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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 (5×10^4 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

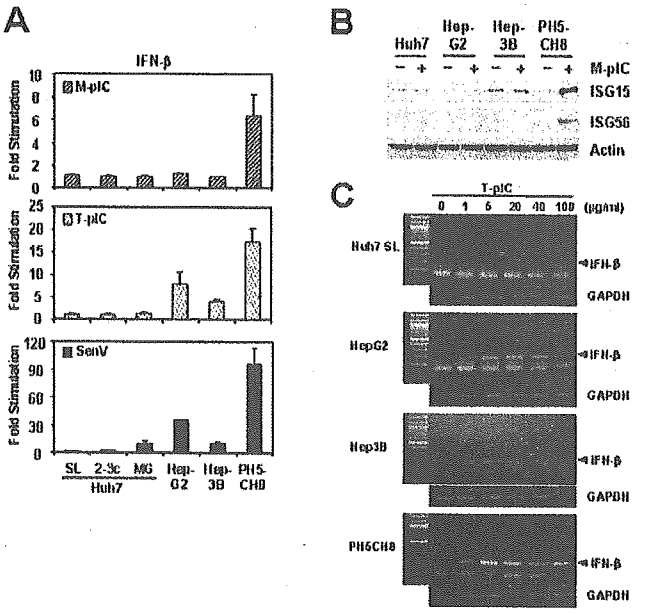

FIG. 1. **Activation of IFN-β transcription in various hepatocyte cell lines by dsRNA and virus.** *A*, cells grown in 24-well plates were cotransfected with IFN-β Luc and pCMV-β-galactosidase for 24 h before stimulation with 50 μg/ml poly(I-C) directly added to culture medium for 6 h (*M-pIC*, *upper 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

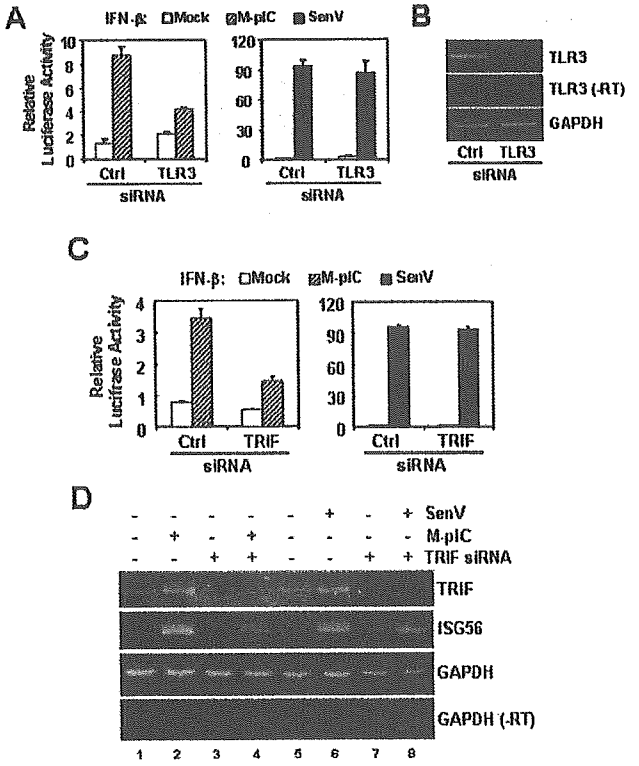

FIG. 2. **Extracellular dsRNA, but not SenV, activates IFN response via TLR3 and TRIF in PH5CH8 hepatocytes.** *A*, TLR3 expression is required for activation of IFN-β promoter by M-pIC but not by SenV. PH5CH8 cells grown in 24-well plates were transfected with control (*Ctrl*) or TLR3 siRNA, and pIFN-β 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.

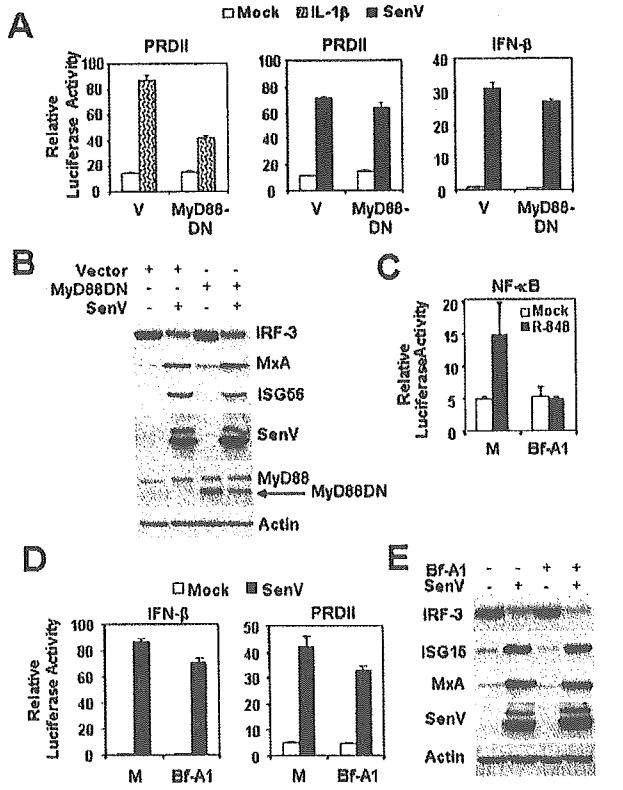

FIG. 3. **SenV activation of type I IFN response in hepatocytes is MyD88-independent and does not require endosomal acidification.** *A*, PH5CH8 cells in 24-well plates were cotransfected with the indicated reporter plasmids (100 ng), pCMV-β-galactosidase (100 ng), and 600 ng of a dominant-negative form 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*.

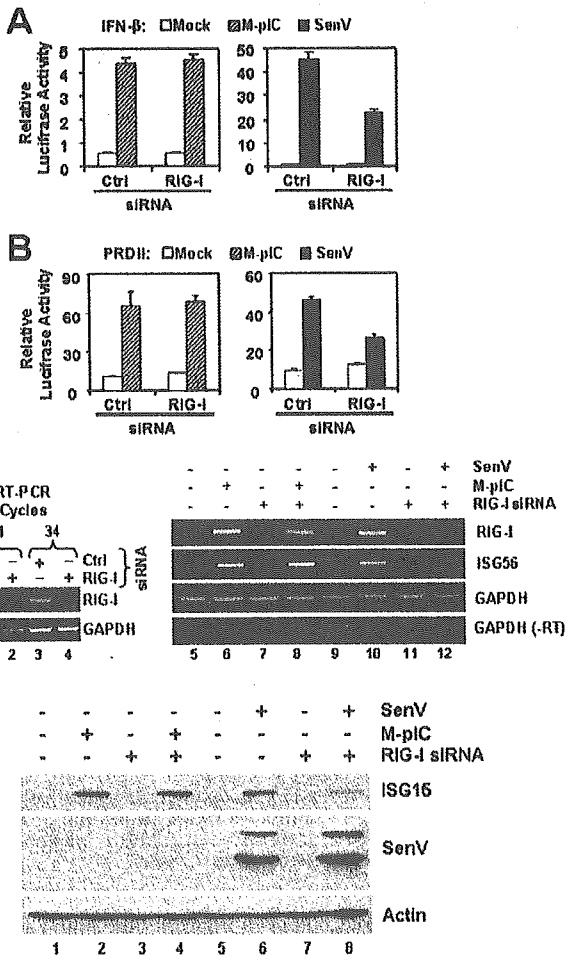

FIG. 4. **SenV, but not TLR3 engagement, activates type I IFN response through RIG-I in hepatocytes.** PH5CH8 cells grown in 24-well plates were transfected with control (*Ctrl*) or RIG-I siRNA, and pIFN-βLuc (*A*) or PRDII Luc (*B*), and pCMV-β-galactosidase. 48 h later, cells were mock-treated or stimulated 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

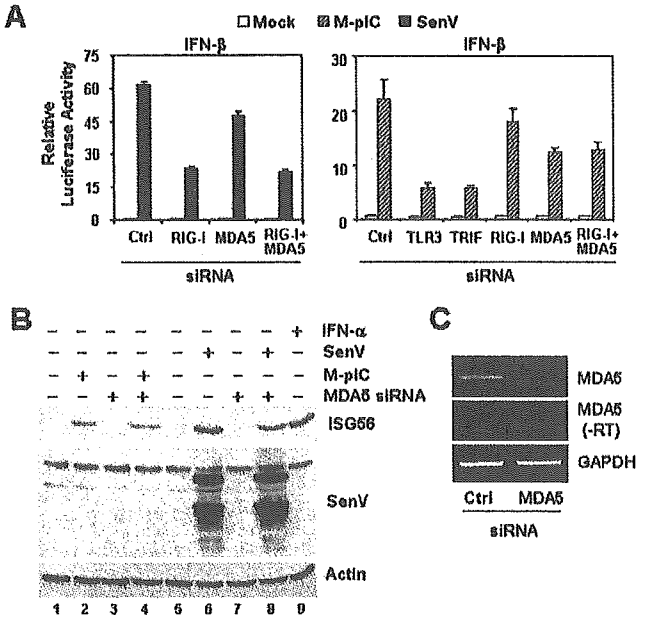

FIG. 5. **Poly(I-C), as well as SenV activation of the IFN-β promoter is partially MDA5-dependent in hepatocytes.** *A*, SenV (*left panel*) and M-pIC (*right panel*)-induced IFN-β promoter activity in PH5CH8 cells transfected with the indicated siRNAs. *B*, immunoblot detection of ISG56 and SenV proteins 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.

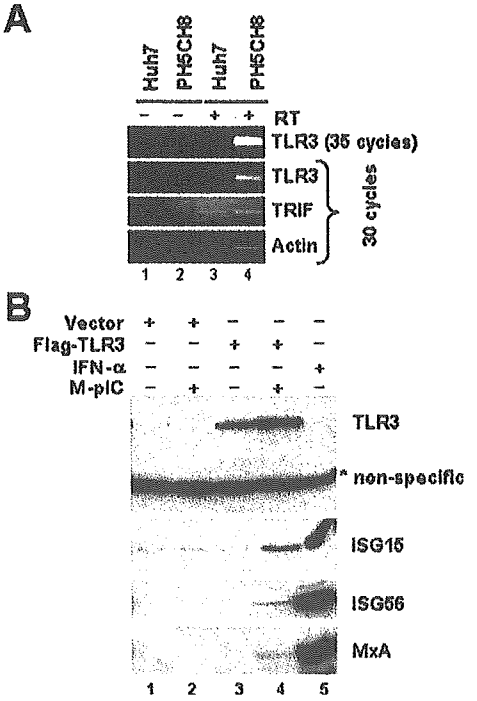

FIG. 6. **The defect of TLR3 signaling in Huh7 cells is because of insufficient expression of TLR3.** *A*, semiquantitative RT-PCR detection of TLR3, TRIF, and β-actin mRNAs in Huh7 and PH5CH8 cells. *B*, ectopic expression a FLAG-tagged TLR3 restored the induction of ISG15, ISG56, and MxA expression 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

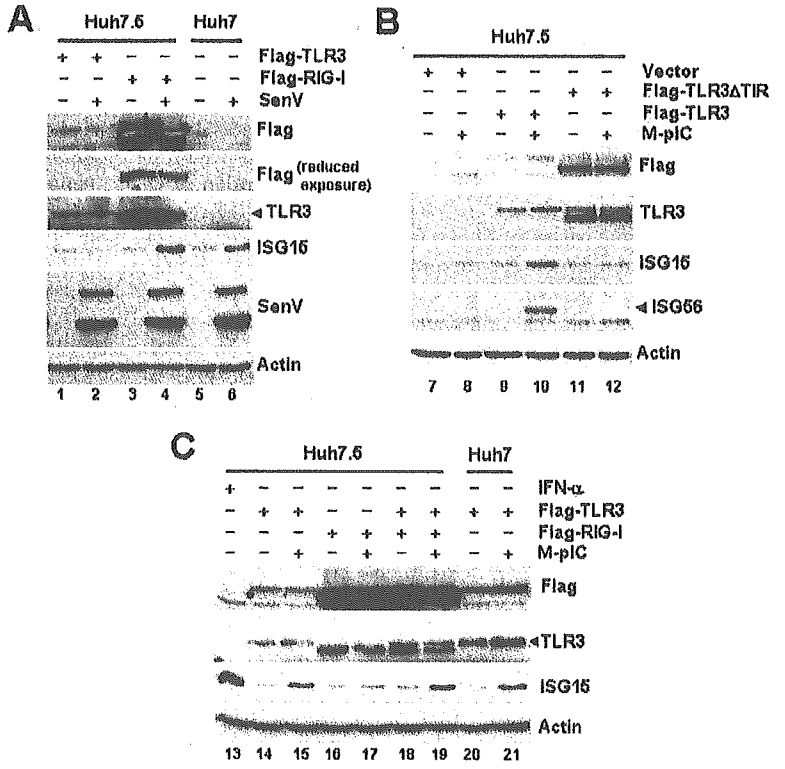

FIG. 7. **TLR3 and RIG-I signaling pathways function independently in Huh7.5 cells.** *A*, the defect of SenV-induced ISG15 expression can be restored by overexpression of RIG-I, but not TLR3 in Huh7.5 cells. *B*, the defect of extracellular dsRNA-induced expression of ISG15 and ISG56 can be restored 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-β 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-β 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-κB 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-α 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-β 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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cDNA microarray analysis to compare HCV subgenomic replicon cells with their cured cells

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Abstract

The hepatitis C virus (HCV) replicon system carrying autonomously replicating HCV subgenomic RNA in human hepatocyte cells is a potent tool for basic studies of HCV, such as viral replication and drug development. Recently, we developed two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively. Since the expression of HCV proteins is thought to affect the host cells' gene expression profiles, we attempted to identify target genes of HCV proteins using microarray analysis (9970 genes) by comparing 50-1 and 1B-2R1 replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with interferon-α. The results showed that HCV replicons could have a variety of expression profiles in human hepatocytes. The results also showed that 2 and 6 genes were commonly up-regulated (more than 2.0-fold) and down-regulated (less than 0.50-fold), respectively, in both 50-1 and 1B-2R1 replicon cells compared with their cured cells. The differential expression profiles of genes selected by the microarray analysis were confirmed with standard RT-PCR and real-time LightCycler PCR. It was noteworthy that the commonly down-regulated genes contained large multifunctional proteases 2 and 7, which are known as catalytic subunits of immunoproteasome, and serine proteinase inhibitor clade C. Our microarray analysis demonstrated that HCV subgenomic replicons can change the gene expression profiles of host cells, and it allowed us to compile the first list of genes that the replicons transcriptionally regulate.

Keywords: Hepatitis C virus; HCV subgenomic replicon; Cured cells; cDNA microarray; Gene expression profile

1. Introduction

Hepatitis C virus (HCV) infection frequently causes chronic hepatitis (Choo et al., 1989; Kuo et al., 1989), which progresses to liver cirrhosis and hepatocellular carcinoma (Ohkoshi et al., 1990; Saito et al., 1990). HCV belongs to the family Flaviviridae, whose genome consists of a positive-stranded RNA molecule of about 9.6 kb and encodes a large polyprotein precursor of about 3000 amino acids (Kato et al., 1990; Tanaka et al., 1995). This precursor protein is cleaved by the host and viral proteases to generate at least ten proteins: the core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. Although many hypotheses have been proposed over the past decade regarding the functions of the viral proteins (Bartenschlager and Lohmann, 2000; Kato, 2001), the lack of reproducible and efficient HCV proliferation in cell cultures (Kato and Shimotohno, 2000) has been a serious obstacle in understanding those proteins' actual functions.

However, in 1999, an HCV replicon system carring autonomously replicating HCV subgenomic RNA containing the NS3-NS5B regions was first established using a human hepatoma cell line, Huh-7 (Lohmann et al., 1999). Since then, several additional replicons have also been established (Blight et al., 2000, 2003; Ikeda et al., 2002; Kato et al., 2003b). In these systems, replicated HCV RNAs were detected by Northern blot analysis, and the HCV proteins pro-

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duced were detected by Western blot analysis. Therefore, the system of HCV replicons has become a powerful tool for basic studies in HCV, such as viral replication and drug development (Bartenschlager, 2002).

Recently, we also established two HCV subgenomic replicons (50-1 and 1B-2R1) derived from two HCV strains, 1B-1 and 1B-2, respectively, using Huh-7 cells (Kato et al., 2003a; Kishine et al., 2002). We demonstrated that the 50-1 and 1B-2R1 subgenomic replicons (Kato et al., 2003a) were sensitive to interferon (IFN)-α, IFN-β and IFN-γ as are the other replicons (Frese et al., 2001, 2002). The nucleotide sequences of the NS3-NS5B regions in the 50-1 subgenomic replicon showed differences of 8.1% from those in the1B-2R1 subgenomic replicon (Kato et al., 2003a), although both the 1B-1 and 1B-2 strains belong to genotype 1b. Although the efficient replication of an HCV subgenomic replicon expressing HCV proteins is considered to affect the gene expression profiles of host cells (Bartenschlager and Lohmann, 2000; Kato, 2001), few reports have demonstrated inclusive searches for HCV's target genes (Zhu et al., 2003). Therefore, we thought a comprehensive search for HCV subgenomic replicon-regulated cellular genes would be important in understanding the molecular interplay exerted by HCV in vivo.

In the present study, to obtain the candidates of HCV's target genes, we performed cDNA microarray analysis by comparing two types of HCV subgenomic replicon cells with their "cured cells", from which the replicons had been eliminated by prolonged treatment with IFN-α. Here we report on the differential gene expression profiles in the replicon cells, and we first provide a list of genes that the replicons transcriptionally regulate.

2. Materials and methods

2.1. Cell cultures

50-1 and 1B-2R1 cells possessing 50-1 and 1B-2R1 subgenomic replicons, respectively, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 mg/ml; Geneticine, Invitrogen). The 50-1 and 1B-2R1 cells were known to possess the G418-resistant phenotype, because neomycin phophotransferase (NEOR) was produced by the efficient replication of HCV subgenomic replicon in the cells. When an HCV subgenomic replicon is excluded from the cells or its level is decreased, the cells are killed by the presence of G418. Therefore, the cured cells obtained from 50-1 and 1B-2R1 cells were maintained in the absence of G418.

2.2. IFN treatment

To prepare the cured cells, 50-1 and 1B-2R1 cells (each 1×10^6) were plated onto 10-cm plates and were cultured for 1 day immediately before IFN treatment. Human IFN-α (Sigma) was added to the cells at a final concentration of 3000 IU/ml as described previously (Kato et al., 2003a). The incubation in the absence of G418 was continued for 3 weeks with the addition of IFN-α (3000 IU/ml) at 4-day intervals. The cured cells obtained from 50-1 and 1B-2R1 cells were named 50-1C and 1B-2R1C cells, respectively.

2.3. Northern blot analysis

Total RNAs from the cultured cells were prepared using the RNeasy extraction kit (Qiagen). Three micrograms of total RNA was used to detect the HCV replicon RNA and β-actin. Northern blotting and hybridization were performed as described previously (Ikeda et al., 2002; Kato et al., 2003a). As a molecular length marker, replicon RNA synthesized in vitro from replicon cassette plasmid pNSS1RZ2RU (Kato et al., 2003a) was also utilized.

2.4. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting analysis with a polyvinylidene difluoride membrane were performed as previously described (Hijikata et al., 1993). The antibodies used in this study were those against NS3 (Novacastra Laboratories, UK), NS5B (a generous gift from M. Kohara, Tokyo Metropolitan Institute of Medical Science), and β-actin (Sigma). Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin-Elmer Life Sciences).

2.5. cDNA microarray analysis

The 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells (each 1×10^6 cells) were plated onto 10 cm plates, and each plate was cultured for 5 days in the absence of G418. The confluent cells were harvested and total RNAs were prepared using the RNeasy extraction kit (Qiagen). Using the obtained total RNAs, cDNA microarray analysis (CodeLink™, Uniset human I containing 9970 spots of 30-mer oligonucleotides; Amersham Biosciences) was performed by Kurabo Industries Ltd. (Osaka, Japan) with the authorization of Amersham Biosciences.

2.6. Analysis of mRNA expression by RT-PCR

The total RNAs (each 2 μg) that were the same as those subjected to cDNA microarray analysis were reverse-transcribed with Superscript II using an oligo dT primer (Invitrogen). One-tenth of the synthesized cDNA was subjected to PCR. The PCR primers are listed in Table 1. After 10 min at 98 °C, PCR was performed with Taq DNA polymerase (TaKaRa, Japan). Each cycle consisted of annealing at 60 °C (64 °C for LMP2 and LMP7 only) for 45 s, primer extension at 72 °C for 1 min, and denaturation at 94 °C for 20 s. The cycle numbers and the size of PCR products were also

Table 1
The primers used for RT-PCR analysis of mRNA expression

Genes	Orientation	Nucleotide sequence	Product (bp)	Cycles
Large multifunctional protease2 (LMP2)	Forward	ATGGAACCCTGGGAGGAATGCTG	145	30
	Reverse	GCAATAGCGTCTGTGGTGAAGCG		
Large multifunctional protease 7 (LMP7)	Forward	CTGGGATAAGAAGGGTCCTGGAC	293	27
	Reverse	TACTGGTGCAGCAGGTCACTGGAC		
Serine proteinase inhibitor (serpin) clade C	Forward	TGGATGAATTGGAGGAGATGATGC	249	25
	Reverse	CAATCACAACAGCGGTACTTGCAG		
S100-type calcium binding protein A14	Forward	CAGAGGATGCTCAGGAATTCAGTG	256	27
	Reverse	CTCTTGGCCGCTTCTCCAATGAG		
Latent transforming growth factor β binding protein 1 (LTBP1)	Forward	GCCTTGGTTGACTTCAGTGAACAG	325	27
	Reverse	CAGAAGGCACGTAGCCTGGCAG		
Weakly similar to zinc finger protein 91	Forward	CCAGAACCACATCCAAACCATCC	299	33
	Reverse	CCATCCCTTCGAAGCTGTGCTC		
Transgelin	Forward	GATTCTGAGCAAGCTGGTGAACAG	254	25
	Reverse	AGTGCCCATCATTCTTGGTCACTG		
Annexin A1	Forward	GATGCCAGGGCCTTGTATGAAGC	264	25
	Reverse	AACACCTTTCATGGCTTGATGAAGC		
Solute carrier family 7	Forward	AGTCCTTCGCTGGAAGAAGCCTG	314	27
	Reverse	CCATGTCCTCATTAGCCTCCTCTG		
Protein phosphatase 1 regulatory subunit 1A	Forward	CCACGGCAACGGAAGAAGATGAC	302	27
	Reverse	GCTCCCTTGGAATCCAGTGGTGG		
Phosphatidylserine-specific phospholipase A1α	Forward	GAGAAACAAGGACACCAACATCGAG	288	28
	Reverse	GTCACACTTGCTTGTAAGTTCACTG		
Oncostatin M receptor	Forward	CAGAAAAGAGTCACTCTGGCCCTG	292	27
	Reverse	GGTGCCTCTACTGGGTTTGTTGG		
Similar to interferon-induced protein 35	Forward	CCGTATGTGAATGGGGAGATCCAG	222	27
	Reverse	GCCTGACTCAGAGGTGAAGACTG		
Caspase 1	Forward	AGAAACACTCTGAGCAAGTCCCAG	278	30
	Reverse	AACATTATCTGGTGTGGAAGAGCAG		
Neutrophil cytosolic factor 2	Forward	GACATGGTGTCTAAGAAACTGGAG	277	27
	Reverse	CTCATAACTGAAGAGTGCCTCCAC		
Putative secred protein ZSIG13	Forward	CTGGTTATGACAATGACCGACCAG	272	25
	Reverse	GCAGATCTGGGCATATTTGAGAGG		
GAPDH	Forward	GACTCATGACCACAGTCCATGC	334	22
	Reverse	GAGGAGACCACCTGGTGCTCAG		

listed in Table 1. The PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. RT-PCR was performed in duplicate experiments. The mRNA levels of target genes were monitored by a ChemiImager 4400 (Alpha Innotech), which measured the intensities of bands stained with ethidium bromide as described previously (Kato et al., 2003a). As an internal control, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was amplified by RT-PCR, and the products were used to normalize the mRNA levels of the target genes.

2.7. LightCycler PCR

One-twentieth of the cDNA synthesized above was subjected to real-time LightCycler PCR as described previously (Nozaki and Kato, 2002; Nozaki et al., 2003). The primers listed in Table 1 were also used for LightCycler PCR. Temperature cycling conditions for each primer set consisted of 10 min at 95 °C followed by 35 cycles for 1 s at 94 °C, 5 s at 60 °C (64 °C for LMP2 and LMP7 only), and 6–14 s (25 bp per second) at 72 °C. All reactions were performed in a LightCycler™ Quick System 330 (Roche) using Fast-Start DNA Master SYBR Green I mix (Roche) according to the manufacturer's instructions. The experiments were performed in at least triplicate. The relative mRNA expression ratios of the target genes were calculated based on crossing-point analysis using a second derivative maximum method (LightCycler analysis software version 3.5). To correct for differences in RNA quality and quantity between the samples, data were normalized using the ratio of the target cDNA concentration to that of GAPDH. This ratio was assessed by a different reaction in the same experimental round.

Table 2
Genes whose expression levels were commonly altered in 1B-2R1 and 50-1 cells compared with their cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Up-regulation (more than 2-fold)			
Phosphatidylserine-specific phospholipase A1α[a]	2.2	2.9	NM_015900
Oncostatin M receptor[a]	2.1	2.2	NM_003999
Down-regulation (less than 0.50-fold)			
LMP2[a]	0.14	0.30	NM_002800
LMP7[a]	0.21	0.44	NM_004159
Similar to interferon-induced protein 35[a]	0.31	0.32	BC001356
Weakly similar to zinc finger protein 91[a]	0.36	0.42	AK027354
Protein phosphatase 1, regulatory subunit 1A[a]	0.40	0.32	NM_006741
Serpin clade C[a]	0.49	0.31	NM_000488

[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

3. Results

3.1. Preparation of the cured cells from 50-1 and 1B-2R1 cells

To obtain cured cells for the microarray analysis, 50-1 and 1B-2R1 cells were cultured with prolonged IFN-α treatment as described Section 2. After 3 weeks of this treatment, we demonstrated by Northern blot analysis that the replicon RNAs were not detected in the IFN-α-treated (50-1C and 1B-2R1C) cells, although approximately 10^8 copies of replicon RNA were detected in the total RNA (3 mg) extracted from 50-1 and 1B-2R1 cells (Fig. 1A). We further confirmed by RT-nested PCR (Mizutani et al., 1996) for the detection of the 5′-untranslated region that the replicon RNAs were absolutely excluded from the cells (data not shown). Western blot analysis also showed that the NS3 and NS5B proteins were no longer detected in 50-1C and 1B-2R1C cells, but were detected in 50-1 and 1B-2R1 cells, as shown in Fig. 1B.

3.2. cDNA microarray analysis

To examine the effects of HCV replicons on gene expression in host cells, cDNA microarray analyses (CodeLink™, Amersham Biosciences; 9970 human genes) were performed by comparing 1B-2R1 with 1B-2R1C cells and 50-1 with 50-1C cells. The majority of genes examined showed only small differences, with ratios ranging between 2.0 and 0.50 (data not shown). There were 55 and 101 up-regulated genes (those

Table 3
Genes whose expression levels were up-regulated (more than 3-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
AU62G04.X1	**8.5**	1.4	AI929792
Homeobox 1(HESX1)	**4.2**	0.50	NM_003865
Microsomal NAD+ dependent retinol dehydrogenase 4	**3.4**	0.92	NM_003708
Advillin	**3.3**	0.61	NM_006576
SSFV proviral integration oncogene Spi1	**3.1**	1.0	NM_003120
Napsin 2 precursor	**3.1**	0.94	AF098485
Transgelin[a]	0.85	**8.5**	NM_003186
Uncharacterized bone marrow protein BM040	0.81	**5.8**	AF217516
Annexin A1[a]	1.0	**4.2**	NM_000700
Putative secreted protein ZSIG13[a]	1.7	**3.9**	AF193611
Protease serine 23	1.2	**3.8**	NM_007173
Colon cancer antigen NY-CO-45	1.3	**3.7**	AF039442
HSPC157 protein	1.1	**3.5**	NM_014179
Uronyl-2-sulfotransferase	1.0	**3.5**	NM_005715
Cadherin, EGF lag seven-pass G-type receptor 2	0.68	**3.5**	NM_001408
Hypothetical protein (LOC51321)	1.1	**3.4**	NM_016627
Kidney-specific membrane protein (NX-17)	1.0	**3.3**	NM_020665
Neutrophil cytosolic factor 2[a]	1.8	**3.2**	NM_000433
Amphiregulin	1.4	**3.1**	NM_001657
Fibrillin 1	0.83	**3.1**	NM_000138
LTBP1[a]	1.6	**3.0**	NM_000627

The numbers of more than 3-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

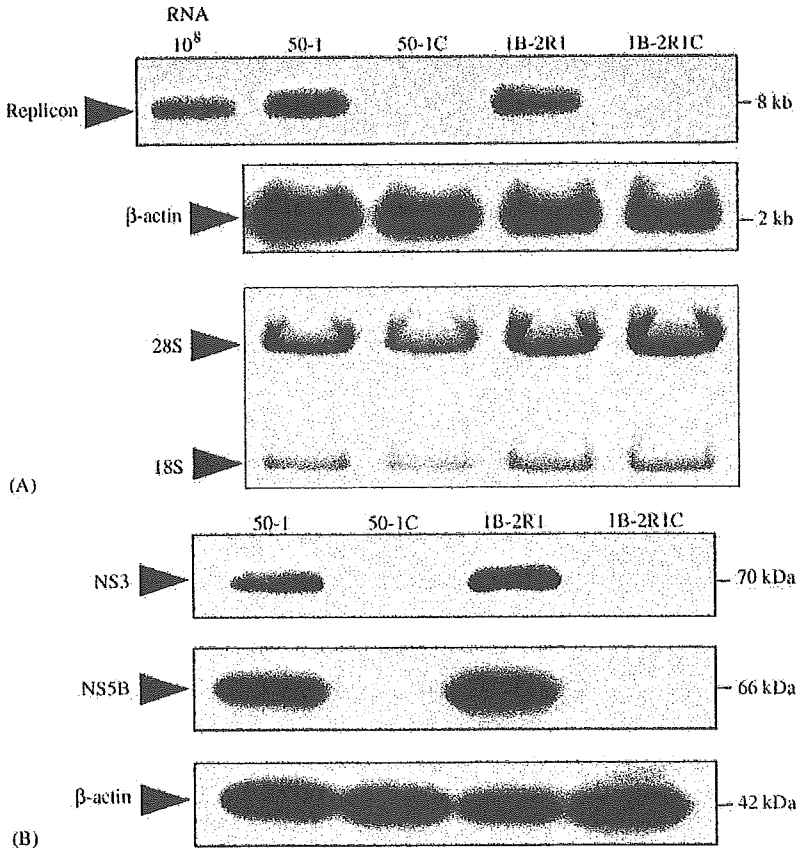

Fig. 1. Characterization of the replicon cells and their cured cells. (A) Northern blot analysis. Total RNAs from 50-1 and 1B-2R1 cells, as well as total RNAs from the cured cells, were analyzed by Northern blotting using a positive-stranded HCV genome-specific RNA probe (upper panel) and a β-actin-specific RNA probe (middle panel). RNA samples were equalized for 28S and 18S ribosomal RNAs stained with ethidium bromide (lower panel). A synthetic RNA transcribed from pNSS1RZ2RU (10^8 genome equivalents spiked into normal cellular RNA) was used as a positive control. (B) Western blot analysis. Productions of NS3 and NS5B in 50-1 and 1B-2R1 cells were analyzed by immunoblotting using anti-NS3 and anti-NS5B antibodies, respectively. 50-1C and 1B-2R1C cells were also analyzed to confirm the lack of NS3 and NS5B proteins. β-actin was used as a control for the amount of protein loaded per lane.

with ratios of more than 2.0) in 1B-2R1 and 50-1 cells, respectively. Between the two types of replicon cells, only two genes were commonly up-regulated. There were 56 and 74 down-regulated genes (those with ratios of less than 0.50) in 1B-2R1 and 50-1 cells, respectively, of which 6 genes were commonly down-regulated in both types of replicon cells. Table 2 summarizes the genes that the replicons commonly affected. Among these genes, it is noteworthy that large multifunctional proteases 2 (LMP2) and LMP7, which have been known as catalytic subunits in immunoproteasome (Akiyama et al., 1994; Tanaka and Kasahara, 1998), and serine proteinase inhibitor (serpin) clade C (Gettins, 2002) were down-regulated in both types of replicon cells (discussed below). However, no common genes were directly linked to the transformation of the cells. Since the standard of selection seemed to be rather strict, we further selected the genes whose expression levels were up-regulated or down-regulated with ratios of more than 3.0 or less than 0.33, respectively, in either 1B-2R1 or 50-1 cells. By this method, we selected 6 and 15 genes as up-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 3); and 6 and 9 genes as down-regulated genes in 1B-2R1 and 50-1 cells, respectively (as shown in Table 4). These selections allowed us to find several additional genes, including latent transforming growth factor β binding protein 1 (LTBP1) and caspase 1, that were commonly regulated in both types of replicons.

3.3. RT-PCR confirmation of the alteration of gene expression by HCV replicons

To confirm the results of our microarray selection, we examined the levels of several mRNAs by RT-PCR in duplicate. As shown by the stars in Tables 2-4, 16 genes (7 up-regulated and 9 down-regulated) were subjected to RT-PCR analysis. As shown in Fig. 2, RT-PCR confirmed that the expressions of most of these genes changed. This result suggests that the relative mRNA expression ratio obtained by the microarray analysis reflects the differential expres-

Table 4
Genes whose expression levels were down-regulated (less than 0.33-fold) in either 1B-2R1 or 50-1 cells compared with the cured cells

Genes	Relative mRNA expression ratio		Accession no.
	1B-2R1/1B-2R1C	50-1/50-1C	
Hephaestin	**0.14**	1.7	NM_014799
Solute carrier family 7[a]	**0.15**	0.62	NM_003982
Caspase 1[a]	**0.18**	0.65	NM_033292
Protease inhibitor 3	**0.19**	1.1	NM_002638
Collagen type II α1	**0.31**	1.6	NM_033150
C-terminal binding protein 2	**0.31**	0.71	NM_022802
ATPase α polypeptide (ATP 12A)	0.57	**0.26**	NM_001676
Hypothetical protein FLJ20043	0.79	**0.27**	NM_017637
CM2-HT0948-070900-368-D08 cDNA	1.0	**0.28**	BF089733
S100-type calcium binding protein A14[a]	0.62	**0.30**	NM_020672
Hypothetical protein MGC2827	0.65	**0.31**	NM_023940
EGFL6	2.4	**0.32**	NM_015507
ISL1 transcription factor	0.94	**0.32**	NM_002202
Pre-α globulin inhibitor	1.2	**0.32**	NM_002217
Regulator of G-protein signalling 16	0.65	**0.33**	NM_002928

The numbers of less than 0.33-fold were indicated by bold letters.
[a] RT-PCR analysis was performed to confirm the result of microarray analysis.

sion profiles of the replicon and its cured cells. Of the 16 genes, 9 (4 up-regulated and 5 down-regulated) were further subjected to real-time LightCycler PCR analysis in order to obtain the actual ratios of mRNA expression. As shown in Table 5, the resultant relative mRNA expression ratios actually correlated with those obtained by our microarray analysis. Regarding the selected genes in this study, we confirmed by RT-PCR the reproducibility of the relative mRNA ratios using different lots of RNA specimens derived from 1B-2R1 and 1B-2R1C cells (data not shown). Taken together, our results suggest that these altered mRNA expressions are caused by the multiplication of HCV subgenomic replicons.

4. Discussion

This study yielded evidence of alterations in gene expression by HCV subgenomic replicons in human hepatocytes, as observed through microarray analysis (9970 genes), and first provided a list of genes including LMP2, LMP7, and serpin clade C that the replicons transcriptionally regulate.

To date, only one report of cDNA microarray analysis (832 cytokine-related genes) has been conducted by comparing HCV subgenomic replicon cells with parental Huh-7 cells (Zhu et al., 2003). That analysis obtained 14 up-regulated genes (those with ratios of more than 2.0) in the replicon cells. However, the parental Huh-7 cells may not be appropriate for use as control cells in such microarray analyses, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid the clone-based differences for microarray analysis. From this principal reason, we used two types of cured cells derived from 50-1 and 1B-2R1 cells as the control cells for our microarray analysis. The cured cells are considered to have the same background as the replicon cells. The possibility remains that the genes selected in this study were obtained by the effect of IFN-α that was used to

Table 5
LightCycler RT-PCR analysis of genes whose expression levels were altered by HCV replicons

Genes	Relative mRNA expression ratio (mean ± S.D.)	
	1B-2R1/1B-2R1C	50-1/50-1C
Up-regulation		
Phosphatidylserine-specific phospholipase A1α	2.03 ± 0.09	3.09 ± 0.74
Oncostatin M receptor	2.58 ± 0.20	2.46 ± 0.49
Transgelin	0.83 ± 0.11	13.72 ± 0.56
Annexin A1	1.19 ± 0.17	4.23 ± 0.72
Down-regulation		
LMP2	0.06 ± 0.00	0.40 ± 0.12
LMP7	0.09 ± 0.02	0.33 ± 0.08
Serpin clade C	0.39 ± 0.11	0.37 ± 0.11
Solute carrier family 7	0.13 ± 0.08	0.77 ± 0.18
S100-type calcium binding protein A14	0.37 ± 0.21	0.32 ± 0.17

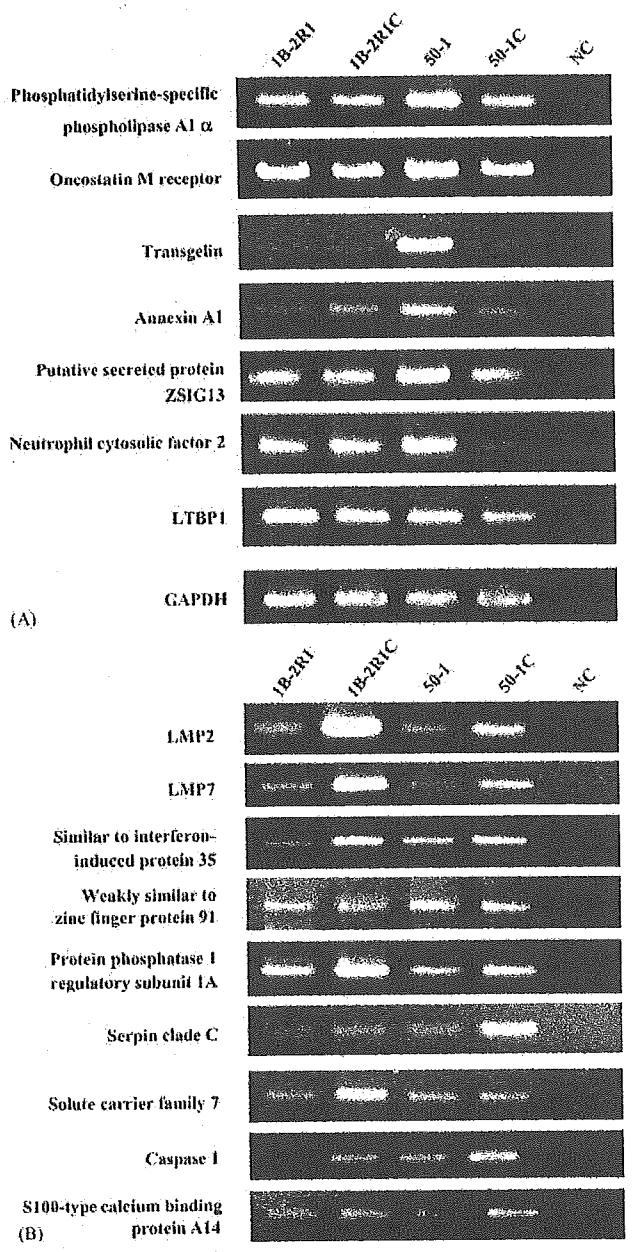

Fig. 2. RT-PCR analysis of mRNA expression of selected genes based on the microarray results. The total RNAs extracted from 50-1, 50-1C, 1B-2R1, and 1B-2R1C cells were subjected to RT-PCR, and the mRNA levels of target genes were monitored as described in Section 2. The primers used, PCR product lengths, and PCR cycle numbers are listed in Table 1. GAPDH was used as a control. (A) Up-regulated genes. (B) Down-regulated genes.

eliminate replicon RNA. However, this possibility is quite low because most of the genes regulated by IFN-α were not transcriptionally altered according to our microarray analysis, and it is unlikely that IFN-α treatment irreversibly altered the expression of genes that it either induced or suppressed. In actuality, most genes selected by our cDNA microarray analysis were not related to the genes regulated by IFN-α, although the selected genes in this study did not show any common characteristics involved in the progression of hepatic diseases. Very recently, after most of the present study was finished, a cDNA microarray analysis using full-length HCV RNA replicating cells and their cured cells was reported by Scholle et al. (2004). Although those authors found dozens of genes whose expression levels were altered by the replication of HCV RNA derived from HCV strain N, we had no information on the genes they had selected and therefore we could not compare them with ours. Therefore, we are currently establishing full-length HCV RNA replicating cells derived from 1B-1 and 1B-2 HCV strains. Further cDNA microarray analysis using such full-length HCV RNA replicating cells will help to identify HCV's target genes.

Among the genes selected in this study, LMP2 and LMP7 are quite interesting. Both are known as important catalytic subunits in immunoproteasome induced by IFN-γ (Akiyama et al., 1994; Tanaka and Kasahara, 1998). In the presence of IFN-γ, the three catalytic subunits of vertebrate proteasomes are replaced by their homologous subunits, LMP2, LMP7, and MECL1, to form immunoproteasome, which increased the ability to produce peptides with a proper motif for efficient MHC binding (Fehling et al., 1994; Van Kaer et al., 1994). A number of peptides that were poorly processed by the standard proteasome were recently found to be more effectively produced by the immunoproteasome (Van den Eynde and Morel, 2001). Therefore, down-regulation of LMP2 and LMP7 expressions in HCV subgenomic replicon cells will reduce the production efficiency of viral antigenic peptides presented to CD8+ T cells (Van den Eynde and Morel, 2001), and may subsequently help to cause the persistent viral infection. In contrast with the expression of LMP2 and LMP7, that of the MECL1 gene was not altered regardless of the presence of HCV replicon. The molecular mechanism by which HCV replicon cells suppress LMP2 and LMP7 remains unknown. This phenomenon is considered to be caused by one of the HCV NS proteins in the replicon cells. As a first step toward identifying the responsible NS protein, we carried out a preliminary experiment using 1B-2R1C cells that stably expressed NS3, NS4A, NS4B, NS5A, NS5B, or NS3-NS5B protein by retrovirus-mediated gene transfer. Unfortunately, however, this experiment failed to identify the responsible NS protein. This result suggests that either the replication of replicon RNA or replicon RNA itself is necessary to suppress LMP2 and LMP7 gene expression. To clarify this point, further analysis will be necessary, using HCV subgenomic replicon cells derived from the other HCV strains or HCV subgenomic replicon cells re-established by the transfection of 50-1 or 1B-2R1 subgenomic replicon RNA.

A third interesting gene obtained in this study was serpin clade C. Although the expression of the serpin clade C gene was down-regulated to approximately one-third in HCV subgenomic replicon cells, those of the other eight clades of the serpin family were not quite altered. Since serpins are a unique class of proteinase inhibitors that irreversibly neu-

tralize target proteinases by a mechanism that conformationally distorts the proteinase (Gettins, 2002), the relationship between serpin clade C and HCV serine proteinase is interesting. To clarify this relationship, further analysis, such as that of the compulsory expression of serpin clade C in the replicon cells, will be necessary.

In this study, we demonstrated that microarray analysis to compare HCV subgenomic replicon cells with their cured cells was useful for screening and selecting HCV's target genes. Also, we compiled the first list of genes transcriptionally regulated by the multiplication of HCV subgenomic replicons. Although we need to clarify the mechanisms underlying transcriptional regulation by HCV subgenomic replicons, we believe that the genes involved in viral replication and multiplication are among the genes listed in this study. Further analysis using new experimental systems, such as the full-length HCV RNA replicating system, will be useful to clarify this point.

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Original article

Efficient formation of vesicular stomatitis virus pseudotypes bearing the native forms of hepatitis C virus envelope proteins detected after sonication

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Abstract

Hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in addition to acute hepatitis. The HCV genome encodes two envelope glycoproteins, E1 and E2. To investigate the role of E1 and E2 in HCV infection, we used a recombinant vesicular stomatitis virus (VSV), VSVΔG*, harboring the green fluorescent protein gene instead of the VSV G envelope protein gene. It was complemented with the native form of E1 and E2, or E1 or E2 alone, to make HCV pseudotypes VSVΔG*(HCV), VSVΔG*(E1), and VSVΔG*(E2). Neither E1 nor E2 expression was detected on the cell surface, as reported. Unlike previous reports, infectious activities of VSVΔG*(HCV), VSVΔG*(E1) and VSVΔG*(E2) pseudotypes were detected under conditions where VSV was completely neutralized by anti-VSV. We could enhance the infectious titers 100-fold by sonication upon virus harvest. Bovine lactoferrin efficiently inhibited infection by VSVΔG*(HCV) as well as VSVΔG*(E2), as the interaction between E2 and lactoferrin has been thought to contribute to the inhibition of HCV infectivity. VSVΔG*(HCV) infected many adherent cell lines, including hepatic cell lines, but not most hematopoietic cell lines. Treatment of cells with trypsin, tunicamycin, or sulfated polysaccharides before infection reduced the infectivity of VSVΔG*(HCV) by about 90%, suggesting that a cell surface protein(s) with sugar chains plays an important role in HCV infection. The VSV pseudotypes developed here would be useful for analyzing the early stages of HCV infection.

Keywords: HCV; Pseudotype; Envelope; Sonication; Glycosylation

1. Introduction

Hepatitis C virus (HCV) has been one of the major causative agents of posttransfusion and sporadic hepatitis [1]. At present, transfusion-associated hepatitis C has been virtually eliminated in developed countries, and risk factors that most are strongly correlated with HCV infection there are illegal drug use and high-risk sexual behavior. Current worldwide estimations suggest that more than 200 million people are infected with HCV [2]. The infection frequently develops into chronic hepatitis, which further leads to the development of liver cirrhosis and hepatocellular carcinoma [3,4]. The mechanisms involved in HCV infection and HCV-mediated disease progression are not well understood, and a therapy effective for most HCV-infected patients is not yet available.

HCV is an enveloped, positive-stranded RNA virus belonging to the Flaviviridae family [2]. The viral genome contains

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a single open-reading frame of approximately 9.5 kb that codes for a large polyprotein precursor of 3000 amino acids (aa) [5,6] (Fig. 1a). Structural proteins are located in the N-terminal of the precursor polyprotein, which is to be cleaved by cellular signal peptidases. The core protein (C) is followed by two putative envelope proteins, E1 and E2. A small protein, p7, is produced by the cleavage of the E2 protein. Downstream of the structural proteins, non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are located [7,8]. The E1 and E2 envelope proteins form a non-covalently linked heterodimer, which probably represents the native prebudding complex, in the endoplasmic reticulum (ER) [9].

The binding of the virus to the host cell surface receptor(s) is the first step in the infection process. As in most enveloped viruses, E1 and E2 are believed to be the major viral attachment proteins in HCV. There has been no clear evidence as to which protein, E1 or E2, defines the interaction with human cells, because of the lack of a suitable experimental system for HCV entry. It has been reported that a truncated, soluble form of E2 binds to human CD81 (hCD81) and human scavenger receptor class B type I (SR-IB), suggesting that

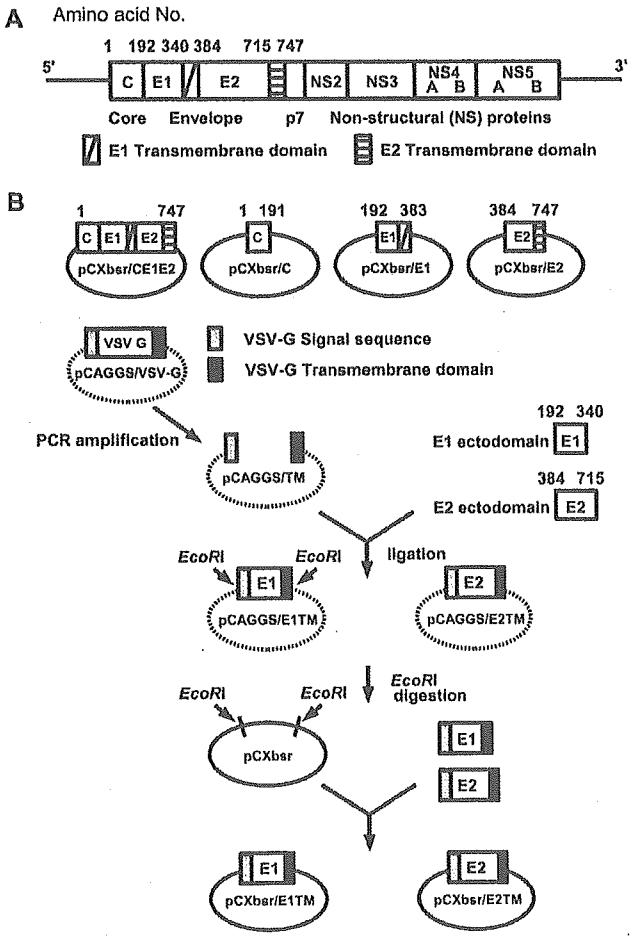

Fig. 1. Preparation of plasmids expressing HCV envelope proteins. (a) Structure of HCV genome. (b) Schematic representation of the plasmids and the chimeric gene constructs for the expression of HCV envelope glycoproteins. TM represents the signal sequence and the transmembrane domain of VSV G protein.

hCD81 or SR-IB is a candidate cellular receptor for HCV [10,11]. Furthermore, HCV particles have also been reported to utilize the LDL receptor for binding and their entry into the cells [12,13]. But it is still unknown whether they serve as functional receptors, since the expression of neither hCD81 nor the LDL receptor is restricted in hepatocytes, and hCD81 transgenic mice are resistant to HCV infection [14]. There may also be a functional difference between the native form of HCV envelope protein and the soluble form of E2. The main obstacle to clarifying these points is the lack of suitable tools with which to evaluate the attachment to and entry into the target cells quantitatively. Recently, to analyze virus entry mediated by HCV envelopes, systems for the production of vesicular stomatitis virus (VSV) pseudotypes bearing modified HCV envelope proteins have been reported [15–17]. To express the HCV envelope proteins on the cell surface to incorporate them into VSV virions, chimeric HCV E1 and E2 proteins containing the transmembrane domain and cytoplasmic tail of VSV G glycoprotein (VSV G) were generated. Otherwise, E1 or E2 is not expressed on the cell surface. There is the possibility that these pseudotypes may show a different infectivity from the viruses bearing the native forms of HCV envelopes.

In this study, we developed a system to prepare VSV pseudotypes expected to bear the native HCV envelope proteins, E1E2, E1 or E2. That is, cells were transfected with the native structural protein genes, and then infected with a recombinant VSV, VSVΔG*G, containing the green fluorescent protein (GFP) gene as a reporter instead of VSV G [18]. Unlike previous reports [15–17], we could detect pseudotype virus activities after transfection of not only the native structural protein gene C–E1–E2, but also the unmodified E1 or E2 gene. These pseudotype virus-like activities were not neutralized by any sera from chronic hepatitis C patients, as previously reported using pseudotypes with chimeric HCV envelopes [17,19]. These infectious activities were inhibited by treatment with bovine lactoferrin, as we reported using PCR for detection of HCV infection [20,21]. Using these new VSV pseudotypes, we further examined the mechanism involved in HCV infection. That is, the infectivity of pseudotype viruses was studied in various cell lines, and the effects of chemical reagents on the infection were tested.

2. Materials and methods

2.1. Cells

293T is derived from the human embryonic kidney cell line 293 and contains the SV40 large T-antigen [22]. The other cell lines used in this study and their derivations are listed in Table 3. BALL-1, C8166, C91/PL, Daudi, HEL, HL-60, K562, Molt-4, MT-2, Raji, TALL-1, U937 and Wil2NS were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS). 293T, A172, HepG2, HOS and Huh7 were maintained in Dulbecco's modi-