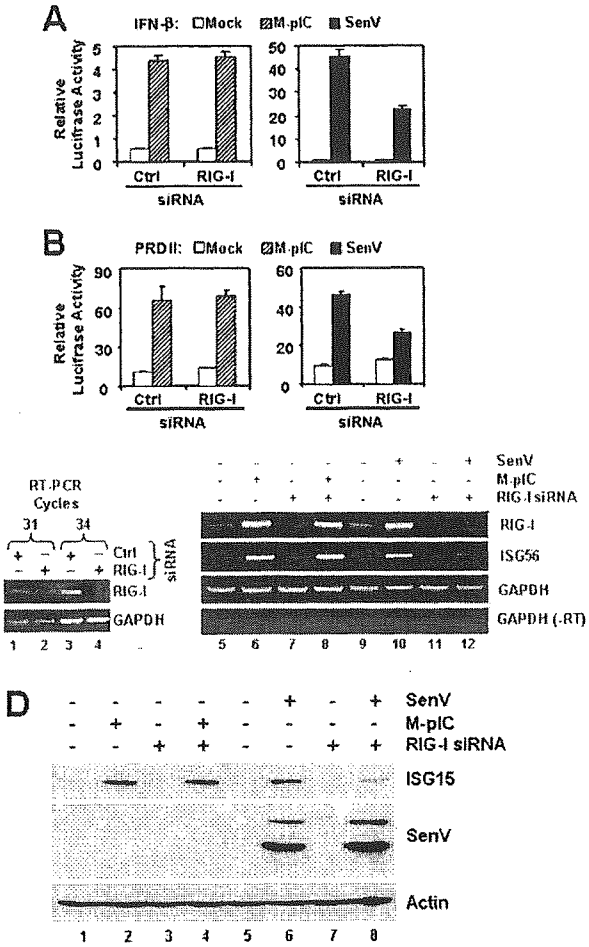


FIG. 4. **SenV, but not TLR3 engagement, activates type I IFN response through RIG-I in hepatocytes.** PH5CH8 cells grown in 24-well plates were transfected with control (*Ctrl*) or RIG-I siRNA, and pIFN-βLuc (*A*) or PRDII Luc (*B*), and pCMV-β-galactosidase. 48 h later, cells were mock-treated or stimulated with 50 μg/ml M-pIC for 6 h or infected with SenV (100 HAU/ml) for 16 h. *C*, semiquantitative RT-PCR detection of RIG-I, ISG56, and GAPDH mRNAs in PH5CH8 cells under conditions of *A* and *B*. The *left panel* shows efficient knockdown of the basal expression of RIG-I in PH5CH8 cells by RIG-I siRNA determined by increasing cycles of RT-PCR. *D*, immunoblot analysis of ISG15 and SenV protein expression in PH5CH8 cells under conditions of *panels A* and *B*. Actin was included as a loading control.

*SenV Activates Type I IFN Responses through a RIG-I-dependent Pathway in Hepatocytes*—Studies with knock-out mice have indicated that both virus infection and dsRNA can trigger type I IFN responses via TLR3-independent mechanisms (7, 8). Recently, Yoneyama and colleagues (19) demonstrated that RIG-I, a putative DExD/H box RNA helicase containing an N-terminal sequence with CARD-like homology domains, is essential for IFN-β production induced in response to infection with Newcastle disease virus. To determine whether SenV induces IFN-β promoter activity in PH5CH8 cells through a RIG-I-dependent pathway, we utilized RNA interference to knockdown RIG-I expression prior to virus challenge. Transfection of a RIG-I-specific siRNA, but not a scrambled control siRNA, reproducibly caused a ~50% reduction in SenV-induced activation of both the IFN-β (Fig. 4*A*, *right panel*) and PRDII promoters (Fig. 4*B*, *right panel*). Semiquantitative RT-PCR confirmed that the transfection of RIG-I siRNA efficiently knocked down the basal expression of RIG-I (Fig. 4*C*, *lanes 2* and *4* in the *left panel* and *lanes 7* and *11*, in *right panel*), and also significantly blunted the up-regulation of this IFN-induced protein upon SenV infection (Fig. 4*C*, *right panel*, compare *lanes 9* and *10* with *lanes 11* and *12*). SenV-induced transcription of ISG56 mRNA was also significantly reduced in cells transfected with RIG-I siRNA (Fig. 4*C*, *lanes 9–12*). Therefore, RIG-I is an essential component in the TLR-independent pathway by which PH5CH8 cells sense SenV infection and initiate a type I IFN response.

In contrast to these results, PH5CH8 cells transfected with the RIG-I siRNA responded normally when poly(I-C) were added to the media, in terms of activation of both the IFN-β (Fig. 4*A*, *left panel*) and NF-κB-dependent PRDII (Fig. 4*B*, *left panel*) promoters. In contrast to SenV-induced signaling, the poly(I-C) induction of RIG-I transcription was not dramatically reduced by transfection of the RIG-I siRNA (Fig. 4*C*, compare *lanes 5* and *6* with *lanes 7* and *8*). Similarly, there was no inhibition of poly(I-C) induction of ISG56 transcription (Fig. 4*C*, *lanes 5–8*). We also confirmed the RT-PCR results by immunoblot analysis of ISG15 expression under these conditions (Fig. 4*D*). These data indicate that TLR3- and RIG-I-mediated signaling function independently of each other in PH5CH8

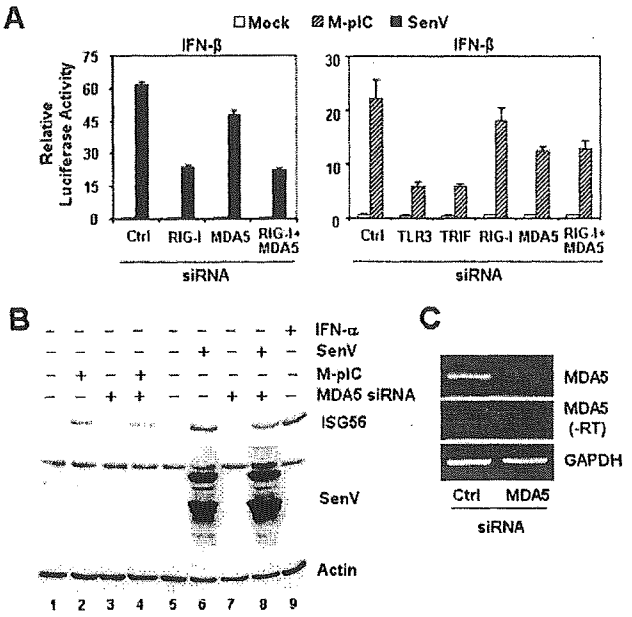


FIG. 5. **Poly(I-C), as well as SenV activation of the IFN-β promoter is partially MDA5-dependent in hepatocytes.** *A*, SenV (*left panel*) and M-pIC (*right panel*)-induced IFN-β promoter activity in PH5CH8 cells transfected with the indicated siRNAs. *B*, immunoblot detection of ISG56 and SenV proteins in PH5CH8 cells transfected with Ctrl (*lanes 1, 2,* and *5, 6*) or MDA5 siRNA (*lanes 3, 4,* and *7, 8*). Where indicated, cells were treated with 50 μg/ml M-pIC for 8 h or infected with 100 HAU/ml SenV for 16 h. In *lane 9*, cells were treated with 500 units/ml of IFN-α2b as a control for ISG56 expression. *C*, semiquantitative RT-PCR detection of MDA5 and GAPDH mRNAs in control (*Ctrl*)/MDA5 siRNA-transfected PH5CH8 cells.

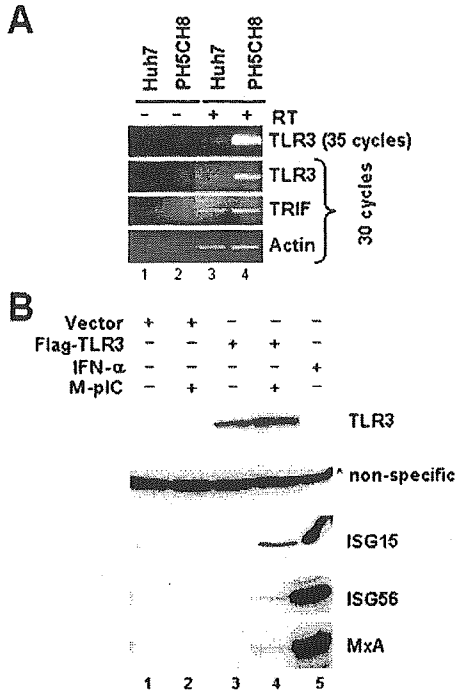


FIG. 6. **The defect of TLR3 signaling in Huh7 cells is because of insufficient expression of TLR3.** *A*, semiquantitative RT-PCR detection of TLR3, TRIF, and β-actin mRNAs in Huh7 and PH5CH8 cells. *B*, ectopic expression a FLAG-tagged TLR3 restored the induction of ISG15, ISG56, and MxA expression by M-pIC (50 μg/ml) determined by immunoblot analysis. A nonspecific band detected by the TLR3 antibody serves as a loading control. In *lane 5*, cells were treated with 500 units/ml of IFN-α2b as a positive control for ISG expression.

cells, similar to what has been reported previously for epithelial HeLa cells (19).

*Poly(I-C) Activation of the IFN-β Promoter Is Partially MDA5-dependent*—Because siRNA knockdown of RIG-I did not ablate but only partially reduced the IFN-β promoter response to SenV infection in PH5CH8 cells (Fig. 4*A*, *right panel*), we carried out similar experiments to determine whether the human melanoma differentiation associated gene-5 product (MDA5) participates in this signaling pathway, possibly in a redundant role with respect to RIG-I. MDA5, another DExD/H box RNA helicase, is an IFN-inducible protein that shares a subdomain architecture and considerable sequence homology with RIG-I (47). It has been associated with the induction of apoptosis, but not clearly identified as playing a role in activation of IRF-3 similar to that of RIG-I. siRNA-mediated knockdown of MDA5 expression had only a minor, but reproducible, suppressive effect on SenV induction of the IFN-β promoter. The magnitude of the reduction in promoter activity was substantially less than that observed with RIG-I knockdown (Fig. 5*A*, *left panel*), but it was associated nonetheless within a modest reduction in SenV-induced expression of ISG56 (Fig. 5*B*, compare *lanes 6 versus 8*). The suppressive effect of MDA5 knockdown was not additive with RIG-I knockdown, as the degree of suppression of promoter activity was not increased when both RIG-I and MDA5 siRNAs were cotransfected into PH5CH8 cells. The MDA5 knockdown had no effect on SenV protein expression (Fig. 5*B*).

Interestingly, MDA5 knockdown caused ~30% reduction in the induction of IFN-β promoter activity by extracellular poly(I-C) (Fig. 5*A*, *right panel*). This effect was less than that observed with TLR3 or TRIF knockdown, yet nonetheless reproducible and significant. Consistent with this, MDA5 knockdown also caused a moderate reduction in poly(I-C)-induced ISG56 expression (Fig. 5*B*, compare *lanes 2* and *4*). We conclude from these results that MDA5, like RIG-I, may contribute to viral activation of the IFN-β promoter but, unlike RIG-I, plays a greater role in poly(I-C) activation of the promoter than in SenV-induced responses. Whether this occurs in association with, or independent of, the TLR3-TRIF pathway remains to be determined.

*TLR3 Overexpression Reconstitutes Poly(I-C)-induced ISG Expression in Huh7 Cells*—As shown in Fig. 1, all three Huh7 cell sublines studied were defective in poly(I-C) signaling. This lack of poly(I-C) responsiveness may be explained by the fact that Huh7 cells express a negligible abundance of TLR3 mRNA, in contrast to PH5CH8 cells that demonstrate robust TLR3 expression (Fig. 6*A*). In contrast, TRIF mRNA abundance was approximately equal in these cell lines. Overexpression of FLAG-tagged TLR3 effectively restored the ability of Huh7 cells to respond to poly(I-C) when added to the culture medium (50 μg/ml), as indicated by expression of ISG15, ISG56, and MxA (Fig. 6*B*, compare *lanes 2* and *4*). Thus, Huh7 cells are normally deficient in their ability to respond to externally applied poly(I-C) because of a lack of sufficient expression of TLR3.

*TLR3- and RIG-I-mediated Signaling Function Independently in Huh7.5 Cells*—TLR3 signaling has been reported not to be adversely affected by overexpression of a dominant-negative form of RIG-I in HeLa cells (19). Consistent with this, the siRNA knockdown experiments shown in Figs. 2 and 4 suggest that these pathways function largely independently of each other in PH5CH8 cells. To confirm this in another hepatocyte-derived cell line, we utilized a Huh7 subline, Huh7.5, which is highly permissive for replication of HCV RNA replicons (48) and which has recently been shown to be defective for RIG-I

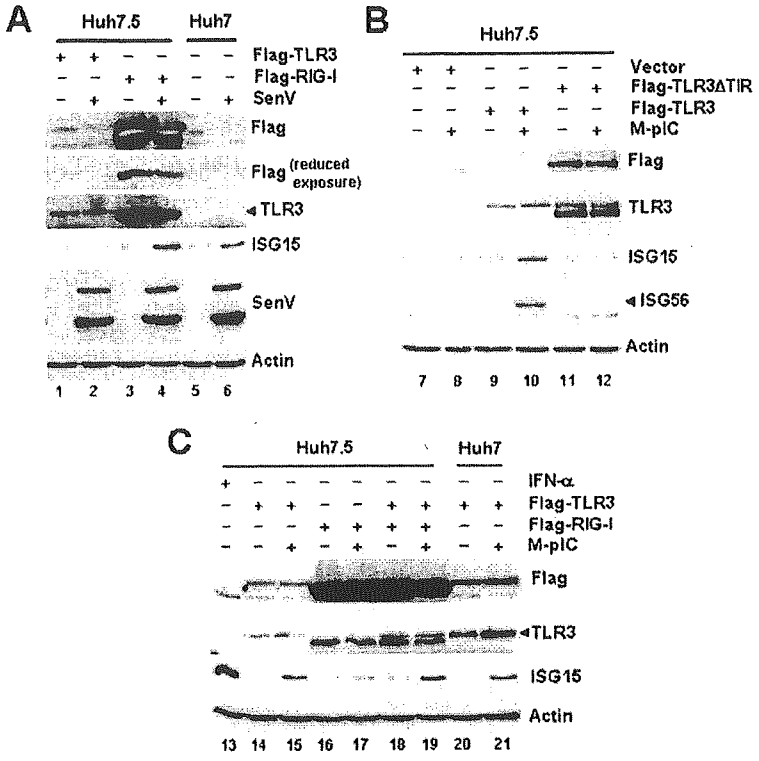


FIG. 7. **TLR3 and RIG-I signaling pathways function independently in Huh7.5 cells.** *A*, the defect of SenV-induced ISG15 expression can be restored by overexpression of RIG-I, but not TLR3 in Huh7.5 cells. *B*, the defect of extracellular dsRNA-induced expression of ISG15 and ISG56 can be restored by overexpression of TLR3, but not a mutant TLR3 with the TIR domain deleted (ΔTIR) in Huh7.5 cells. *C*, ectopic expression of TLR3, but not RIG-I, restored the response to M-pIC in Huh7.5 cells. All panels shown were immunoblot analysis. Where indicated, cells were treated with 50 μg/ml M-pIC for 12 h, or 100 HAU/ml SenV for 16 h, or 500 units/ml of IFN-α2b. Please note that the TLR3 antibody detects transfected FLAG-RIG-I for unknown reasons. The superintense bands of RIG-I detected by anti-FLAG antibody is likely because of the 2× FLAG tag *versus* 1× FLAG tag in TLR3.

signaling because of a point mutation within its CARD-like homology domain (49). Thus, in contrast to PH5CH8 cells in which both TLR3 and RIG-I pathways are intact, neither pathway is functional in Huh7.5 cells. By reconstituting expression of either TLR3 or a functional RIG-I molecule in Huh7.5 cells, we were thus able to assess the role played by each signaling pathway independently in recognition of viral infection while determining the extent to which these pathways act independently of each other.

We first confirmed previous observations (49) that SenV-induced signaling through RIG-I is deficient in Huh7.5 cells, by demonstrating that SenV-induced expression of ISG15 was dependent upon ectopic expression of RIG-I in these cells (Fig. 7*A*). In contrast, ectopic expression of TLR3 did not rescue SenV-induced ISG15 expression in Huh7.5 cells, suggesting that the products of SenV infection do not engage TLR3, or that optimal TLR3 signaling requires functional RIG-I. The latter is unlikely, however, as RIG-I knockdown had no effect on IFN induction by extracellular poly(I-C) in PH5CH8 cells (Fig. 4). Moreover, ectopic expression of TLR3 alone was able to restore responsiveness to extracellular poly(I-C) in Huh7.5 cells (Fig. 7*B*, *lanes 8 versus 10*), to a degree similar to that in normal Huh7 cells (Fig. 7*C*, *lanes 15 versus 21*). The rescue of poly(I-C) responsiveness was specific for TLR3, as overexpression of a TIR domain-deleted TLR3 mutant failed to restore expression of ISG15 and ISG56 in response to poly(I-C) (Fig. 7*B*, *lanes 10 versus 12*), nor did RIG-I (Fig. 7*C*, *lanes 15 versus 17*). Dual ectopic expression of TLR3 and RIG-I only slightly increased the ISG15 induction in response to poly(I-C) (Fig. 7*C*, *lanes 18* and *19*). Taken together, the data suggest that there is little if any cross-talk between the TLR3- and RIG-I pathways in hepatocytes.

## DISCUSSION

We have shown here that cultured hepatoma cells generally have impaired poly(I-C) and viral-activated IFN responses, compared with PH5CH8 cells that are derived from normal hepatocytes (Fig. 1). These data are thus in agreement with a previous report (50) showing that hepatoma cells have impaired antiviral responses. HepG2 and Hep3B cells are deficient in signaling in response to extracellular poly(I-C), and appear to have diminished responses to either intracellularly delivered poly(I-C) or SenV infection. As described by others (51, 52), we found that Huh7 cells also failed to respond to poly(I-C), irregardless of whether it was added to the culture medium or introduced into cells via transfection. In addition, only a weak response was observed in some Huh7 cell sublines (Huh7 2–3c and MG) after infection with SenV, a more potent IFN inducer. Poly(I-C) signaling could be restored to Huh7 cells by ectopic expression of TLR3 (Fig. 6), indicating that the lesion in Huh7 cells is at the level of the PRR molecule, and that the downstream pathway involving TRIF is intact. In contrast, the non-neoplastic PH5CH8 cells retain robust IFN-β responses to extracellular and intracellular poly(I-C) as well as SenV infection, and thus may be more representative of the antiviral signaling pathways present within hepatocytes *in vivo*.

dsRNA is commonly expressed during the replication of most RNA viruses, and has long been considered a candidate viral PAMP. Treatment of mammalian cells with the synthetic dsRNA analog, poly(I-C), induces type I IFN production through activation of transcription factors IRF-3 and NF-κB (53). TLR3 is well characterized as a PRR that is engaged specifically by dsRNA in many cell types. We have shown here that it is expressed in non-neoplastic hepatocytes, and that it plays an important role in these cells in activation of the IFN-β promoter following exposure to extracellular poly(I-C) (Fig. 2*A*). Furthermore, we have shown that poly(I-C) signaling through TLR3 leading to the induction of ISG expression is dependent upon the adaptor protein TRIF in these cells, as might be expected (Fig. 2*C*). Thus, the data presented demonstrate clearly that the TLR3-TRIF pathway is functional in the non-neoplastic hepatocyte-derived PH5CH8 cells, although it is

generally absent in cultured cells derived from hepatocellular carcinomas (Fig. 1).

Importantly, neither RNA interference directed silencing of TLR3 nor TRIF resulted in any impairment of IFN-$\beta$ promoter activation or ISG expression in SenV-infected PH5CH8 cells (Fig. 2). This indicates that dsRNA, or possibly another PAMP produced during SenV replication, triggers activation of the IFN-$\beta$ promoter in these hepatocytes through a distinctly different signaling pathway. This is consistent with several recent reports that dsRNA and/or virus infection can initiate antiviral signaling through TLR3-independent pathways (7, 8). SenV could potentially activate signaling through recognition of viral ssRNA by endosomally located TLR7/8, as reported for murine plasmacytoid dendritic cells and B lymphocytes (7). However, neither endosomal acidification inhibitors nor expression of a dominant-negative MyD88 mutant affected the response in SenV-infected PH5CH8 cells (Fig. 3). Thus, SenV appears to activate a TLR-independent intracellular signaling pathway in hepatocytes that is distinct from the TLR3-TRIF pathway activated by extracellular poly(I-C).

Consistent with a previous study carried out in mouse fibroblasts and human epithelial cells (19), we found that RIG-I is an essential component of this TLR-independent antiviral signaling pathway in cells derived from non-neoplastic hepatocytes (Fig. 4). Similarly, we have recently shown that SenV activation of IRF-3 is dependent upon RIG-I expression in hepatoma cells as well (49). RIG-I is a cytoplasmic RNA helicase that contains tandem motifs near its N terminus with limited homology to CARD domains and a downstream DExD/H-box helicase domain. It putatively binds viral dsRNA within its helicase domain, resulting in activation of IRF-3 and NF-$\kappa$B through signaling involving the N-terminal CARD-like homology domains (19). MDA5 is a closely related DExD/H box helicase with similar domain architecture, and has been associated with apoptotic signaling in terminally differentiating melanoma cells (47). Its expression is induced by IFN-$\alpha$ as well as infection with human immunodeficiency virus (47, 54). We found that it contributes to the induction of IFN signaling by extracellular poly(I-C) (Fig. 5), but has only a minor, yet clearly discernible, contribution to SenV induction of IFN-$\beta$ promoter activity and downstream ISG expression. Whether it functions in a fashion similar to that proposed for RIG-I, as a PRR molecule binding dsRNA through its helicase domain, seems likely but remains to be demonstrated. It is also not known whether RIG-I and MDA5 are evolved to preferentially recognize dsRNAs derived from viruses. The relationship of MDA5 to the TLR3-TRIF pathway also remains to be defined.

Although HCV replicon RNAs expressing selectable markers are capable of replicating in some alternative cell types (55), Huh7 cells have proven to be nearly unique in their ability to support the autonomous replication of these viral RNAs (34). In addition, we previously demonstrated that Huh7 cells are uniquely permissive for self-amplification of subgenomic replicons derived from hepatitis A virus, another positive-strand RNA virus (56). Huh7.5, a Huh7 subline that is highly permissive for HCV RNA replication (48), has a lethal mutation in the RIG-I CARD-like homology domain that renders it unresponsive to structured HCV RNA or SenV induced signaling (49). Whereas this accounts for the highly permissive phenotype of Huh7.5 cells, it does not fully explain why parental Huh7 cells are already more permissive than other cell types. Although relatively little is known regarding the cellular factors associated with permissiveness for HCV (34, 57–59), it is intriguing to speculate that the enhanced permissiveness for positive-strand virus RNA replication in normal Huh7 cells may relate to the absence of significant TLR3-mediated antiviral responses in these cells. It remains to be determined, however, whether HCV RNA replication results in specific engagement of TLR3 and activation of the downstream signaling pathway, and whether the TLR3 signaling cascade acts to limit HCV replication. Recent data indicate that expression of the HCV NS3/4A protease inhibits poly(I-C)-induced, TLR3-dependent signaling by directing the proteolytic cleavage of TRIF in osteosarcoma cells as well as in HeLa cells supporting replication of subgenomic HCV RNA replicons (26). However, for reasons that remain unclear, the ectopic expression of NS3/4A does not appear to block TLR3 signaling in PH5CH8 cells.[2]

In summary, hepatocytes contain two distinct antiviral signaling pathways leading to expression of type I IFNs, one dependent upon TLR3 and the other on RIG-I, and with little evidence of significant cross-talk between them. However, although the RIG-I and TLR3 pathways function independently in hepatocytes (Figs. 4 and 7), it is very likely that during viral infection *in vivo* both pathways are activated in a coordinate manner to enhance innate immune responses. Activation of the RIG-I pathway by the intracellular presentation of dsRNA or another viral PAMP produced by replicating viruses may contribute to the initial induction of type I IFNs. Subsequently, viral dsRNA released by lysis of cells in later stages of the infection may engage TLR3 and induce TRIF-dependent signaling, resulting in further amplification of the antiviral response (53). Importantly, type I IFNs induced through either pathway would have a positive feedback on signaling through both pathways, as both RIG-I and TLR3 are ISGs (19, 60).

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Short Communication

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

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

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

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

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

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

Supplementary material is available in JGV Online.

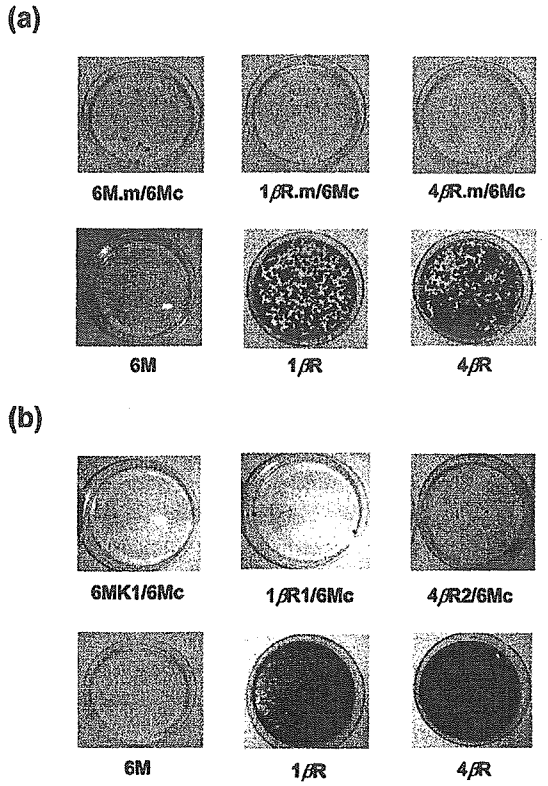


**Fig. 1.** IFN sensitivities of various HCV replicon-harbouring cells. (a) 6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc cells obtained as G418-resistant mixed colonies were treated with IFN-α (400 IU $ml^{-1}$) for 3 weeks in the presence of G418 (300 μg $ml^{-1}$). 6M, 1βR and 4βR cells were also used for control experiments. G418-resistant colonies were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004). (b) IFN sensitivities of 6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc cells were examined as described in (a) except that they were treated with IFN-β (200 IU $ml^{-1}$).

3 weeks). Although many colonies were found to have survived in 1βR and 4βR cells after IFN-α treatment, only a few colonies survived in 1βR.m/6Mc and 4βR.m/6Mc cells or in 6M and 6M.m/6Mc cells (Fig. 1a). Similar results were obtained when these replicon-harbouring cells were treated with IFN-β (400 IU $ml^{-1}$) (data not shown), although two colonies (named 6βR and 7βR, described below) derived from 6M.m/6Mc and 4βR.m/6Mc cells, respectively, proliferated as highly IFN-resistant clones.

We further examined the IFN sensitivity of replicon-harbouring cells (6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc) established by the transfection of *in vitro*-synthesized replicon RNAs (6MK1, 1βR1 and 4βR2 obtained from 6M, 1βR and 4βR cells, respectively) (Namba *et al.*, 2004; Kato *et al.*, 2005) into 6Mc cells. The results revealed that few or no colonies survived in 1βR1/6Mc and 4βR2/6Mc cells, as in 6M and 6MK1/6Mc cells, whereas many colonies survived in 1βR and 4βR cells (Fig. 1b). These results suggested that cellular factors rather than viral factors contributed to the highly IFN-resistant phenotype of HCV replicon-harbouring cells. However, the present results obtained under a high concentration of IFN do not necessarily rule out a possible role for HCV mutations in conferring low degrees of IFN resistance, because effects of HCV mutations on IFN activity are presumably weaker than those of the cellular factors.

To obtain evidence in favour of the idea that alterations in cellular factor(s) are involved in the emergence of an IFN-resistant phenotype, we attempted to prepare cured cells from the replicon-harbouring cells possessing a highly IFN-resistant phenotype. Since phosphorylation of signal transducer and activator of transcription 1 (STAT1) occurred in the 1βR and 4βR cells treated with IFN-γ (Fig. 2a), these replicon-harbouring cells were treated with IFN-γ (500 IU $ml^{-1}$) for 3 weeks, and cured 1βRc and 4βRc cells were obtained. Western blot (Fig. 2b) and RT-PCR (data not shown) analyses showed that no replicons were detected in either type of cured cells. Analysis of a luciferase reporter assay indicated that the complete defect of the IFN-α/β signalling was not restored in the cured 1βRc and 4βRc cells (Fig. 2c).

To clarify whether or not the signalling defect in these replicon-harbouring cells was restricted to type I IFN, we examined the phosphorylation status of STAT3 in 6M, 1βR and 4βR cells treated with interleukin-6 (IL6). Since it has been reported that STAT3 is also activated by IFN-α treatment (Pfeffer *et al.*, 1997), the phosphorylation status of STAT3 in these replicon-harbouring cells after IFN-α treatment was also examined. Our results revealed that STAT3 was not phosphorylated in 1βR and 4βR cells treated with IFN-α, while phosphorylation of STAT3 was observed in 6M, 1βR and 4βR cells treated with IL6 and in 6M cells treated with IFN-α (Fig. 2d), indicating that only type I IFN signalling was defective in 1βR and 4βR cells. These results suggested that the initial reaction following the addition of IFN-α/β was defective in replicon-harbouring cells possessing a highly IFN-resistant phenotype.

Following up this suggestion, we examined the genetic status of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1). Sequence analysis of TYK2 and JAK1 cDNAs obtained from 1βR and 4βR cells was performed after cloning into the pCXbsr vector (Akagi *et al.*, 2000), as described previously (Nozaki *et al.*, 2003). However, the results showed no mutations in these cDNAs (data not shown). We next focused on type I IFN receptors (IFNAR1 and IFNAR2c). Our results showed that the mRNA levels of the two receptors were almost equal among all examined replicon-harbouring cells including 6Mc cells (see Supplementary Fig. S1a, available in JGV Online). More than three independent clones of each cDNA (1708 bp for IFNAR1 and 1582 bp for IFNAR2c) were sequenced as described above. Table 1 shows a summary of sequence analysis of IFNAR1

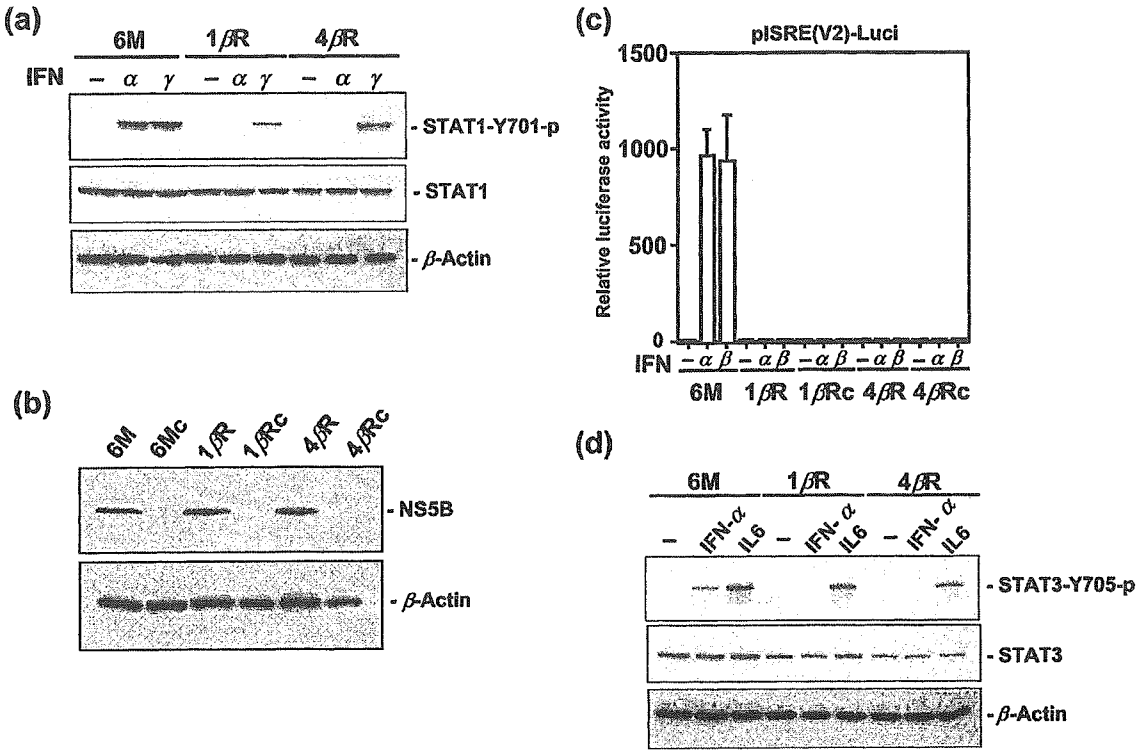


**Fig. 2.** Cellular factors rather than HCV replicons contribute to defects in type I IFN signalling. (a) Western blot analysis of STAT1 in 6M, 1$\beta$R and 4$\beta$R cells treated with IFN-$\alpha$ or -$\gamma$. The replicon-harbouring cells were treated with or without IFN-$\alpha$ or -$\gamma$ (500 IU ml$^{-1}$ each) for 30 min, and Western blot analysis for STAT1 and its phosphorylation status (Tyr-701) was then performed as previously described (Hijikata *et al.*, 1993). $\beta$-Actin was used as a control for the amount of protein loaded per lane. (b) Western blot analysis of NS5B. Anti-NS5B antibody was used for immunoblotting. $\beta$-Actin was used as described in (a). (c) Analysis of IFN signal transduction. Dual luciferase assays using pISRE(V2)-Luci (Dansako *et al.*, 2003) were performed as previously described (Naganuma *et al.*, 2000). Cells were treated with IFN-$\alpha$ or -$\beta$ (500 IU ml$^{-1}$ each) for 6 h. (d) The defect in the signalling pathway in 1$\beta$R and 4$\beta$R cells is restricted to type I IFN. Replicon-harbouring cells were left untreated or treated with IFN-$\alpha$ (500 IU ml$^{-1}$) or IL6 (100 ng ml$^{-1}$) for 30 min. Western blot analysis for STAT3 and its phosphorylation status (Tyr-705) was performed as described in (a).

and IFNAR2c mRNAs. Surprisingly, we found that nt 475 of IFNAR2c mRNA from 1$\beta$R cells had a U substituted for G in 13/13 clones, resulting in a nonsense mutation at codon 159 from glutamic acid (GAG) to the termination codon UAG (see Supplementary Fig. S1b). Furthermore, nt 319 of IFNAR1 mRNA from 4$\beta$R cells was also found to have a U substituted for G in 12/12 clones, resulting in a nonsense mutation at codon 107 from glutamic acid (GAA) to the termination codon UAA (see Supplementary Fig. S1c). However, interestingly, IFNAR1 and IFNAR2c mRNAs obtained from 1$\alpha$R and 4$\alpha$R cells, which were derived from clone 1 and clone 4 and were counterparts of 1$\beta$R and 4$\beta$R cells, respectively (see Supplementary Fig. S2, available in JGV Online), did not possess the nonsense mutations found in the mRNAs from 1$\beta$R and 4$\beta$R cells. In addition, several kinds of deletion and another nonsense mutation (lysine to a termination codon at codon 458) were found in approximately half of IFNAR1 cDNA clones obtained from 3$\beta$R and 5$\beta$R cells. In contrast to the finding of frequent mutations and deletions in IFNAR mRNAs from the $\beta$R series, such genetic abnormalities in IFN receptors were quite rare in the $\alpha$R series (Table 1).

To evaluate the possibility that genetic mutants might pre-exist in the cloned replicon-harbouring cells (clones 1, 3, 4 and 5 shown in supplementary Fig. S2) or that genetic mutants had appeared during the IFN-$\beta$ treatment of the cloned replicon-harbouring cells, we repeated IFN-$\beta$ treatment of cloned 1, 3, 4 and 5 cells following the method described previously (Namba *et al.*, 2004). The result was almost identical to that obtained previously (Namba *et al.*, 2004), indicating the good reproducibility of the experiment with IFN-$\beta$ treatment (data not shown). In the present study, each of the three colonies showing resistance to IFN-$\beta$ was isolated and proliferated (see Supplementary Fig. S2) and we then performed sequence analysis of IFNAR mRNAs

**Table 1.** Genetic alterations of type I IFN receptors in HCV replicon-harbouring cell lines possessing IFN-resistant phenotypes

The determined nucleotide sequences were compared with those of 6M and 6Mc cells, which confirmed that the deduced amino acid sequences were identical to the human IFNAR1 (GenBank accession no. NM_000629) and IFNAR2c (GenBank accession no. L41942) sequences. NM, Not mutated.

| Cell line | IFNAR1 mRNA | | | IFNAR2c mRNA | | |
|---|---|---|---|---|---|---|
| | Nucleotide change and position | Effect on protein | Mutation frequency* | Nucleotide change and position | Effect on protein | Mutation frequency* |
| 6M | NM | None | 0/3 | NM | None | 0/3 |
| 1βR | NM | None | 0/3 | G to U at nt 475 | E to stop at codon 159 | 13/13 |
| 3βR | Deletion of 5 nt at nt 376 | Truncated (135 aa) | 2/11 | NM | None | 0/3 |
| | A to U at nt 1372 | K to stop at codon 458 | 3/11 | | | |
| 4βR | G to U at nt 319 | E to stop at codon 107 | 12/12 | NM | None | 0/3 |
| 5βR | Deletion of 176 nt at nt 201 | Truncated (78 aa) | 4/10 | NM | None | 0/3 |
| | Deletion of 79 nt at nt 201 | Truncated (67 aa) | 2/10 | | | |
| 1αR | NM | None | 0/3 | NM | None | 0/3 |
| 3αR | NM | None | 0/3 | NM | None | 0/3 |
| 4αR | Deletion of 5 nt at nt 376 | Truncated (135 aa) | 1/3 | NM | None | 0/3 |
| 5αR | Deletion of 79 nt at nt 201 | Truncated (67 aa) | 1/3 | NM | None | 0/3 |
| αRmix | NM | None | 0/3 | NM | None | 0/3 |
| 6Mc | NM | None | 0/3 | NM | None | 0/3 |

*Number of mutated or truncated clones/number of examined clones.

as described above. The results revealed that the nonsense mutations or deletions identified at this time (see Supplementary Table S1, available in JGV Online) were quite different from those obtained from the βR series (Table 1). Therefore, it is unlikely that the identified IFNAR mutants pre-existed in cloned 1, 3, 4 and 5 cells when these cells were obtained as colonies surviving IFN-α treatment.

To examine whether or not additional HCV replicon cell lines possessing the IFN-resistant phenotype could be obtained from HCV replicon-harbouring cells other than the parental replicon-harbouring cells used for the isolation of the αR and βR series, 6M.m/6Mc, 1βR.m/6Mc, 4βR.m/6Mc and 50-1 replicon-harbouring cells were treated with IFN-β (see Supplementary Fig. S2). Finally, we obtained four replicon-harbouring cell lines (6βR obtained from the 4βR.m/6Mc cells, 7βR obtained from the 6M.m/6Mc cells, and 8βR and 9βR obtained from the 50-1 cells) showing resistance to IFN-β. These results indicated that HCV replicon-harbouring cells showing the IFN-resistant phenotype were obtained from HCV replicon-harbouring cells established immediately. By sequence analysis of IFNAR1 and IFNAR2c cDNAs as described above, the E107stop nonsense mutation in IFNAR1 cDNA, which was the same mutation found in the 4βR cells, was found again in the 8βR and 9βR cells, while no IFNAR mutations were detected in the 6βR and 7βR cells (see Supplementary Table S1). The observation that IFNAR mutations occurred preferentially after IFN-β treatment is interesting. Since a variety of mutations and deletions in the IFN receptors were obtained from the cloned replicon-harbouring cells surviving after IFN-β treatment, such genetic alterations might occur accidentally in order to impair the antiviral states caused after IFN-β treatment. Thereafter, only replicon-harbouring cells possessing the IFNAR mutants might be able to proliferate in the presence of G418, resulting in the βR series.

To clarify whether or not the IFNAR mutations found in the βR series were determinants for the IFN sensitivity of HCV replicons, we prepared 4βR cells (possessing the IFNAR1 mutant) stably expressing wild-type IFNAR1 and examined its IFN sensitivity. Analysis of a luciferase reporter assay (see Supplementary Fig. S3a, available in JGV Online) clearly showed that IFN signalling in 4βR cells was restored by the expression of wild-type IFNAR1 in comparison with those of 4βR cells expressing the IFNAR1 mutant (see Supplementary Fig. S3b). The quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-β clearly showed that the level of 4βR replicon in cells expressing wild-type IFNAR1 was drastically decreased after IFN-β treatment, as was the level of 6M replicon in cells expressing wild-type IFNAR1 (see Supplementary Fig. S3c). In summary, we demonstrated that the IFNAR mutation found in 4βR cells was a major determinant for a strongly IFN-resistant phenotype of 4βR cells, suggesting that IFNAR mutations, which lead to the impairment of IFN signalling, convert HCV replicon-harbouring cells from an IFN-sensitive phenotype to a highly IFN-resistant phenotype.

IFNAR1 and IFNAR2c belonging to the class II cytokine receptor superfamily are structurally conserved transmembrane receptors located on the cell surface (see Supplementary Fig. S4a, available in JGV Online). However, since both the IFNAR1 E107stop mutant and the IFNAR2c E159stop mutant found in 4$\beta$R and 1$\beta$R cells, respectively, were N-terminally truncated and probably soluble forms, these truncated proteins may not be functional as IFN receptors or may act as dominant-negative inhibitors, and will lead to the interception of IFN signalling (see Supplementary Fig. S4b). Thus, the cause of the IFN-resistant phenotype of 1$\beta$R or 4$\beta$R cells appeared to be the functional disruption of IFNAR. The present results suggest that the downstream JAK/STAT pathway is intact, at least in 4$\beta$R cells.

Although for the most part we could clarify the mechanism underlying a highly IFN-resistant phenotype of HCV replicon-harbouring cells, at least in the case of 4$\beta$R cells, the mechanism underlying a partially IFN-resistant phenotype remains unclear, because IFNAR mutations were rare in the $\alpha$R series. Since the expression levels of IFNAR, TYK2 and JAK1 were not decreased in the $\alpha$R series, a functional deficiency of other cellular factor(s) involved in the IFN signalling may contribute to the acquisition of IFN resistance. Alternatively, certain HCV mutation(s) may account for the partially IFN-resistant phenotype of the $\alpha$R series.

Since Machida *et al.* (2004a, b) recently reported that the frequency of genetic mutation was enhanced by HCV replication in *in vitro*-infected B cells and that the HCV core and NS3 were involved in the induction of a mutator phenotype mediated through the activation of inducible nitric oxide synthase, we cannot exclude the possibility that persistent HCV replication induces some irreversible genetic mutations. To clarify whether or not HCV acts as a mutagen for cellular factors, further study using an HCV RNA replication system (Ikeda *et al.*, 2002, 2005; Naka *et al.*, 2005) will also be necessary.

The HCV replicon-harbouring cells including 1$\beta$R and 4$\beta$R, in which IFN signalling is impaired, used or obtained in the present study may be useful for future studies, not only of the mechanism(s) underlying the IFN resistance of the replicons but also of the functional characterization of IFN receptors. Furthermore, these replicon cells may also be useful for screening novel anti-HCV reagents that act by mechanisms unrelated to IFN signalling.

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Short Communication

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

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

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

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

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

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

Supplementary material is available in JGV Online.

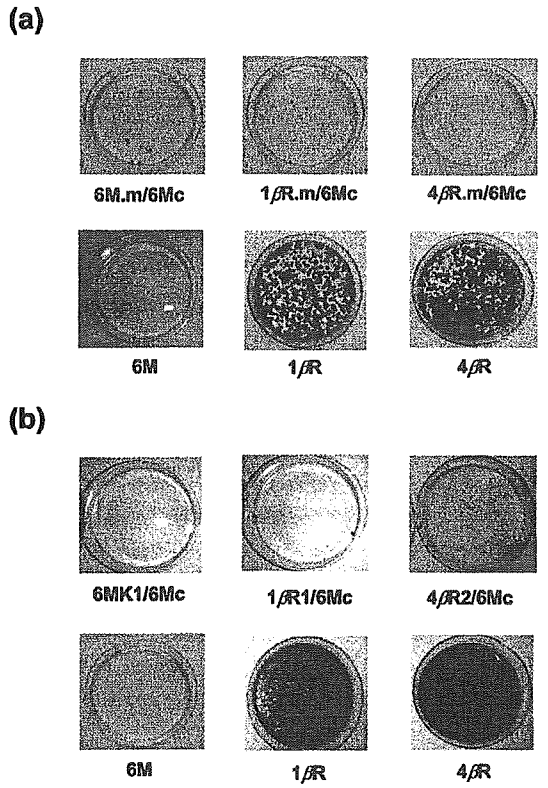


**Fig. 1.** IFN sensitivities of various HCV replicon-harbouring cells. (a) 6M.m/6Mc, 1βR.m/6Mc and 4βR.m/6Mc cells obtained as G418-resistant mixed colonies were treated with IFN-α (400 IU $ml^{-1}$) for 3 weeks in the presence of G418 (300 μg $ml^{-1}$). 6M, 1βR and 4βR cells were also used for control experiments. G418-resistant colonies were stained with Coomassie brilliant blue as described previously (Naganuma *et al.*, 2004). (b) IFN sensitivities of 6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc cells were examined as described in (a) except that they were treated with IFN-β (200 IU $ml^{-1}$).

3 weeks). Although many colonies were found to have survived in 1βR and 4βR cells after IFN-α treatment, only a few colonies survived in 1βR.m/6Mc and 4βR.m/6Mc cells or in 6M and 6M.m/6Mc cells (Fig. 1a). Similar results were obtained when these replicon-harbouring cells were treated with IFN-β (400 IU $ml^{-1}$) (data not shown), although two colonies (named 6βR and 7βR, described below) derived from 6M.m/6Mc and 4βR.m/6Mc cells, respectively, proliferated as highly IFN-resistant clones.

We further examined the IFN sensitivity of replicon-harbouring cells (6MK1/6Mc, 1βR1/6Mc and 4βR2/6Mc) established by the transfection of *in vitro*-synthesized replicon RNAs (6MK1, 1βR1 and 4βR2 obtained from 6M, 1βR and 4βR cells, respectively) (Namba *et al.*, 2004; Kato *et al.*, 2005) into 6Mc cells. The results revealed that few or no colonies survived in 1βR1/6Mc and 4βR2/6Mc cells, as in 6M and 6MK1/6Mc cells, whereas many colonies survived in 1βR and 4βR cells (Fig. 1b). These results suggested that cellular factors rather than viral factors contributed to the highly IFN-resistant phenotype of HCV replicon-harbouring cells. However, the present results obtained under a high concentration of IFN do not necessarily rule out a possible role for HCV mutations in conferring low degrees of IFN resistance, because effects of HCV mutations on IFN activity are presumably weaker than those of the cellular factors.

To obtain evidence in favour of the idea that alterations in cellular factor(s) are involved in the emergence of an IFN-resistant phenotype, we attempted to prepare cured cells from the replicon-harbouring cells possessing a highly IFN-resistant phenotype. Since phosphorylation of signal transducer and activator of transcription 1 (STAT1) occurred in the 1βR and 4βR cells treated with IFN-γ (Fig. 2a), these replicon-harbouring cells were treated with IFN-γ (500 IU $ml^{-1}$) for 3 weeks, and cured 1βRc and 4βRc cells were obtained. Western blot (Fig. 2b) and RT-PCR (data not shown) analyses showed that no replicons were detected in either type of cured cells. Analysis of a luciferase reporter assay indicated that the complete defect of the IFN-α/β signalling was not restored in the cured 1βRc and 4βRc cells (Fig. 2c).

To clarify whether or not the signalling defect in these replicon-harbouring cells was restricted to type I IFN, we examined the phosphorylation status of STAT3 in 6M, 1βR and 4βR cells treated with interleukin-6 (IL6). Since it has been reported that STAT3 is also activated by IFN-α treatment (Pfeffer *et al.*, 1997), the phosphorylation status of STAT3 in these replicon-harbouring cells after IFN-α treatment was also examined. Our results revealed that STAT3 was not phosphorylated in 1βR and 4βR cells treated with IFN-α, while phosphorylation of STAT3 was observed in 6M, 1βR and 4βR cells treated with IL6 and in 6M cells treated with IFN-α (Fig. 2d), indicating that only type I IFN signalling was defective in 1βR and 4βR cells. These results suggested that the initial reaction following the addition of IFN-α/β was defective in replicon-harbouring cells possessing a highly IFN-resistant phenotype.

Following up this suggestion, we examined the genetic status of tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1). Sequence analysis of TYK2 and JAK1 cDNAs obtained from 1βR and 4βR cells was performed after cloning into the pCXbsr vector (Akagi *et al.*, 2000), as described previously (Nozaki *et al.*, 2003). However, the results showed no mutations in these cDNAs (data not shown). We next focused on type I IFN receptors (IFNAR1 and IFNAR2c). Our results showed that the mRNA levels of the two receptors were almost equal among all examined replicon-harbouring cells including 6Mc cells (see Supplementary Fig. S1a, available in JGV Online). More than three independent clones of each cDNA (1708 bp for IFNAR1 and 1582 bp for IFNAR2c) were sequenced as described above. Table 1 shows a summary of sequence analysis of IFNAR1

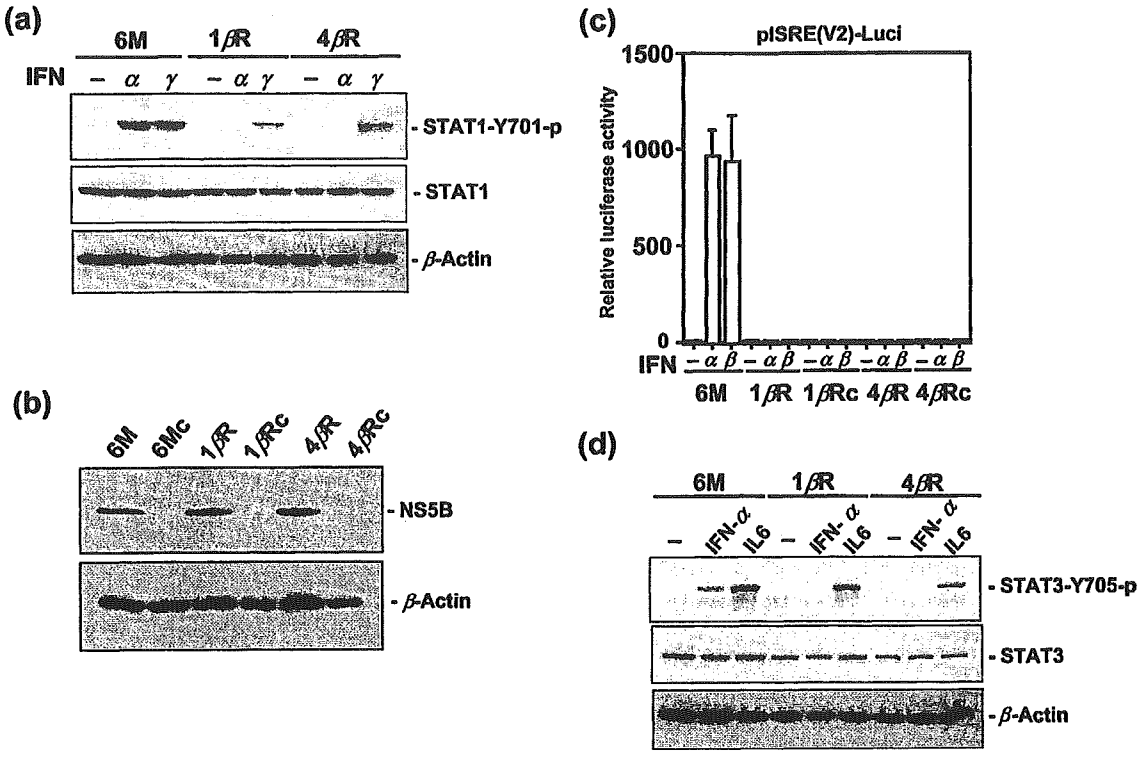


**Fig. 2.** Cellular factors rather than HCV replicons contribute to defects in type I IFN signalling. (a) Western blot analysis of STAT1 in 6M, 1$\beta$R and 4$\beta$R cells treated with IFN-$\alpha$ or -$\gamma$. The replicon-harbouring cells were treated with or without IFN-$\alpha$ or -$\gamma$ (500 IU ml$^{-1}$ each) for 30 min, and Western blot analysis for STAT1 and its phosphorylation status (Tyr-701) was then performed as previously described (Hijikata *et al.*, 1993). $\beta$-Actin was used as a control for the amount of protein loaded per lane. (b) Western blot analysis of NS5B. Anti-NS5B antibody was used for immunoblotting. $\beta$-Actin was used as described in (a). (c) Analysis of IFN signal transduction. Dual luciferase assays using pISRE(V2)-Luci (Dansako *et al.*, 2003) were performed as previously described (Naganuma *et al.*, 2000). Cells were treated with IFN-$\alpha$ or -$\beta$ (500 IU ml$^{-1}$ each) for 6 h. (d) The defect in the signalling pathway in 1$\beta$R and 4$\beta$R cells is restricted to type I IFN. Replicon-harbouring cells were left untreated or treated with IFN-$\alpha$ (500 IU ml$^{-1}$) or IL6 (100 ng ml$^{-1}$) for 30 min. Western blot analysis for STAT3 and its phosphorylation status (Tyr-705) was performed as described in (a).

and IFNAR2c mRNAs. Surprisingly, we found that nt 475 of IFNAR2c mRNA from 1$\beta$R cells had a U substituted for G in 13/13 clones, resulting in a nonsense mutation at codon 159 from glutamic acid (GAG) to the termination codon UAG (see Supplementary Fig. S1b). Furthermore, nt 319 of IFNAR1 mRNA from 4$\beta$R cells was also found to have a U substituted for G in 12/12 clones, resulting in a nonsense mutation at codon 107 from glutamic acid (GAA) to the termination codon UAA (see Supplementary Fig. S1c). However, interestingly, IFNAR1 and IFNAR2c mRNAs obtained from 1$\alpha$R and 4$\alpha$R cells, which were derived from clone 1 and clone 4 and were counterparts of 1$\beta$R and 4$\beta$R cells, respectively (see Supplementary Fig. S2, available in JGV Online), did not possess the nonsense mutations found in the mRNAs from 1$\beta$R and 4$\beta$R cells. In addition, several kinds of deletion and another nonsense mutation (lysine to a termination codon at codon 458) were found in approximately half of IFNAR1 cDNA clones obtained from 3$\beta$R and 5$\beta$R cells. In contrast to the finding of frequent mutations and deletions in IFNAR mRNAs from the $\beta$R series, such genetic abnormalities in IFN receptors were quite rare in the $\alpha$R series (Table 1).

To evaluate the possibility that genetic mutants might pre-exist in the cloned replicon-harbouring cells (clones 1, 3, 4 and 5 shown in supplementary Fig. S2) or that genetic mutants had appeared during the IFN-$\beta$ treatment of the cloned replicon-harbouring cells, we repeated IFN-$\beta$ treatment of cloned 1, 3, 4 and 5 cells following the method described previously (Namba *et al.*, 2004). The result was almost identical to that obtained previously (Namba *et al.*, 2004), indicating the good reproducibility of the experiment with IFN-$\beta$ treatment (data not shown). In the present study, each of the three colonies showing resistance to IFN-$\beta$ was isolated and proliferated (see Supplementary Fig. S2) and we then performed sequence analysis of IFNAR mRNAs

**Table 1.** Genetic alterations of type I IFN receptors in HCV replicon-harbouring cell lines possessing IFN-resistant phenotypes

The determined nucleotide sequences were compared with those of 6M and 6Mc cells, which confirmed that the deduced amino acid sequences were identical to the human IFNAR1 (GenBank accession no. NM_000629) and IFNAR2c (GenBank accession no. L41942) sequences. NM, Not mutated.

| Cell line | IFNAR1 mRNA | | | IFNAR2c mRNA | | |
|---|---|---|---|---|---|---|
| | Nucleotide change and position | Effect on protein | Mutation frequency* | Nucleotide change and position | Effect on protein | Mutation frequency* |
| 6M | NM | None | 0/3 | NM | None | 0/3 |
| 1βR | NM | None | 0/3 | G to U at nt 475 | E to stop at codon 159 | 13/13 |
| 3βR | Deletion of 5 nt at nt 376 | Truncated (135 aa) | 2/11 | NM | None | 0/3 |
| | A to U at nt 1372 | K to stop at codon 458 | 3/11 | | | |
| 4βR | G to U at nt 319 | E to stop at codon 107 | 12/12 | NM | None | 0/3 |
| 5βR | Deletion of 176 nt at nt 201 | Truncated (78 aa) | 4/10 | NM | None | 0/3 |
| | Deletion of 79 nt at nt 201 | Truncated (67 aa) | 2/10 | | | |
| 1αR | NM | None | 0/3 | NM | None | 0/3 |
| 3αR | NM | None | 0/3 | NM | None | 0/3 |
| 4αR | Deletion of 5 nt at nt 376 | Truncated (135 aa) | 1/3 | NM | None | 0/3 |
| 5αR | Deletion of 79 nt at nt 201 | Truncated (67 aa) | 1/3 | NM | None | 0/3 |
| αRmix | NM | None | 0/3 | NM | None | 0/3 |
| 6Mc | NM | None | 0/3 | NM | None | 0/3 |

*Number of mutated or truncated clones/number of examined clones.

as described above. The results revealed that the nonsense mutations or deletions identified at this time (see Supplementary Table S1, available in JGV Online) were quite different from those obtained from the βR series (Table 1). Therefore, it is unlikely that the identified IFNAR mutants pre-existed in cloned 1, 3, 4 and 5 cells when these cells were obtained as colonies surviving IFN-α treatment.

To examine whether or not additional HCV replicon cell lines possessing the IFN-resistant phenotype could be obtained from HCV replicon-harbouring cells other than the parental replicon-harbouring cells used for the isolation of the αR and βR series, 6M.m/6Mc, 1βR.m/6Mc, 4βR.m/6Mc and 50-1 replicon-harbouring cells were treated with IFN-β (see Supplementary Fig. S2). Finally, we obtained four replicon-harbouring cell lines (6βR obtained from the 4βR.m/6Mc cells, 7βR obtained from the 6M.m/6Mc cells, and 8βR and 9βR obtained from the 50-1 cells) showing resistance to IFN-β. These results indicated that HCV replicon-harbouring cells showing the IFN-resistant phenotype were obtained from HCV replicon-harbouring cells established immediately. By sequence analysis of IFNAR1 and IFNAR2c cDNAs as described above, the E107stop nonsense mutation in IFNAR1 cDNA, which was the same mutation found in the 4βR cells, was found again in the 8βR and 9βR cells, while no IFNAR mutations were detected in the 6βR and 7βR cells (see Supplementary Table S1). The observation that IFNAR mutations occurred preferentially after IFN-β treatment is interesting. Since a variety of mutations and deletions in the IFN receptors were obtained from the cloned replicon-harbouring cells surviving after IFN-β treatment, such genetic alterations might occur accidentally in order to impair the antiviral states caused after IFN-β treatment. Thereafter, only replicon-harbouring cells possessing the IFNAR mutants might be able to proliferate in the presence of G418, resulting in the βR series.

To clarify whether or not the IFNAR mutations found in the βR series were determinants for the IFN sensitivity of HCV replicons, we prepared 4βR cells (possessing the IFNAR1 mutant) stably expressing wild-type IFNAR1 and examined its IFN sensitivity. Analysis of a luciferase reporter assay (see Supplementary Fig. S3a, available in JGV Online) clearly showed that IFN signalling in 4βR cells was restored by the expression of wild-type IFNAR1 in comparison with those of 4βR cells expressing the IFNAR1 mutant (see Supplementary Fig. S3b). The quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-β clearly showed that the level of 4βR replicon in cells expressing wild-type IFNAR1 was drastically decreased after IFN-β treatment, as was the level of 6M replicon in cells expressing wild-type IFNAR1 (see Supplementary Fig. S3c). In summary, we demonstrated that the IFNAR mutation found in 4βR cells was a major determinant for a strongly IFN-resistant phenotype of 4βR cells, suggesting that IFNAR mutations, which lead to the impairment of IFN signalling, convert HCV replicon-harbouring cells from an IFN-sensitive phenotype to a highly IFN-resistant phenotype.

IFNAR1 and IFNAR2c belonging to the class II cytokine receptor superfamily are structurally conserved transmembrane receptors located on the cell surface (see Supplementary Fig. S4a, available in JGV Online). However, since both the IFNAR1 E107stop mutant and the IFNAR2c E159stop mutant found in 4$\beta$R and 1$\beta$R cells, respectively, were N-terminally truncated and probably soluble forms, these truncated proteins may not be functional as IFN receptors or may act as dominant-negative inhibitors, and will lead to the interception of IFN signalling (see Supplementary Fig. S4b). Thus, the cause of the IFN-resistant phenotype of 1$\beta$R or 4$\beta$R cells appeared to be the functional disruption of IFNAR. The present results suggest that the downstream JAK/STAT pathway is intact, at least in 4$\beta$R cells.

Although for the most part we could clarify the mechanism underlying a highly IFN-resistant phenotype of HCV replicon-harbouring cells, at least in the case of 4$\beta$R cells, the mechanism underlying a partially IFN-resistant phenotype remains unclear, because IFNAR mutations were rare in the $\alpha$R series. Since the expression levels of IFNAR, TYK2 and JAK1 were not decreased in the $\alpha$R series, a functional deficiency of other cellular factor(s) involved in the IFN signalling may contribute to the acquisition of IFN resistance. Alternatively, certain HCV mutation(s) may account for the partially IFN-resistant phenotype of the $\alpha$R series.

Since Machida *et al.* (2004a, b) recently reported that the frequency of genetic mutation was enhanced by HCV replication in *in vitro*-infected B cells and that the HCV core and NS3 were involved in the induction of a mutator phenotype mediated through the activation of inducible nitric oxide synthase, we cannot exclude the possibility that persistent HCV replication induces some irreversible genetic mutations. To clarify whether or not HCV acts as a mutagen for cellular factors, further study using an HCV RNA replication system (Ikeda *et al.*, 2002, 2005; Naka *et al.*, 2005) will also be necessary.

The HCV replicon-harbouring cells including 1$\beta$R and 4$\beta$R, in which IFN signalling is impaired, used or obtained in the present study may be useful for future studies, not only of the mechanism(s) underlying the IFN resistance of the replicons but also of the functional characterization of IFN receptors. Furthermore, these replicon cells may also be useful for screening novel anti-HCV reagents that act by mechanisms unrelated to IFN signalling.

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