2001; Taniguchi *et al.*, 2001; Taniguchi & Takaoka, 2002). ISRE-dependent gene expression is also mediated by binding of the ISRE by molecules such as IRF-1, IRF-3 and IRF-7 (Kanazawa *et al.*, 2004). IRF-3 is a transducer of virus-mediated signalling and plays a critical role in the induction of cellular antiviral responses (Lin *et al.*, 1998; Sato *et al.*, 2000; Taniguchi *et al.*, 2001; Yoneyama *et al.*, 1998). Transcriptional activation and suppression of IRF-3 are inversely correlated with the level of HCV replication *in vitro* (Yamashiro *et al.*, 2006). Following virus infection, IRF-3 is phosphorylated by two cytoplasmic kinases, TBK1 and IKKε (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003). The phosphorylated IRF-3 forms a homodimer, translocates to the nucleus and predominantly activates expression of the IFN-β gene and certain ISGs (Doyle *et al.*, 2002; Nakaya *et al.*, 2001; Taniguchi & Takaoka, 2002).

RIG-I is a recently identified cytoplasmic DExD/H box RNA helicase that participates in recognition of virus-related dsRNA as a pathogen-related molecular pattern (Yoneyama *et al.*, 2005). RIG-I contains two caspase-recruitment domains (CARDs) in the N terminus and a DExD/H box RNA helicase in the C terminus. MDA5 has been identified as another CARD-containing DExD/H box RNA helicase (Andrejeva *et al.*, 2004). More recently, an adaptor molecule of RIG-I and MDA5, Cardif (also known as IPS-I, MAVS and VISA), has been identified by four independent groups (Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005; Xu *et al.*, 2005). On association with dsRNA, RIG-I or MDA5 causes conformational changes and homo-oligomerization, and binds the CARD of Cardif (Saito *et al.*, 2007). Cardif subsequently recruits the kinases TBK1 and IKKε, which catalyse phosphorylation and activation of IRF-3 (Yoneyama *et al.*, 1998).

The IRF-3-mediated IFN-β induction pathway could be a target for viruses to counteract antiviral responses and promote their replication in host cells. Ebola virus, bovine viral diarrhea virus (BVDV) and influenza A virus interfere with the activation of IRF-3 through interactions of their virus-encoded proteins (Basler *et al.*, 2003; Schweizer & Peterhans, 2001; Talon *et al.*, 2000). There are several reports that HCV proteins interact with IFN-mediated antiviral systems. The NS5A and E2 proteins have been reported to interfere with the action of IFN by inhibiting the activity of PKR (He & Katze, 2002). It was reported recently that the HCV NS34A protease blocks virus-induced activation of IRF-3, possibly by proteolytic cleavage of Cardif (Foy *et al.*, 2003; Meylan *et al.*, 2005).

The HCV subgenomic replicon is an *in vitro* model that simulates autonomous cellular replication of HCV genomic RNA (Lohmann *et al.*, 1999). Expression of the HCV replicon can be abolished by treatment with small amounts of type I and type II IFNs (Blight *et al.*, 2000; Frese *et al.*, 2002; Guo *et al.*, 2001), suggesting intact IFN receptor-mediated cellular responses. In contrast, viral expression persists in the absence of the exogenous IFN. Baseline expression levels of ISG were substantially decreased in cells expressing the HCV replicon compared with parental Huh7 cells (Kanazawa *et al.*, 2004). These findings led us to speculate that intracellular virus-induced antiviral responses are attenuated or caused to malfunction by the expression of viral proteins.

In this study, we investigated cell lines that support subgenomic HCV replication and HCV cell culture for the dsRNA-induced cellular IFN expression pathway. Here, we report that RIG-I- and Cardif-mediated IFN gene activation is uniformly attenuated in several replicon-expressing cell lines of different lineages and, more importantly, that the HCV NS4B protein is involved in the suppression of antiviral IFN responses.

METHODS

Plasmids. Plasmids pEF-flagRIG-I and ΔRIG-I expressed full-length and C-terminally truncated RIG-I protein, respectively (Yoneyama *et al.*, 2004). The plasmid pER-flagRIG-IKA (RIG-IKA) has a point mutation in the putative ATP-binding site of the RIG-I helicase domain and was used as a negative control for ΔRIG-I and RIG-I full transfection assays. Expression plasmids for full-length Cardif (Cardif), Cardif CARD (CARD) and CARD-truncated Cardif (ΔCARD) were provided by Dr J. Tschopp (University of Lausanne, Switzerland) (Meylan *et al.*, 2005). Expression plasmids for toll-like receptor 3 (TLR3) and TIR domain-containing adaptor inducing IFN-β (TRIF), the transmembrane receptor of dsRNA and the adaptor molecule of TLR3, respectively, were provided by Dr S. Akira (Osaka University, Japan). Plasmids expressing HCV NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B were amplified from HCV pCV-J4-L4S (Yanagi *et al.*, 1997) by PCR and subcloned. The DNA fragments were inserted into the vector pcDNA4/TO/*myc*-His (Invitrogen). Nucleotide sequences were confirmed by sequencing. Plasmids TOPO-NS34A (HCV N), TOPO-NS4B (HCV N) and pcDNA-NS4B (HCV JFH1) expressed Myc-tagged NS34A and NS4B proteins derived from the HCV N (Beard *et al.*, 1999) and HCV JFH1 (Wakita *et al.*, 2005) strains, as indicated. Plasmid pISRE-TA-Luc (Invitrogen) contained five copies of consensus ISRE motifs upstream of the firefly luciferase gene. Plasmid pIFNβ-Fluc was constructed by cloning the human IFN-β promoter region, spanning nt −110 to −36, upstream of the firefly luciferase gene of pGL3 Basic (Promega). Plasmid pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega), which expressed the *Renilla* luciferase protein, was used for correction of transfection efficiency.

Cell culture. HCV strain JFH1-infected Huh7.5.1, Huh7, Huh7.5.1 (kindly provided by Dr F. Chisari, The Scripps Institute, CA, USA; Zhong *et al.*, 2005), HeLa and HEK293 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37 °C with 5% CO_2. Cells expressing the HCV replicon were cultured in medium containing 100 μg G418 (Wako) ml^{-1}.

HCV replicon constructs and transfected cell lines. An HCV subgenomic replicon plasmid, pHCVIbneo-delS (designated pRep-N), was derived from an HCV clone of strain N, genotype 1b, and pSGR-JFH1 was derived from HCV JFH1, genotype 2a (Guo *et al.*, 2001; Wakita *et al.*, 2005). These replicons were reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising *Renilla* luciferase and neomycin phosphotransferase to construct pRep-Reo-1b and pRep-Reo-2a, respectively (Tanabe *et al.*, 2004; Yokota *et al.*, 2003). RNA was synthesized from the replicons using T7 polymerase (Promega) and transfected into Huh7,

HeLa and HEK293 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/1bReo, Huh7/2aReo, HeLa/2aReo and 293/2aReo). We have previously reported that firefly luciferase activities of Feo-replicon-expressing cells correlate well with HCV NS3, NS4A and NS5A protein expression levels and with the levels of replicon RNA (Yokota *et al.*, 2003).

Transient transfection. Transient DNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. ISRE reporter assays were carried out as previously described (Nakagawa et al., 2004). To analyse IFN expression in HCV JFH1 cell cultures, a total of 1×10^5 Huh7.5.1, JFH-1 infected Huh7.5.1 and IFN-treated Huh7.5.1 cells were seeded into 24-well plates the day before transfection. Plasmids pISRE-TA-Luc and ΔRIG-I (200 ng each) were transfected using 1 μl Lipofectamine 2000. RIG-IKA was used as a control. Luciferase assays were performed on day 3 post-transfection.

For further study, 400 ng of each non-structural protein was added to 1×10^4 Huh7 or HEK293 cells that had been seeded into 96-well plates the day before transfection. pISRE-TA-Luc and ΔRIG-I (40 ng each) were transfected using 0.5 μl Lipofectamine 2000. RIG-IKA was used as a control.

Western blotting. Preparation of the cytoplasmic and nuclear fractions of cell lysates was carried out as described previously (Tanabe *et al.*, 2004). Protein (20 μg) was separated using NuPAGE 4–12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon PVDF membrane (Roche). The membrane was immunoblotted with anti-IRF-3 (Santa Cruz) and detected by chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

RT-PCR. Interleukin (IL)-8 mRNA was detected by RT-PCR as described previously (Itsui *et al.*, 2006). The primers used were IL8-S (5′-GCACAAACTTTCAGAGACAGCAGAGCACAC-3′) and IL8-AS (5′-CAGAGCTGCAGAAATCAGGAAGGCTGCCAA-3′).

Indirect immunofluorescence assay. Cells seeded onto tissue culture chamber slides were fixed with cold acetone. The cells were incubated with anti-protein disulphide isomerase (PDI) or anti-Myc antibodies and subsequently with Alexa 488- or Alexa 568-labelled secondary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories) and visualized by fluorescence microscopy (BZ-8000; Keyence).

Luciferase reporter assays. Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX; PerkinElmer) using a Bright-Glo Luciferase Assay System (Promega) or a Dual Luciferase Assay System (Promega). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assay. To evaluate cell viabilities, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to manufacturer's instructions.

Statistical analyses. Statistical analyses were performed using an unpaired, two-tailed Student's *t*-test. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

IRF-3 dimer formation is attenuated in cells expressing the HCV replicon

In the HCV replicon-expressing cell lines Huh7/Rep-Reo-2a, Hela/Rep-Reo-2a and 293/Rep-Reo-2a, replicon expression levels corresponded well to internal *Renilla* luciferase activities. Expression of the HCV replicon was suppressed by IFN in a dose-dependent manner (data not shown).

Activation of RIG-I or MDA5 induces phosphorylation and homodimerization of IRF-3. Following transfection of poly(I:C) into Huh7, HeLa or HEK293 cells, IRF-3 dimers were detected (Fig. 1). However, in cells supporting HCV replicons, IRF-3 dimer formation was almost completely abolished. These findings showed that expression of HCV proteins blocked activation of dsRNA-mediated IFN expression and that these effects were consistently found in several cell lines of different origin.

The HCV replicon suppresses RIG-I/Cardif-induced IFN responses

ISRE reporter activities did not increase in naïve Huh7, HeLa or HEK293 cells following transfection of poly(I:C), whilst overexpression of full-length RIG-I increased poly(I:C)-mediated ISRE reporter activity in Huh7 and HEK293 cells (data not shown). In RIG-I-overexpressing Huh7 cells, transduction with an HCV replicon abolished the poly(I:C)-induced ISRE activation, and elimination of the replicon by IFN treatment restored these ISRE responses (Fig. 2a). Consistent results were obtained by overexpression of ΔRIG-I, a constitutively active form. Transfection of ΔRIG-I in Huh7 and HEK293 cells induced ISRE activation, whilst these responses were abolished or significantly suppressed in cell lines expressing HCV replicons and were recovered by eliminating the replicon by IFN treatment (data not shown). Similarly, ISRE activation by overexpression of Cardif, an adaptor molecule of RIG-I, was almost completely blocked in replicon-expressing cells and was recovered by eliminating the replicon from the cells (data not shown). The RIG-I-mediated IFN response was

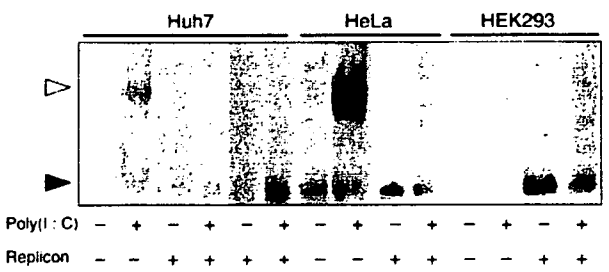

Fig. 1. Double-stranded RNA-induced IRF-3 dimer formation in cell lines that support HCV subgenomic replication. Poly(I:C) was transfected into naïve Huh7, HeLa and HEK293 cells, and into corresponding cell lines expressing the HCV replicon. Six hours after transfection, cell lysates were prepared, separated in polyacrylamide gels and blotted onto PVDF membrane. The membrane was immunoblotted with anti-IRF-3 and visualized by chemiluminescence (see Methods). The positions of the IRF-3 dimer (open arrowhead) and monomer (closed arrowhead) are indicated.

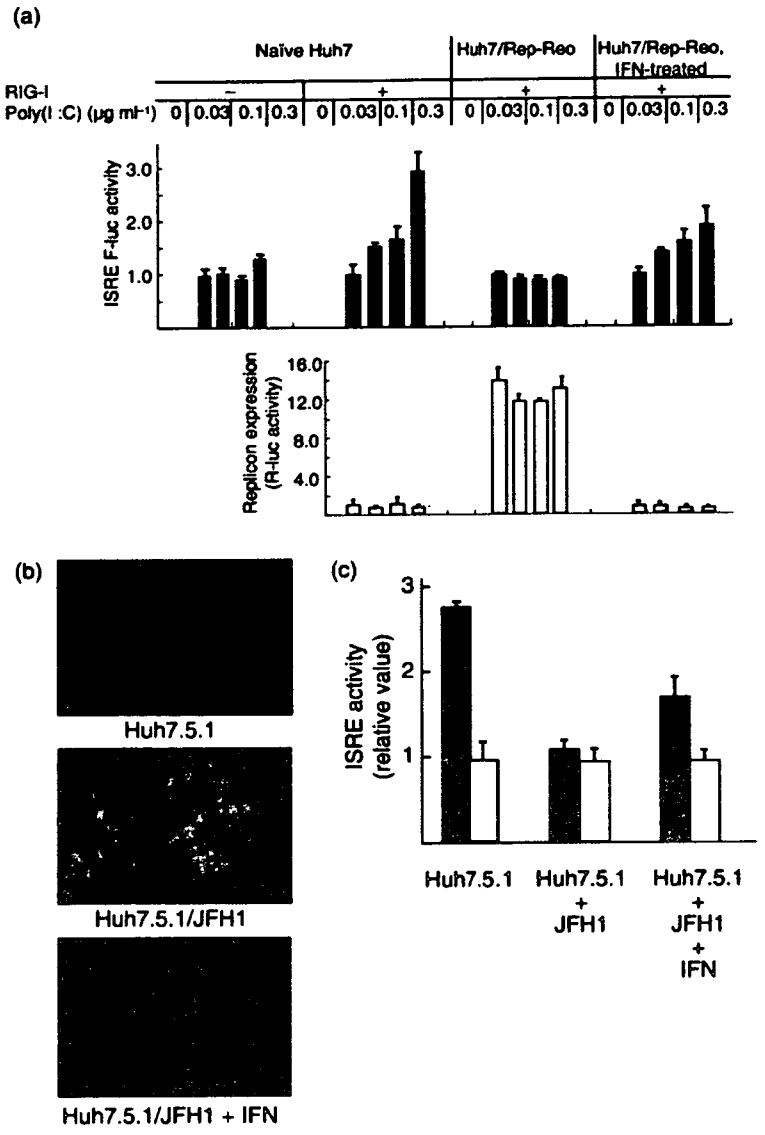

Fig. 2. Suppression of dsRNA-induced, RIG-I-mediated ISRE activation by HCV replication. (a) The HCV replicon suppresses transcriptional activation after poly(I : C) stimulation. The RIG-I expression plasmid and pISRE-TA-Luc were transiently transfected into the cell lines indicated. The following day, the amounts of poly(I : C) indicated were transfected into the corresponding cell lines and dual luciferase assays were carried out 8 h after transfection. Filled bars indicate ISRE-regulated firefly luciferase (F-luc) activities and open bars indicate *Renilla* luciferase (R-luc) activities representing replicon expression levels. In both graphs, scales for the *y*-axis are shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD. (b) Immunofluorescence microscopy results. Huh7.5.1 cells infected with HCV JFH1 (Huh7.5.1/JFH1) and JFH1-infected cells from which the virus had been eliminated by IFN treatment (Huh7.5.1/JFH1 + IFN) were incubated with anti-core primary antibodies followed by Alexa Fluor-conjugated secondary antibody (green). Nuclei were stained with DAPI (blue). (c) ISRE activation by ΔRIG-I overexpression. The plasmid pISRE-TA-Luc was co-transfected with ΔRIG-I (filled bars) or RIG-IKA (empty bars) into naïve Huh7.5.1, Huh7.5.1/JFH1 or Huh7.5.1/JFH1 + IFN cells. Luciferase assays were carried out 8 h after transfection. The *y*-axis indicates ISRE-regulated luciferase activity shown as relative values. Assays were carried out in triplicate and results are shown as means ± SD.

also suppressed in HCV JFH1 virus cell culture. In JFH1-infected Huh7.5.1 cells, ΔRIG-I-induced ISRE reporter activation was significantly suppressed, but was recovered in IFN-treated, virus-eliminated cells (Fig. 2b and c). These results demonstrated that RIG-I- and Cardif-mediated antiviral responses were substantially suppressed by both subgenomic and genomic viral replication in both hepatocyte- and non-hepatocyte-derived host cells.

NS34A and NS4B are responsible for suppressing RIG-I-mediated IFN responses

We next sought to define which HCV proteins were responsible for inhibition of the RIG-I- and IRF-3-mediated IFN induction pathway. We constructed expression plasmids that expressed the non-structural proteins NS345, NS3, NS34A, NS4A, NS4B, NS5A and NS5B (Fig. 3a). We transfected each expression plasmid with simultaneous activation of the RIG-I pathway by overexpression of ΔRIG-I, Cardif, TBK1 and IKKε (Fig. 3b–e). Expression of full-length non-structural (NS345) and NS34A proteins inhibited ISRE activation mediated by expression of RIG-I and Cardif but not that mediated by TBK1 and IKKε. Interestingly, it was found that NS4B also inhibited ISRE activation mediated by expression of RIG-I and Cardif, but not by TBK1 and IKKε. Consistent with Fig. 3(b), overexpression of NS4B significantly suppressed ΔRIG-I-induced activation of the authentic IFN-β promoter (Fig. 3f).

Another group has studied IFN antagonism of flavivirus non-structural proteins and has reported that HCV NS4B did not affect IFN responses (Muñoz-Jordán *et al.*, 2005).

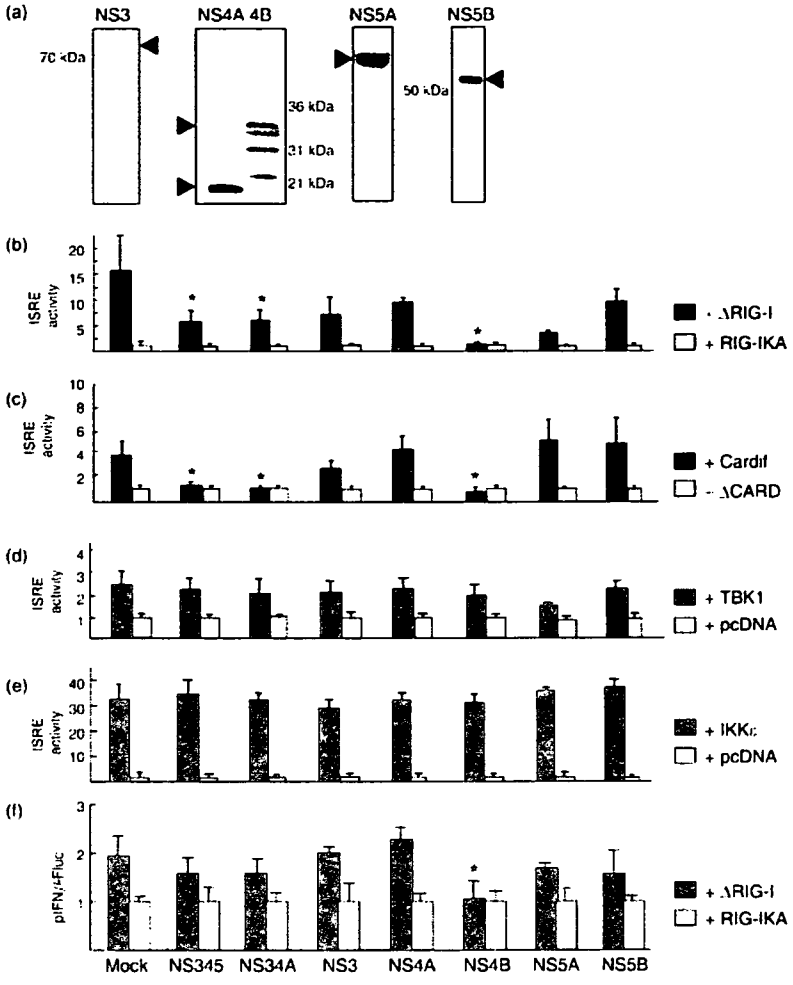

Fig. 3. Co-transfection analyses using plasmids that express individual HCV non-structural proteins. (a) Western blotting. Plasmids expressing the indicated Myc-tagged HCV proteins were transfected into Huh7 cells. Western blotting was carried out using anti-Myc antibody. (b–e) The following plasmids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

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

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

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

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

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

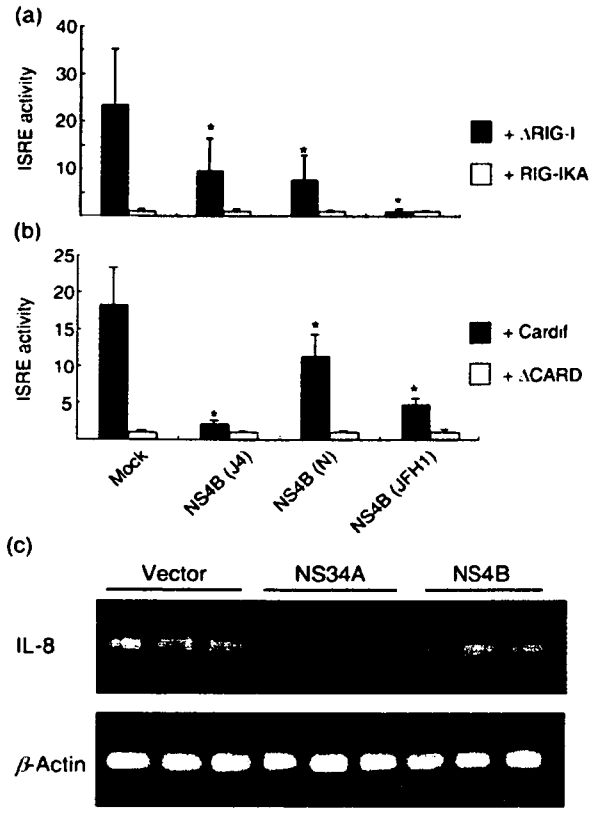

Fig. 4. Co-transfection analyses of HCV NS4B proteins of different origins. NS4B (J4), NS4B (N) and NS4B (JFH1) denote HCV NS4B proteins that were cloned from HCV strains J4, N and JFH1, respectively. The NS4B plasmids indicated were co-transfected with plasmids expressing ΔRIG-I (a) or Cardif (b). Luciferase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

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

DISCUSSION

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

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

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

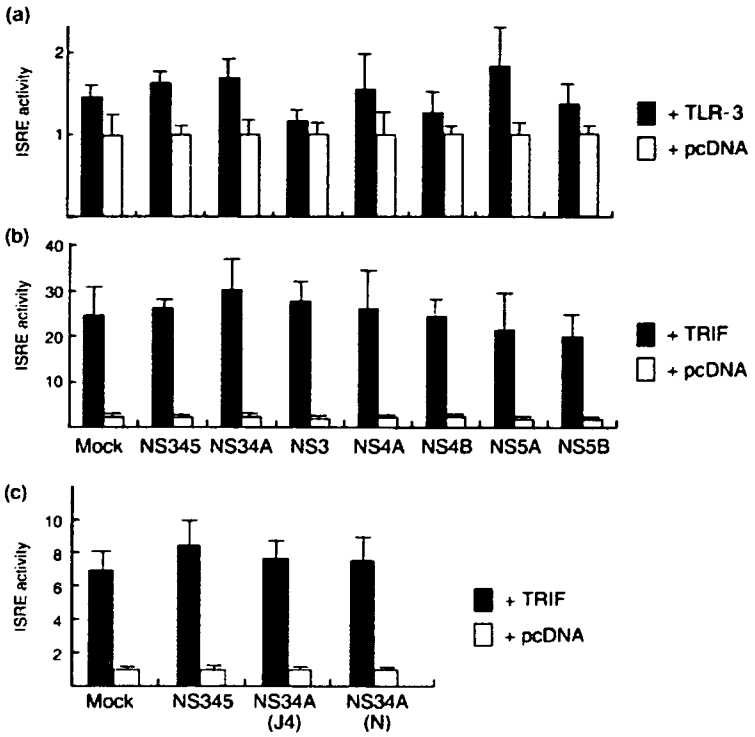

Fig. 5. Co-transfection analyses of HCV non-structural proteins and plasmid expressing TLR-3 or TRIF. (a, b). The following plasmids were co-transfected into Huh7 cells: pISRE-TA-Luc, pRL-CMV, plasmids expressing TLR-3 or TRIF and plasmids expressing individual HCV non-structural proteins, as indicated.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

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

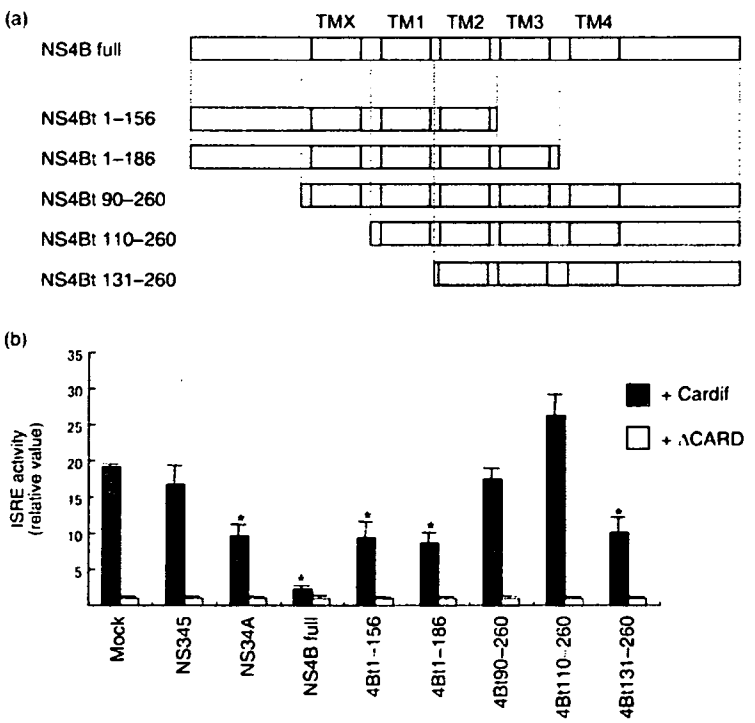

Fig. 6. Co-transfection analyses using truncated NS4B expression constructs. (a) Truncated constructs of NS4B. The protein-coding frame of NS4B was truncated in five constructs corresponding to the five transmembrane domains, as reported by Lundin *et al.* (2003). (b) The truncated NS4B plasmids, pISRE-TA-Luc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

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

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

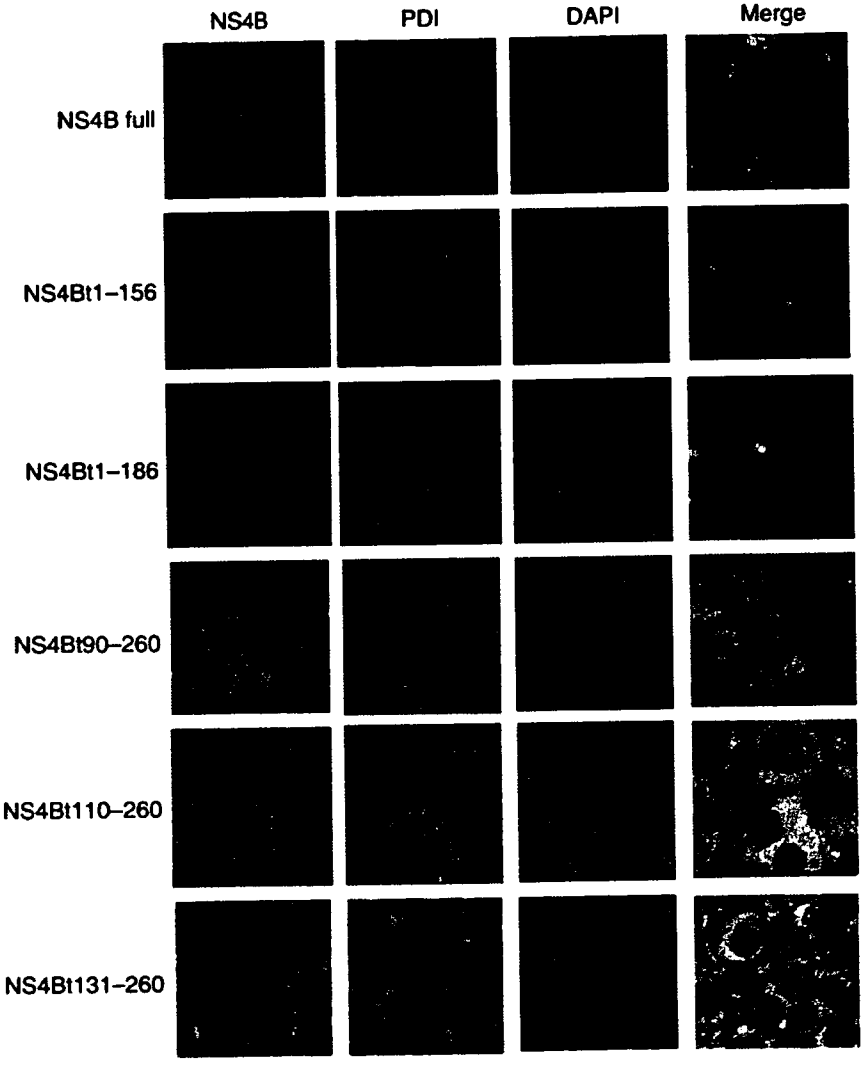

Fig. 7. Indirect immunofluorescence analysis of truncated NS4B proteins. The NS4B constructs indicated were transiently transfected into Huh7 cells. After 48 h, cells were labelled with anti-Myc or anti-PDI antibody. NS4B proteins were immunostained with Alexa Fluor 488-labelled goat anti-mouse IgG,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

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

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

ACKNOWLEDGEMENTS

We are indebted to Dr Jürg Tschopp for providing Cardif, ΔCARD and CARD, and to Dr Frank Chisari for providing the Huh7.5.1 cell line. This study was supported by grants from the Japan Society for the Promotion of Science, Miyakawa Memorial Research Foundation and Viral Hepatitis Research Foundation of Japan.

REFERENCES

Alter, M. J. (1997). Epidemiology of hepatitis C. *Hepatology* **26**, 62S–65S.

Andrejeva, J., Childs, K. S., Young, D. F., Carlos, T. S., Stock, N., Goodbourn, S. & Randall, R. E. (2004). The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the *IFN-β* promoter. *Proc Natl Acad Sci U S A* **101**, 17264–17269.

Basler, C. F., Mikulasova, A., Martinez-Sobrido, L., Paragas, J., Muhlberger, E., Bray, M., Klenk, H. D., Palese, P. & Garcia-Sastre, A. (2003). The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. *J Virol* **77**, 7945–7956.

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D. V. & Lemon, S. M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* **30**, 316–324.

Bigger, C. B., Brasky, K. M. & Lanford, R. E. (2001). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *J Virol* **75**, 7059–7066.

Blight, K. J., Kolykhalov, A. A. & Rice, C. M. (2000). Efficient initiation of HCV RNA replication in cell culture. *Science* **290**, 1972–1974.

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J. & Meurs, E. F. (2005). Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKε. *J Virol* **79**, 3969–3978.

Doyle, S., Vaidya, S., O'Connell, R., Dadgostar, H., Dempsey, P., Wu, T., Rao, G., Sun, R., Haberland, M. & other authors (2002). IRF3 mediates a TLR3/TLR4-specific antiviral gene program. *Immunity* **17**, 251–263.

Elazar, M., Liu, P., Rice, C. M. & Glenn, J. S. (2004). An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J Virol* **78**, 11393–11400.

Ferreon, J. C., Ferreon, A. C., Li, K. & Lemon, S. M. (2005). Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. *J Biol Chem* **280**, 20483–20492.

Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M. & Maniatis, T. (2003). IKKε and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* **4**, 491–496.

Foy, E., Li, K., Wang, C., Sumpter, R., Jr, Ikeda, M., Lemon, S. M. & Gale, M., Jr (2003). Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* **300**, 1145–1148.

Frese, M., Schwärzle, V., Barth, K., Krieger, N., Lohmann, V., Mihm, S., Haller, O. & Bartenschlager, R. (2002). Interferon-γ inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology* **35**, 694–703.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Häussinger, D., Diago, M., Garosi, G. & other authors (2002). Peginterferon α-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* **347**, 975–982.

Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M. & Rice, C. M. (1993). Expression and identification of hepatitis C virus polyprotein cleavage products. *J Virol* **67**, 1385–1395.

Grassmann, C. W., Isken, O., Tautz, N. & Behrens, S. E. (2001). Genetic analysis of the pestivirus nonstructural coding region: defects in the NS5A unit can be complemented in *trans*. *J Virol* **75**, 7791–7802.

Gretton, S. N., Taylor, A. I. & McLauchlan, J. (2005). Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J Gen Virol* **86**, 1415–1421.

Guo, J. T., Bichko, V. V. & Seeger, C. (2001). Effect of alpha interferon on the hepatitis C virus replicon. *J Virol* **75**, 8516–8523.

Hanley, K. A., Manlucu, L. R., Gilmore, L. E., Blaney, J. E., Jr, Hanson, C. T., Murphy, B. R. & Whitehead, S. S. (2003). A trade-off in replication in mosquito versus mammalian systems conferred by a point mutation in the NS4B protein of dengue virus type 4. *Virology* **312**, 222–232.

He, Y. & Katze, M. G. (2002). To interfere and to anti-interfere: the interplay between hepatitis C virus and interferon. *Viral Immunol* **15**, 95–119.

Itsui, Y., Sakamoto, N., Kurosaki, M., Kanazawa, N., Tanabe, Y., Koyama, T., Takeda, Y., Nakagawa, M., Kakinuma, S. & other authors (2006). Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. *J Viral Hepat* **13**, 690–700.

Kanazawa, N., Kurosaki, M., Sakamoto, N., Enomoto, N., Itsui, Y., Yamashiro, T., Tanabe, Y., Maekawa, S., Nakagawa, M. & other authors (2004). Regulation of hepatitis C virus replication by interferon regulatory factor 1. *J Virol* **78**, 9713–9720.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T. & other authors (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101–105.

Katze, M. G., He, Y. & Gale, M. (2002). Viruses and interferon: a fight for supremacy. *Nature Reviews* **2**, 675–687.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., Ishii, K. J., Takeuchi, O. & Akira, S. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* **6**, 981–988.

Khromykh, A. A., Sedlak, P. L. & Westaway, E. G. (2000). *cis*- and *trans*-acting elements in flavivirus RNA replication. *J Virol* **74**, 3253–3263.

Li, Y. & McNally, J. (2001). Characterization of RNA synthesis and translation of bovine viral diarrhea virus (BVDV). *Virus Genes* **23**, 149–155.

Lin, R., Heylbroeck, C., Pitha, P. M. & Hiscott, J. (1998). Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol* **18**, 2986–2996.

Lindström, H., Lundin, M., Häggström, S. & Persson, M. A. (2006). Mutations of the hepatitis C virus protein NS4B on either side of the ER membrane affect the efficiency of subgenomic replicons. *Virus Res* **121**, 169–178.

Lohmann, V., Körner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110–113.

Lohmann, V., Hoffmann, S., Herian, U., Penin, F. & Bartenschlager, R. (2003). Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. *J Virol* **77**, 3007–3019.

Lundin, M., Monné, M., Widell, A., Von Heijne, G. & Persson, M. A. (2003). Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* **77**, 5428–5438.

Lundin, M., Lindström, H., Grönwall, C. & Persson, M. A. (2006). Dual topology of the processed hepatitis C virus protein NS4B is influenced by the NS5A protein. *J Gen Virol* **87**, 3263–3272.

Meylan, E., Curran, J., Hofmann, K., Moradpour, D., Binder, M., Bartenschlager, R. & Tschopp, J. (2005). Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **437**, 1167–1172.

Mori, K. (2000). Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* **101**, 451–454.

Muñoz-Jordán, J. L., Laurent-Rolle, M., Ashour, J., Martínez-Sobrido, L., Ashok, M., Lipkin, W. I. & García-Sastre, A. (2005). Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. *J Virol* **79**, 8004–8013.

Nakagawa, M., Sakamoto, N., Enomoto, N., Tanabe, Y., Kanazawa, N., Koyama, T., Kurosaki, M., Maekawa, S., Yamashiro, T. & other authors (2004). Specific inhibition of hepatitis C virus replication by cyclosporin A. *Biochem Biophys Res Commun* **313**, 42–47.

Nakaya, T., Sato, M., Hata, N., Asagiri, M., Suemori, H., Noguchi, S., Tanaka, N. & Taniguchi, T. (2001). Gene induction pathways mediated by distinct IRFs during viral infection. *Biochem Biophys Res Commun* **283**, 1150–1156.

Polyak, S. J., Khabar, K. S., Paschal, D. M., Ezelle, H. J., Duverlie, G., Barber, G. N., Levy, D. E., Mukaida, N. & Gretch, D. R. (2001). Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol* **75**, 6095–6106.

Qu, L., McMullan, L. K. & Rice, C. M. (2001). Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. *J Virol* **75**, 10651–10662.

Saito, T., Hirai, R., Loo, Y. M., Owen, D., Johnson, C. L., Sinha, S. C., Akira, S., Fujita, T. & Gale, M., Jr (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proc Natl Acad Sci U S A* **104**, 582–587.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clin Microbiol Rev* **14**, 778–809 (Table of Contents).

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., Nakaya, T., Katsuki, M., Noguchi, S. & other authors (2000). Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-α/β gene induction. *Immunity* **13**, 539–548.

Schweizer, M. & Peterhans, E. (2001). Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. *J Virol* **75**, 4692–4698.

Seth, R. B., Sun, L., Ea, C. K. & Chen, Z. J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-κB and IRF 3. *Cell* **122**, 669–682.

Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R. & Hiscott, J. (2003). Triggering the interferon antiviral response through an IKK-related pathway. *Science* **300**, 1148–1151.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. (1998). How cells respond to interferons. *Annu Rev Biochem* **67**, 227–264.

Sumpter, R., Jr, Wang, C., Foy, E., Loo, Y. M. & Gale, M., Jr (2004). Viral evolution and interferon resistance of hepatitis C virus RNA replication in a cell culture model. *J Virol* **78**, 11591–11604.

Talon, J., Horvath, C. M., Polley, R., Basler, C. F., Muster, T., Palese, P. & García-Sastre, A. (2000). Activation of interferon regulatory factor 3 is inhibited by the influenza A virus NS1 protein. *J Virol* **74**, 7989–7996.

Tanabe, Y., Sakamoto, N., Enomoto, N., Kurosaki, M., Ueda, E., Maekawa, S., Yamashiro, T., Nakagawa, M., Chen, C. H. & other authors (2004). Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-α. *J Infect Dis* **189**, 1129–1139.

Taniguchi, T. & Takaoka, A. (2002). The interferon-α/β system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. *Curr Opin Immunol* **14**, 111–116.

Taniguchi, T., Ogasawara, K., Takaoka, A. & Tanaka, N. (2001). IRF family of transcription factors as regulators of host defense. *Annu Rev Immunol* **19**, 623–655.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**, 791–796.

Xu, L. G., Wang, Y. Y., Han, K. J., Li, L. Y., Zhai, Z. & Shu, H. B. (2005). VISA is an adapter protein required for virus-triggered IFN-β signaling. *Mol Cell* **19**, 727–740.

Yamashiro, T., Sakamoto, N., Kurosaki, M., Kanazawa, N., Tanabe, Y., Nakagawa, M., Chen, C. H., Itsui, Y., Koyama, T. & other authors (2006). Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3. *J Gastroenterol* **41**, 750–757.

Yanagi, M., Purcell, R. H., Emerson, S. U. & Bukh, J. (1997). Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci U S A* **94**, 8738–8743.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K. & other authors (2003). Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* **4**, 602–608.

Yoneyama, M., Suhara, W., Fukuhara, Y., Fukuda, M., Nishida, E. & Fujita, T. (1998). Direct triggering of the type I interferon system by

virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J* 17, 1087–1095.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S. & Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5, 730–737.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr & other authors (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175, 2851–2858.

Zheng, Y., Gao, B., Ye, L., Kong, L., Jing, W., Yang, X., Wu, Z. & Ye, L. (2005). Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. *J Microbiol* 43, 529–536.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & Chisari, F. V. (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* 102, 9294–9299.

Zhu, Q., Guo, J. T. & Seeger, C. (2003). Replication of hepatitis C virus subgenomes in nonhepatic epithelial and mouse hepatoma cells. *J Virol* 77, 9204–9210.

Scavenger Receptor Class B Type I Is a Key Host Factor for Hepatitis C Virus Infection Required for an Entry Step Closely Linked to CD81

Mirjam B. Zeisel,[1-3] George Koutsoudakis,[4] Eva K. Schnober,[1-3,5] Anita Haberstroh,[1-3] Hubert E. Blum,[3] François-Loïc Cosset,[6-8] Takaji Wakita,[9] Daniel Jaeck,[10] Michel Doffoel,[11] Cathy Royer,[1,2] Eric Soulier,[1,2] Evelyne Schvoerer,[1,2] Catherine Schuster,[1,2] Françoise Stoll-Keller,[1,2] Ralf Bartenschlager,[4] Thomas Pietschmann,[4] Heidi Barth,[3] and Thomas F. Baumert[1-3,11]

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Scavenger receptor class B type I (SR-BI) has been shown to bind HCV envelope glycoprotein E2, participate in entry of HCV pseudotype particles, and modulate HCV infection. However, the functional role of SR-BI for productive HCV infection remains unclear. In this study, we investigated the role of SR-BI as an entry factor for infection of human hepatoma cells using cell culture–derived HCV (HCVcc). Anti–SR-BI antibodies directed against epitopes of the human SR-BI extracellular loop specifically inhibited HCVcc infection in a dose-dependent manner. Down-regulation of SR-BI expression by SR-BI–specific short interfering RNAs (siRNAs) markedly reduced the susceptibility of human hepatoma cells to HCVcc infection. Kinetic studies demonstrated that SR-BI acts predominately after binding of HCV at an entry step occurring at a similar time point as CD81–HCV interaction. Although the addition of high-density lipoprotein (HDL) enhanced the efficiency of HCVcc infection, anti–SR-BI antibodies and SR-BI–specific siRNA efficiently inhibited HCV infection independent of lipoprotein. *Conclusion:* Our data suggest that SR-BI (i) represents a key host factor for HCV entry, (ii) is implicated in the same HCV entry pathway as CD81, and (iii) targets an entry step closely linked to HCV–CD81 interaction. (HEPATOLOGY 2007;46: 1722-1731.)

Abbreviations: cDNA, complementary DNA; CHO, Chinese hamster ovary; HCV, hepatitis C virus; HCVcc, cell culture–derived HCV; HCVpp, HCV pseudotype particles; HDL, high-density lipoprotein; HRP, horseradish peroxidase; IgG, immunoglobulin G; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient human serum; siRNA, small interfering RNA; SR-BI, scavenger receptor class B type I.

From [1]Institut National de la Santé et de la Recherche Médicale (INSERM), U748, Strasbourg, France; [2]Université Louis Pasteur, Strasbourg, France; the [3]Department of Medicine II, University of Freiburg, Germany; the [4]Department for Molecular Virology, University of Heidelberg, Germany; the [5]Faculty of Biology, University of Freiburg, Germany; [6]INSERM, U758, Lyon, France; [7]Ecole Normale Supérieure de Lyon, Lyon, France; [8]IFR128 BioSciences Lyon-Gerland, Lyon, France; the [9]Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan; the [10]Centre de Chirurgie Viscérale, Hépatique et de Transplantation Multiorganes, Université Louis Pasteur, Strasbourg, France; and the [11]Service d'Hépatogastroentérologie, Hôpitaux Universitaires de Strasbourg, Strasbourg, France.

Received April 5, 2007; accepted August 25, 2007.

Supported by grants of INSERM, France, the European Union (VIRGIL Network of Excellence, LSHM-CT-2004-503359.) Belgium, the chair of excellence program of the Agence Nationale de la Recherche (ANR-05-CEXC-008), France, the Agence Nationale de la Recherche sur le SIDA et les Hépatites Virales (ANRS Grant #06221), France, the Deutsche Forschungsgemeinschaft (Ba1417/11-2), Germany, France. M.B.Z. was supported by the INSERM Poste Vert program in the frame work of Inserm European Associated Laboratory Freiburg-Strasbourg. T.P. was supported by an Emmy Noether fellowship from the Deutsche Forschungsgemeinschaft (Pl 734/1-1), and a grant from the Ministry of Science, Research and the Arts of Baden-Württemberg (Az. 23-7532.24-22-21-12/1) awarded to T.P. and R.B. T.W. was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan as well as Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

Dr. Wakita is currently affiliated with the National Institute of Infectious Diseases, Department of Virology II, Tokyo, Japan. Dr. Pietschmann is currently affiliated with TWINCORE—Center for Experimental and Clinical Infection Research, Department Experimental Virology, Hannover, Germany. Dr. Barth is currently affiliated with Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD.

Address reprint requests to: Thomas F. Baumert, M.D., INSERM Unité 748, Université Louis Pasteur, 3 Rue Koeberlé, F-67000 Strasbourg, France. E-mail: Thomas.Baumert@viro-ulp.u-strasbg.fr; fax: (33) 3-90-24-37-24.

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.21994

Potential conflict of interest: Nothing to report.

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health.[1] The liver is the primary target organ of HCV, and the hepatocyte is its primary target cell. Attachment of the virus to the cell surface followed by viral entry is the first step in a cascade of interactions between the virus and the target cell that is required for successful entry into the cell and initiation of infection.[2] Using recombinant HCV envelope glycoproteins[3] and HCV pseudotype particles (HCVpp),[4,5] several cell surface molecules have been identified interacting with HCV during viral binding and entry. These include the tetraspanins CD81[3] and claudin-1,[6] highly sulfated heparan sulfate,[7] the low-density lipoprotein (LDL) receptor,[8] and scavenger receptor class B type I (SR-BI).[9]

SR-BI is a 509–amino acid glycoprotein with a large extracellular loop anchored to the plasma membrane at both the N- and C- termini by transmembrane domains with short extensions into the cytoplasm.[10] SR-BI is involved in bidirectional cholesterol transport at the cell membrane and can bind both native high-density lipoprotein (HDL) and LDL as well as modified lipoproteins such as oxidized LDL. SR-BI is highly expressed in liver and steroidogenic tissues[10] as well as antigen-presenting cells.[11] Furthermore, SR-BI and its splicing variant SR-BII, have been found to mediate binding and uptake of a broad range of bacteria into human epithelial cells overexpressing SR-BI and SR-BII,[12,13] suggesting that class B scavenger receptors may serve as pattern recognition receptors for bacteria.

Cross-linking studies using recombinant C-terminally truncated HCV envelope glycoprotein E2 isolated SR-BI as a cellular protein binding envelope glycoprotein E2.[9] Antibodies directed against cell surface expressed SR-BI partially inhibited cellular binding of recombinant envelope glycoproteins[14] as well as HCVpp entry.[15-17] Moreover, it has been shown that physiological SR-BI ligands, such as HDL or oxidized LDL, can modulate HCV infection either by enhancing or by inhibiting HCVpp entry, respectively.[18-20]

Recently, several laboratories succeeded in establishing a model for the efficient production of infectious HCV particles in cell culture (HCVcc),[21-23] now allowing determining of the role of cell surface molecules involved in HCV infection. Recent evidence suggests that SR-BI and CD81 may act in a cooperative manner for the initiation of HCVcc infection[24] and that overexpression of SR-BI can modulate HCVcc infection.[25] However, the functional role of SR-BI in productive HCV infection still remains elusive. In particular, it is unclear whether the impact of SR-BI for HCV entry is of key importance or optional, whether SR-BI and CD81 are involved in the same pathways of HCV entry, and which HCV entry step is targeted by SR-BI.

Therefore, in this study, we used the HCVcc system to analyze the functional role of SR-BI for productive HCV infection of human hepatoma cells. Using novel anti–SR-BI antibodies and SR-BI–specific short interfering RNAs (siRNAs), we demonstrate that SR-BI (i) represents a key host factor for HCV entry, (ii) is most likely implicated in the same HCV entry pathway as CD81, and (iii) mediates an entry step occurring postbinding and closely linked to HCV–CD81 interaction.

Materials and Methods

Cells. Human embryonic kidney cells 293T, Chinese hamster ovary cells CHO, and Huh7.5 have been described.[4,7,26,27] Primary human hepatocytes were isolated and cultured as described.[28]

Antibodies. Antibodies directed against the extracellular loop of SR-BI were raised by genetic immunization of Wistar rats and Balb/c mice using a pcDNA-expression vector containing the full-length human SR-BI complementary DNA (cDNA) (pcDNA SR-BI/CLA-1) (Genovac GmbH, Freiburg, Germany).[29] In brief, animals received 4 applications of 50 μg pcDNA SR-BI intradermally using a GeneGun (BioRad) at 2-week intervals. Pre-immune control serum was collected from the same animal bled before immunization. To analyze specificity of the produced anti–SR-BI polyclonal serum, CHO cells were transfected with pcDNA (control vector) or pcDNA SR-BI using liposome-mediated gene transfer (Lipofectamine; Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. CHO cells were then incubated with anti–SR-BI polyclonal serum or pre-immune control serum and analyzed for cell surface SR-BI expression by flow cytometry as described.[14] R-phycoerythrin–conjugated goat anti-rat immunoglobulin G (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Because of the small amounts of pre-immune sera from individual animals used for immunization, commercially available nonimmune rat serum (PAN Biotech) was used as an additional negative control serum for experiments. Rabbit anti–SR-BI antibody (NB 400-104) was obtained from Novus Biologicals (Littleton, CO). This antibody is directed against an epitope within the SR-BI cytoplasmic C-terminal domain (CSPAAKGTVLQEAKL, corresponding to amino acids 496 through 509). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG antibodies were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), mouse anti–

β-actin antibody was from Sigma and anti-CD81 (JS-81) from BD Biosciences (Heidelberg, Germany).

RNA Interference Assay. Commercially available siRNA pools targeting SR-BI, CD81, and CD13 as well as control nontargeting siRNAs were purchased from Dharmacon (Pierce) and transfected into Huh7.5 cells using DharmaFect solution (Pierce) following the manufacturer's protocol. Silencing of SR-BI expression was assessed by western blot and flow cytometry 72 hours after transfection as described previously.[14] Seventy-two hours after transfection, cells were incubated with HCVcc, and HCV infection was assessed as described in the following sections.

Production of Viral Stocks and Infection Assays. Plasmids pJFH1, pFK-Jc1, and pFK-Luc-Jc1 have been described[21,30,31] and encode the full-length HCV Japanese fulminant hepatitis (JFH) cDNA or the chimeric HCV genome designated Jc1, which consists of J6CF and JFH1 segments. The latter construct (pFK-Luc-Jc1) represents a bicistronic reporter virus carrying a firefly-luciferase reporter gene.[30] *In vitro* HCV RNA synthesis[30] and RNA transfection was performed as described.[21,29] To study the effect of HDL on HCVcc infection, JFH1 HCVcc were also generated in lipoprotein-deficient human serum (LPDS), and HDL (30 μg/mL)[33] was added extemporaneously for infection experiments. Culture supernatants from transfected cells were cleared and concentrated as previously described using Amicon Ultra 15 (Millipore, Billerica, MA)[21] and used directly or stored at 4°C or −80°C. Viruses were titered by using the limiting dilution assay on Huh7.5 cells with a few minor modifications, and 50% tissue culture infective dose was calculated based on the method described.[22] siRNA expressing cells and naïve cells were seeded 24 hours before infection experiments in 12-well tissue culture plates at a density of 5×10^4 cells/well. Cells were preincubated in the presence or absence of anti–SR-BI serum or control serum for 1 hour at 37°C and then infected at 37°C for 3 hours with JFH1 HCVcc challenge virus titers ranging from 1×10^7 to 5×10^9 copies/mL or 4 hours with Luc-Jc1 HCVcc at low multiplicity of infection. Alternatively, Huh7.5 were inoculated with a high-titer Luc-Jc1 stock for 1 hour at 4°C in the presence or absence of anti–SR-BI serum or control serum, heparin, anti-CD81 monoclonal antibodies, or concanamycin A at concentrations indicated in the text. Subsequently, cells were washed 3 times with ice-cold phosphate-buffered saline, supplied with fresh culture fluid prewarmed to 37°C and supplemented with the respective inhibitors and shifted to 37°C. Finally, 4 hours later, cells were washed with prewarmed phosphate-buffered saline, supplied with fresh culture fluid without inhibitors, and cultured an additional 48 hours at 37°C. Depending on the experiment, cells were then washed with ice-cold phosphate-buffered saline and RNA extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). Alternatively, cells were lysed for luciferase assay as previously described.[30] HCV RNA was quantitated using VERSANT HCV-RNA 3.0 Assay (bDNA) (Bayer Corporation Diagnostic, Tarrytown, NY) or TaqMan real-time polymerase chain reaction as described.[34]

Results

Production of Antibodies Directed Against the Extracellular Loop of SR-BI Expressed on Human Hepatocytes. To assess the functional role of SR-BI for initiation of HCV infection, we first generated polyclonal anti–SR-BI sera directed against the extracellular loop of SR-BI by genetic immunization. After completion of immunization, antibodies were selected for their ability to bind to human SR-BI expressed on the cell surface of nonpermeabilized transfected CHO cells. As shown in Fig. 1, incubation of CHO cells expressing human SR-BI with rat polyclonal anti–SR-BI antibodies resulted in a specific interaction of this serum with the extracellular ectodomain of SR-BI (Fig. 1). In contrast, no interaction was present in CHO cells transfected with the pcDNA3 control vector and incubated with rat anti–SR-BI serum or in CHO cells transfected with human SR-BI cDNA and incubated with rat preimmune serum

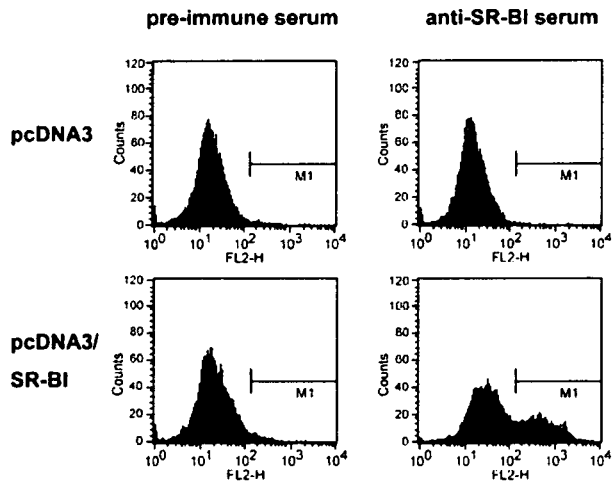

Fig. 1. Production of antibodies directed against the human SR-BI ectodomain by genetic immunization. Specific binding of rat anti-human SR-BI serum to SR-BI expressed in CHO cells. Anti-SR-BI polyclonal serum directed against the SR-BI ectodomain loop was raised by genetic immunization of Wistar rats using a plasmid harboring human SR-BI cDNA. CHO cells were transfected with pcDNA-SR-BI (pCDhSR-BI) or control vector (pcDNA). Flow cytometry of SR-BI or control transfected nonpermeabilized CHO cells incubated with rat anti-human SR-BI polyclonal serum and phycoerythrin-conjugated anti-rat IgG demonstrated specific interaction of anti–SR-BI antibodies with human SR-BI. In contrast, no interaction was present in CHO cells transfected with control vector and incubated with anti–SR-BI serum.

(Fig. 1). To study whether anti-human SR-BI recognizes SR-BI on cells susceptible to HCV infection, human hepatocytes and Huh7.5 hepatoma cells were incubated with the sera and analyzed by flow cytometry. As shown in Fig. 2, incubation of human Huh7.5 cells (Fig. 2A) and human hepatocytes (Fig. 2B) with rat polyclonal anti–SR-BI antibody demonstrated that the antibody recognized SR-BI expressed on HCV target cells, including human hepatocytes. In contrast, no interaction could be detected in the mouse cell line Hepa1.6 (Fig. 2C), confirming the species specificity of the antibody. Similar results were obtained for anti–SR-BI antibodies raised in Balb/c mice (data not shown). Taken together, these data demonstrate that anti–SR-BI sera produced by genetic immunization specifically binds to the ectodomain of human SR-BI expressed on hepatocytes.

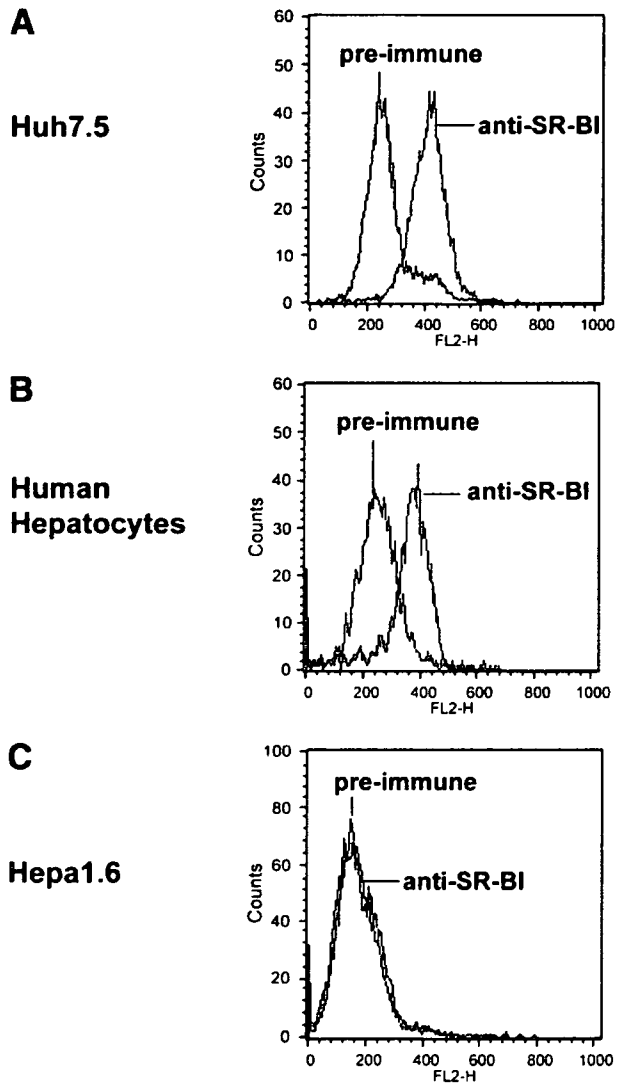

Fig. 2. Interaction of anti-SR-BI antibodies with the SR-BI ectodomain on human hepatocytes and Huh7.5 hepatoma cells. Cell surface expression of SR-BI was determined by flow cytometry using rat anti-human SR-BI serum or control pre-immune serum as described in Fig. 1. Histograms corresponding to cell surface expression of the respective cell surface molecules (open curves) are overlaid with histograms of cells incubated with the appropriate isotype control (gray shaded curves). In contrast to absent interaction on murine Hepa1.6 hepatoma cells, rat anti-human SR-BI serum specifically detected SR-BI on the cell surface of human hepatoma Huh7.5 cells and human primary hepatocytes.

Inhibition of HCV Infection of Different Isolates by Anti–SR-BI Antibodies. To assess the role of SR-BI for HCV infection, we studied JFH1 HCVcc infection of Huh7.5 cells in the presence of anti–SR-BI antibodies directed against epitopes of the SR-BI extracellular loop. Anti–SR-BI polyclonal antibodies markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a dose-dependent manner (Fig. 3A). Fig. 3A shows that anti–SR-BI serum (rat 4) inhibited JFH1 HCVcc infection by more than 70% (Fig. 3A). In contrast, the control pre-immune serum had no inhibitory effect on JFH1 HCVcc infection (Figs. 5, 6). Moreover, mouse anti–SR-BI antibodies generated by genetic immunization of Balb/c mice but not mouse control pre-immune serum were able to reduce JFH1 HCVcc infection of Huh7.5 in a similar manner (data not shown). Taken together, the data demonstrate that antibodies directed against the SR-BI ectodomain efficiently inhibit HCV infection.

To confirm that inhibition of JFH1 HCVcc infection was indeed mediated by anti–SR-BI antibodies, we purified IgG from both rat anti-SRBI (rat 4) and control serum. As shown in Fig. 3B, anti–SR-BI IgG (100 μg/mL) markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a similar manner as anti–SR-BI serum (Fig. 3B). In contrast, control IgG (100 μg/mL) purified from pre-immune serum did not inhibit JFH1 HCVcc infection (Fig. 3B). These data clearly demonstrate that the inhibitory effect of anti–SR-BI serum is mediated by anti–SR-BI antibodies and not by other substances present in the serum (such as oxidized lipoproteins potentially interfering with SR-BI function).

To study whether anti–SR-BI–mediated inhibition of HCV infection applies also to isolates other than JFH1, we performed similar experiments using chimeric J6/CF-JFH1 firefly luciferase reporter virus Luc-Jc1.[30] Figure 3C shows that, similar as for JFH1 HCVcc, both rat (rat 4) and mouse anti–SR-BI antibodies (data not shown) reduced the chimeric reporter virus infectivity in a dose-dependent manner (Fig. 3C), whereas the control pre-immune sera had no inhibitory effect (Fig. 3C and data not shown). Interestingly, we observed variations between the inhibitory effect of anti–SR-BI sera from different rats ranging from 70%-90%. Strongest inhibition was obtained with anti–SR-BI serum from rat 5 (Figs. 5 and 6), demonstrating greater than 90% inhibition of HCVcc infection, with both JFH1 isolate and Luc-Jc1 chimera.

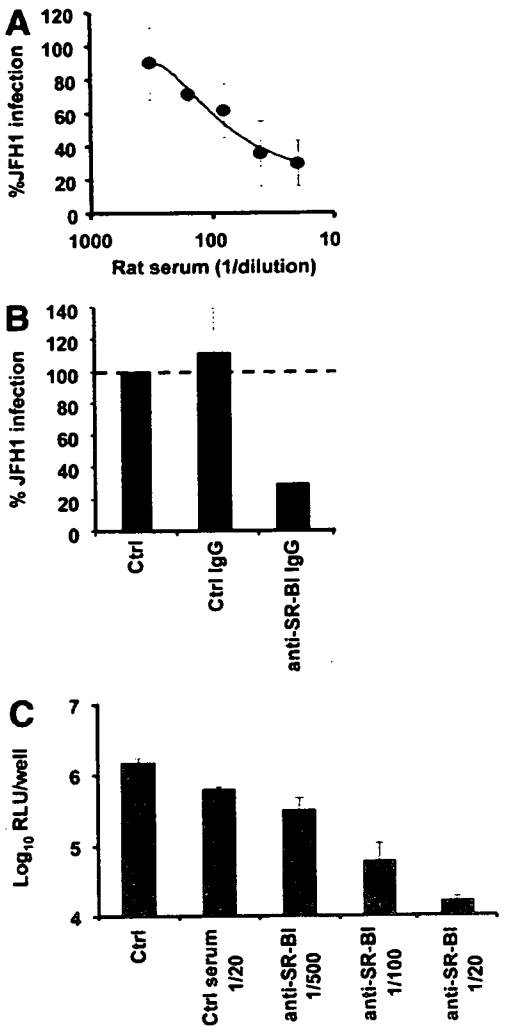

Fig. 3. Inhibition of HCV infection by anti-SR-BI antibodies. (A) Inhibition of JFH1 HCVcc infection by rat polyclonal anti-SR-BI antiserum. Huh7.5 cells were preincubated for 1 hour at 37°C with various dilutions of rat anti-SR-BI or control serum before infection with JFH1 HCVcc for 3 hours at 37°C. HCV infection was assessed by HCV RNA quantitation in lysates of infected Huh7.5 cells 72 hours post-infection. Total RNA was isolated and HCV RNA was quantified as described in Materials and Methods. Results are expressed as mean percentage HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Inhibition of JFH1 HCVcc infection by purified rat anti-SR-BI IgG. Huh7.5 cells were preincubated for 1 hour at 37°C with 100 μg/mL IgG isolated from rat anti-SR-BI or control serum before infection with JFH1 HCVcc. Results are expressed as percent HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (C) Inhibition of Luc-Jc1 HCVcc infection by anti-SR-BI. Huh7.5 cells were preincubated for 1 hour at 37°C with various dilutions of rat anti-SR-BI serum or control serum before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed by measurement of luciferase activity 48 hours after infection in lysates of infected cells. Results are expressed as mean Log_{10} RLU/well (mean ± SD; n = 4).

Silencing of SR-BI Expression Results in Markedly Reduced Susceptibility to HCV Infection. To further investigate the role of SR-BI in HCVcc infection, we silenced SR-BI expression in Huh7.5 cells using siRNAs targeting SR-BI expression. Immunoblot analysis of transfected cells shows that SR-BI–specific siRNA reproducibly down-regulates SR-BI expression, whereas β-actin expression was not affected (Fig. 4A). In contrast, a pool of negative control siRNA as well as siRNA targeting CD81 or CD13 did not significantly modulate SR-BI expression, confirming the specificity of the siRNA used (Fig. 4A). Down-regulation of SR-BI expressed on the cell surface of Huh7.5 cells by SR-BI–specific siRNA was also confirmed by flow cytometry (difference in mean fluorescence intensity (ΔMFI) of SR-BI siRNA-treated cells = 13.98 versus ΔMFI of naïve cells = 148.31). Importantly, down-regulation of SR-BI cell surface expression strongly reduced the susceptibility of human hepatoma cells to infection with HCV (Fig. 4B). As shown in Fig. 4B, siRNA targeting SR-BI or CD81 markedly inhibited JFH1 HCVcc infection of Huh7.5 cells as compared with cells without silenced cell surface molecules (Fig. 4B).

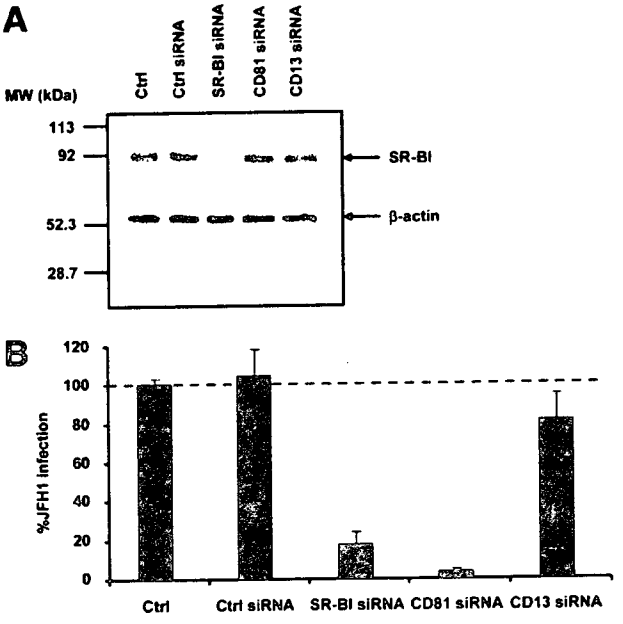

Fig. 4. Silencing of SR-BI expression results in reduced susceptibility to HCV infection. (A) Western blot analysis of siRNA mediated down-regulation of SR-BI expression in Huh7.5 cells. Lysates of control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was performed using rabbit anti-SR-BI antibodies (1/4000) and HRP-conjugated anti-rabbit antibodies (1/1000) or mouse anti-β-actin monoclonal antibody (1/5000) and HRP-conjugated anti-mouse antibodies (1/1000). The presence or absence of SR-BI and β-actin is indicated on the right, and molecular weight (MW) markers (kDa) are indicated on the left. (B) Susceptibility to HCVcc infection is reduced in SR-BI specific siRNA expressing Huh7.5 cells. Control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were incubated with JFH1 HCVcc. Total RNA was extracted 72 hours after infection, and HCV RNA was quantified. Data are expressed as percent HCVcc infectivity of naïve control cells (mean ± SD; n = 4).

In contrast, pools of negative control siRNA as well as CD13-specific siRNA did not significantly reduce JFH1 HCVcc infection (Fig. 4B). Taken together, reduced susceptibility to HCV infection by specific silencing of SR-BI expression clearly demonstrates that SR-BI plays a key role for the establishment of HCV infection of human hepatoma cells.

Impact of Lipoproteins for SR-BI–Mediated HCV Infection. To investigate the impact of lipoproteins on SR-BI–mediated HCV infection, we determined the ability of anti–SR-BI antibodies to inhibit JFH1 HCVcc infection in the absence of HDL, a physiological SR-BI ligand that has been shown to enhance HCVcc infection of human hepatoma cells.[35] To study the role of HDL during inhibition experiments, HCVcc were generated in medium supplemented with LPDS, and HDL was added extemporaneously for infection experiments. Fig. 5A shows that rat anti–SR-BI serum (rat 5) inhibited JFH1 HCVcc infection of Huh7.5 cells in the absence of HDL (Fig. 5A). Interestingly, whereas HDL was able to enhance JFH1 HCVcc infection in control cells and control serum preincubated cells, no such effect was observed in the presence of anti–SR-BI antibodies in concentrations blocking HCVcc infection (Fig. 5A). These results suggest that these antibodies may block both HCV interaction with SR-BI and HDL-mediated enhancing effect on HCVcc infection. To study whether HDL-dependent enhancement of HCVcc infection was dependent on the level of input virus, we repeated experiments using different JFH1 HCVcc preparations with challenge virus titers ranging from 1×10^7 copies/mL to 5×10^9 copies/mL, resulting in similar observations (data not shown). Furthermore, the effects of HDL on HCVcc infection were confirmed by titration experiments using anti–SR-BI antibodies: as shown in Fig. 5A, the enhancing effect of HDL on HCVcc infection appeared to be restored when anti–SR-BI antibodies were used at decreasing concentrations (Fig. 5A). In addition, the role of HDL on JFH1 HCVcc infection was also studied in siRNA-transfected Huh7.5 cells. As shown in Fig. 5B, a minor enhancing effect of HDL was detected in Huh7.5 cells transfected with siRNA targeting SR-BI, suggesting that a low level of SR-BI may still be available for HCV/HDL interplay on these cells. In cells with silenced CD81 expression, no marked enhancing effect of HDL on JFH1 HCVcc infection was observed (Fig. 5B).

SR-BI Mediates an HCV Entry Step Occurring Postbinding and Closely Linked to CD81. Kinetic studies using chimeric JFH1 firefly luciferase reporter virus have demonstrated that glycosaminoglycans predominantly act at the stage of HCV attachment to target cells, whereas CD81 mediates HCV infection at a step postbinding.[31] To map the step targeted by SR-BI during HCV entry, we investigated the inhibitory capacity of

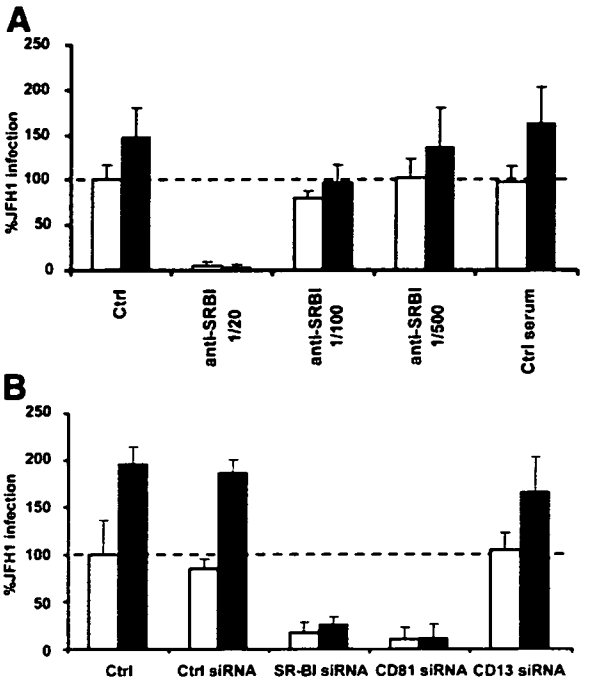

Fig. 5. SR-BI-mediated HCV infection is not dependent on the presence of lipoproteins. (A) Anti-SR-BI inhibits HCVcc infection in the absence of lipoproteins. Huh7.5 cells were preincubated for 1 hour at 37°C with rat anti-SR-BI serum or control serum (diluted 1/20, 1/100, and 1/500) before infection with JFH1 HCVcc generated in LPDS-medium in the presence (black bars) or absence (open bars) of HDL (30 μg/mL). Total RNA was isolated 72 hours after infection, and HCV RNA was quantified. Results are expressed as percent HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Reduced susceptibility to HCVcc infection in SR-BI-specific siRNA expressing Huh7.5 cells is independent of lipoproteins. Control naïve Huh7.5 cells (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were incubated with JFH1 HCVcc generated in LPDS medium in the presence (black bars) or absence (open bars) of HDL (30 μg/mL). Total RNA was extracted 72 hours after infection, and HCV RNA was quantified. Data are expressed as mean percent HCVcc infectivity of naïve control cells (mean ± SD; n = 4).

anti–SR-BI serum (rat 5) and corresponding purified IgG when administered during or after virus binding in side-by-side experiments using heparin—a structural and functional homolog of highly sulfated heparan sulfate and anti-CD81 antibody. Luc-Jc1 HCVcc binding to Huh7.5 cells was performed for 1 hour at 4°C in the presence or absence of inhibitors. Under these conditions, virus attaches to the cells but does not efficiently enter, thus permitting synchronous infection when the inoculum is removed and cells are shifted to 37°C. Therefore, subsequent to virus attachment, unbound virus was washed away, cells were shifted to 37°C to allow entry to proceed, and inhibitors or control medium were added for 4 hours (Fig. 6A). Figure 6B shows that rat anti–SR-BI serum as well as purified anti–SR-BI IgG were able to inhibit Luc-Jc1 HCVcc infection when added following

binding of the virus to the target cell (Fig. 6B). The control serum only had no significant effect on Luc-Jc1 HCVcc infection (Fig. 6B). In contrast, heparin—a homolog of highly sulfated heparan sulfate, inhibited Luc-Jc1 HCVcc infection only when it was present during virus binding but not when added postbinding (Fig. 6B). To further characterize the entry step mediated by SR-BI, anti–SR-BI and anti-CD81 antibodies were added in side-by-side experiments every 20 minutes for up to 120 minutes after viral binding (Fig. 7A). Rat anti–SR-BI serum was able to inhibit Luc-Jc1 HCVcc infection even when added up to 60 minutes after HCVcc binding (Fig. 7B). These data clearly indicate that SR-BI is involved in an entry step occurring after binding. Because almost identical kinetics of inhibition of HCV infection was observed for anti-CD81 antibody assessed in side-by-side

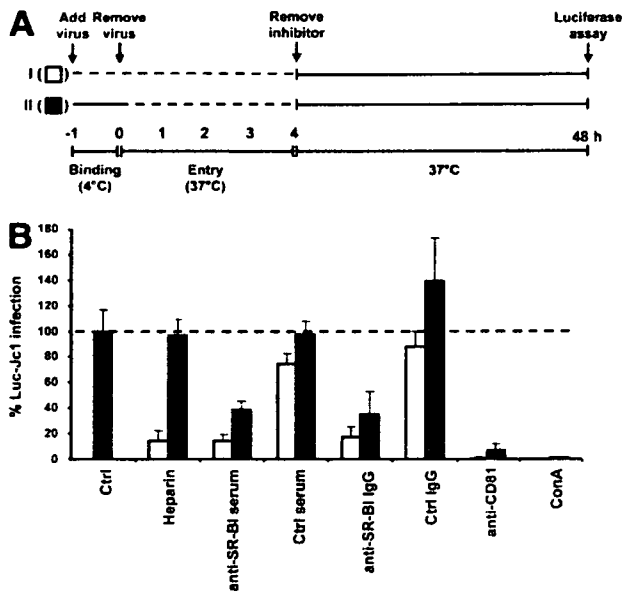

Fig. 6. SR-BI mediates in an HCV entry step occurring postbinding of virions. (A) Schematic drawing of the experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5 cells by rat anti-SR-BI serum or control serum (1/200), anti-SR-BI, or control IgG (100 μg/mL), anti-CD81 monoclonal antibody (10 μg/mL), heparin (250 μg/mL), or concanamycin A (25 nM) was compared using 2 different protocols. Virus binding to target cells was performed in the presence (protocol I) or absence (protocol II) of compounds. Subsequently, in both protocols, cells were washed, supplemented with fresh medium containing the given inhibitors, and shifted to 37°C to allow entry to proceed. Four hours later, cells were again washed and supplied with medium without inhibitors or antibodies. Dashed lines indicate the time intervals where inhibitors or antibodies were present. Luciferase activity was determined 48 hours later and is expressed relative to control infections performed in the same way but without addition of inhibitor. (B) Kinetics of HCVcc entry into human hepatoma cells. The efficiency of infection using the protocols depicted in panel A (protocol I: open bars; protocol II: black bars) was measured as described in (A). Results are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of inhibitory compound or antibody (mean ± SD; n = 4).

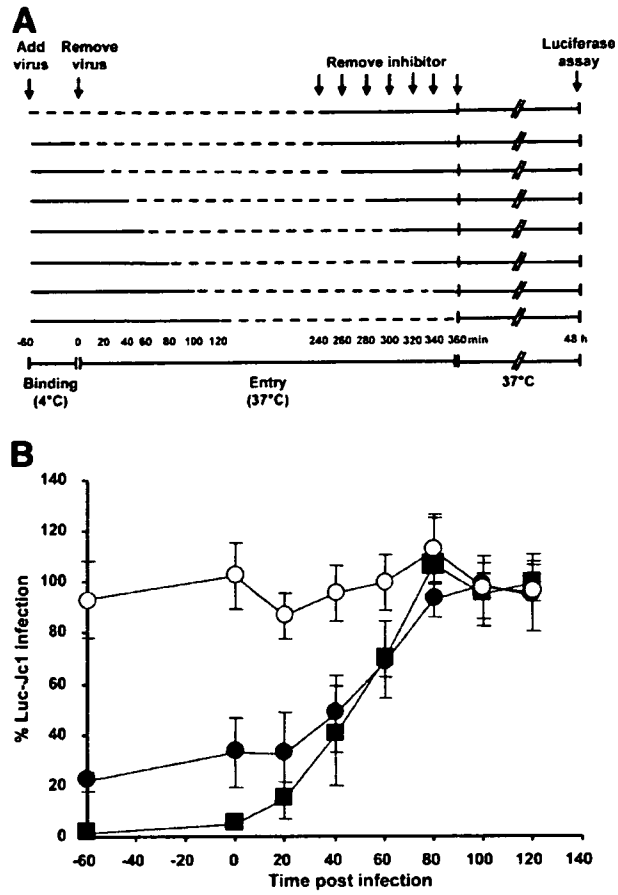

Fig. 7. SR-BI mediates an HCV entry step closely linked to CD81. (A) Schematic drawing of the experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5 cells by rat anti-SR-BI serum or control serum (1/200) as well as anti-CD81 monoclonal antibody (10 μg/mL) was performed as described in the legend to Fig. 6 but inhibitors were added every 20 minutes for 120 minutes after viral binding. Dashed lines indicate the time intervals where inhibitors are present. Luciferase activity was determined 48 hours later and is expressed relative to control infections performed in the same way but without addition of inhibitor. (B) Kinetics of HCVcc entry into human hepatoma cells. The efficiency of infection using rat anti-SR-BI serum (black circle), control serum (white circle), or anti-CD81 antibody (black square) was measured by luciferase assay 48 hours later. Results are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4).

experiments (Fig. 7B), it is likely that the entry steps mediated by SR-BI and CD81 occur during a similar time point and are closely linked to each other. To further address the contribution and interplay of CD81 and SR-BI in HCV entry, we added anti-CD81 and anti–SR-BI IgG simultaneously before Luc-Jc1 HCVcc infection. Figure 8B shows that blocking both CD81 and SR-BI inhibited Luc-Jc1 HCVcc infection more potently than blocking of each receptor alone (Fig. 8A,B). This effect was not observed when control IgG were used in combination with anti-CD81 monoclonal antibody (Fig.

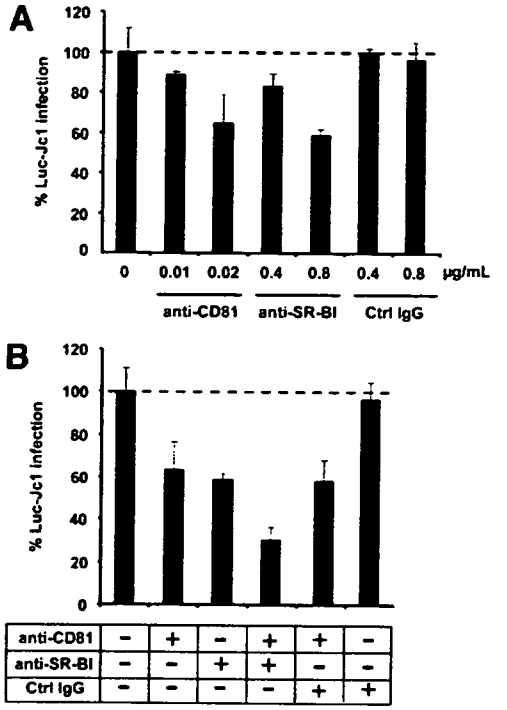

Fig. 8. SR-BI and CD81 act in concert to mediate HCV entry. (A) Dose-dependent inhibition of Luc-Jc1 HCVcc infectivity by anti-SR-BI and anti-CD81 antibodies. Huh7.5 cells were preincubated for 1 hour at 37°C with anti-CD81 monoclonal antibody (0.01 and 0.02 μg/mL), rat anti-SR-BI IgG (0.4 and 0.8 μg /mL) or control IgG (0.4 and 0.8 μg /mL) before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed by measurement of luciferase activity 48 hours after infection. Data are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Additive effect of anti-SR-BI and anti-CD81 antibodies in inhibition of HCVcc entry. Huh7.5 cells were preincubated for 1 hour at 37°C with rat anti-SR-BI IgG (0.8 μg/mL) or control IgG (0.8 μg /mL) either alone or in combination with anti-CD81 monoclonal antibody (0.02 μg/mL) before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed as described in (A). Data are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4).

8B). Taken together, our results suggest that SR-BI and CD81 may act in concert to mediate HCV entry.

Discussion

Using an infectious HCV tissue culture system, we demonstrate that SR-BI (1) represents a key host factor for HCV entry, (2) is implicated in the same HCV entry pathway as CD81, and (3) targets an entry step occurring after binding closely linked to CD81.

SR-BI delivers HDL cholesteryl ester to the liver and steroidogenic tissues by a process termed the "selective uptake pathway."[36-39] This process differs markedly from that of the classic clathrin coated pit-mediated LDL receptor endocytic pathway, in which the entire lipoprotein is internalized and degraded.[40] In the selective uptake pathway, SR-BI binds HDL and the core cholesteryl ester are delivered to the plasma membrane without the endocytosis of the entire HDL particle. SR-BI–mediated selective HDL cholesteryl ester uptake is a 2-step process: the first step involves lipoprotein binding to the extracellular domain of SR-BI and the second step consists in the selective transfer of lipid to the plasma membrane.[41,42]

The marked inhibition of HCV infection of 2 different isolates (JFH1 and Jc1) by anti–SR-BI antibodies and siRNAs suggests that SR-BI plays a key role for establishment of HCV infection. These results extend recently obtained evidence suggesting that SR-Bs modulate HCV infection.[24,25] Extending previous studies, we demonstrate that inhibition of HCVcc infection by anti–SR-BI antibodies or SR-BI–specific siRNA was not dependent on the presence of lipoproteins in the tissue culture medium, suggesting that SR-BI can mediate HCV entry independent from an interaction of HCV or SR-BI with HDL. Lavillette et al.[17] demonstrated that silencing SR-BI expression markedly reduced HCVpp entry independent of HDL, whereas Voisset et al.[19] demonstrated that SR-BI silencing only reduced the HDL-mediated enhancement of HCVpp entry. Using the HCVcc model system and transfected siRNAs, we now demonstrate that silencing of SR-BI expression resulted in a marked down-regulation of susceptibility to HCV infection independent of the presence of lipoproteins, although HDL was able to enhance HCV infection. In our hands, the use of an optimal siRNA delivery system was crucial for the study of HCV infection. Whereas recombinant lentiviral vectors were characterized by interference with HCV infection unrelated to the expressed siRNA (data not shown), the transfection of *in vitro* transcribed SR-BI siRNAs specifically resulted in down-regulation of HCV infection The specific effect of SR-BI siRNAs is demonstrated by the lack of inhibitory effects of various control siRNAs including siRNAs targeting another protein expressed on the cell surface of hepatoma cells (CD13).

Because the presence of HDL did not inhibit but rather enhanced HCV infection, it is unlikely that HCV and HDL compete for the SR-BI HDL binding domain. The highly reproducible enhancement of HCV infection by HDL may point to a more efficient interaction of SR-BI with HCV, for example, as a result of a conformational change induced by HDL. These findings are in line with a previous study demonstrating that HDL is a serum factor that attenuates neutralization by antiviral antibodies of HCVpp or HCVcc.[33] The authors hypothesized that HDL may stimulate cell entry of viral particles by accelerating their endocytosis.[35] In contrast to results of ectodomain blocking by anti-SR-BI, HDL appeared to slightly enhance HCVcc infection in cells with silenced SR-BI. This may not be un-

expected because down-regulation by siRNA, in contrast to ectodomain blocking, most likely leaves some SR-BI accessible for HDL/HCV interplay.

Furthermore, we demonstrate that in target cells with silenced CD81 expression, HDL appeared not to markedly enhance HCVcc infection (Fig. 5B). These findings strongly suggest that the HDL/SR-BI-HCV interaction acts in concert with CD81 within the same entry pathway and does not represent another or redundant route of cell entry. This conclusion is further supported by an additive inhibitory effect of anti–SR-BI and anti-CD81 antibodies on Luc-Jc1 HCVcc infection (Fig. 8B), confirming previous results obtained for JFH1.[24]

Using an HCVcc-based kinetic entry assay (Figs. 6 and 7), we mapped the HCV entry step targeted by SR-BI. As shown in Figs. 6 and 7, anti–SR-BI IgG markedly inhibited HCVcc infection when added up to 60 minutes postbinding of attached virus. These data for the first time directly demonstrate that SR-BI predominantly mediates an HCV entry step occurring after binding of HCV to the hepatocyte cell surface membrane. These findings confirm the hypothesis raised by von Hahn and colleagues[20] based on experiments using oxidized lipoproteins as SR-BI ligands. In contrast to anti–SR-BI and anti-CD81 antibodies, heparin—a homolog of highly sulfated heparan sulfate—was able to inhibit HCVcc infection only when added before HCV binding. Taken together, these data suggest that glycosaminoglycans such as highly sulfated heparan sulfate act predominantly at the stage of viral attachment, whereas SR-BI and CD81 mediate entry steps occurring postbinding. Subsequent steps in HCV entry are most likely mediated by claudin-1, a recently discovered co-host factor for HCV infection.[6]

Kinetic studies using anti–SR-BI and CD81 antibodies in side-by-side experiments demonstrated that SR-BI is required for an entry step occurring at a similar time point as CD81–HCV interaction. Although the magnitude of antibody-mediated inhibition of HCVcc infection was different, the kinetics of inhibition of HCV infection by anti–SR-BI and anti-CD81 antibodies was remarkably similar (Fig. 6). Both anti–SR-BI and anti-CD81 antibodies were able to inhibit HCV infection when added up to 60 minutes after binding and lost their ability to inhibit HCV infection when added 80 minutes after binding. The rate of loss of CD81 antibody HCVcc inhibition overtime in our study appeared to be different from that observed for HCVpp in previously published studies.[6,43] This either may be due to experimental differences or may be a difference between the behaviors of HCVpp and HCVcc. Taken together, our results demonstrate that the entry steps mediated by SR-BI and CD81 occur during a similar time frame and are closely linked to each other.

SR-BI and its splicing variant SR-BII contain an identical extracellular domain. SR-BII is encoded by an alternatively spliced messenger RNA from the SR-BI gene and differs from SR-BI only in the carboxy-terminal cytoplasmic tail, which, as shown previously, must contain a signal that confers predominant intracellular expression and rapid endocytosis of HDL.[44] Scavenger receptor BII, which is expressed at low levels in the liver compared with SR-BI,[45] mediates rapid HDL endocytosis through a clathrin-dependent, caveolae-independent pathway,[44] but is inefficient compared with SR-BI in HDL cholesteryl ester selective uptake.[46] Because our tools (anti–SR-BI antibody and SR-BI siRNAs) also may target SR-BII, we cannot completely exclude a role for SR-BII in HCV infection as most recently shown by other investigators.[25]

In conclusion, our results demonstrate that SR-BI plays a key role for the establishment of HCV infection mediating HCV infection during an entry step occurring postbinding closely linked to the interaction of HCV with CD81. The functional mapping of SR-BI–HCV interaction and its impact for HCV entry has important implications for the understanding of the very first steps of HCV infection and the development of novel antiviral strategies targeting HCV entry.

Acknowledgment: We thank C. M. Rice (The Rockefeller University, New York, NY) for the gift of Huh7.5 cells and T. Huby (INSERM U551, Hôpital de la Pitié, Paris, France) for the gift of pcDNASR-BI/CLA-1. We are grateful to the Division of Clinical Chemistry, University Hospital Freiburg for providing HDL and LPDS. We also would like to acknowledge J. Dubuisson (Centre National de la Recherche Scientifique-UMR 8161, Institut de Biologie de Lille, Lille, France), J. McKeating (University of Birmingham, Birmingham, UK) and J.-L. Mandel (IGBMC, Strasbourg) for helpful discussions.

References

1. Chisari FV. Unscrambling hepatitis C virus-host interactions. Nature 2005;436:930-932.
2. Marsh M, Helenius A. Virus entry: open sesame. Cell 2006;124:729-740.
3. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, et al. Binding of hepatitis C virus to CD81. Science 1998;282:938-941.
4. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudoparticles containing functional E1–E2 envelope protein complexes. J Exp Med 2003;197:633-642.
5. Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, et al. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc Natl Acad Sci U S A 2003;100:7271-7276.
6. Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, et al. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 2007;446:801-805.
7. Barth H, Schäfer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, et al. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J Biol Chem 2003;278:41003-41012.

8. Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci U S A 1999;96:12766-12771.
9. Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 2002;21:5017-5025.
10. Krieger M. Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. J Clin Invest 2001;108:793-797.
11. Yamada E, Montoya M, Schuettler CG, Hickling TP, Tarr AW, Vitelli A, et al. Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets. J Gen Virol 2005;86:2507-2512.
12. Philips JA, Rubin EJ, Perrimon N. Drosophila RNAi screen reveals CD36 family member required for mycobacterial infection. Science 2005;309: 1251-1253.
13. Vishnyakova TG, Kurlander R, Bocharov AV, Baranova IN, Chen Z, Abu-Asab MS, et al. CLA-1 and its splicing variant CLA-2 mediate bacterial adhesion and cytosolic bacterial invasion in mammalian cells. Proc Natl Acad Sci U S A 2006;103:16888-16893.
14. Barth H, Cerino R, Arcuri M, Hoffmann M, Schurmann P, Adah MI, et al. Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes. J Virol 2005;79:5774-5785.
15. Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, et al. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. J Virol 2005;79:6023-6034.
16. Bartosch B, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, et al. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J Biol Chem 2003; 278:41624-41630.
17. Lavillette D, Tarr AW, Voisset C, Donot P, Bartosch B, Bain C, et al. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. HEPATOLOGY 2005;41:265-274.
18. Bartosch B, Verney G, Dreux M, Donot P, Morice Y, Penin F, et al. An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies. J Virol 2005;79:8217-8229.
19. Voisset C, Callens N, Blanchard E, Op De Beeck A, Dubuisson J, Vu-Dac N. High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I. J Biol Chem 2005;280:7793-7799.
20. von Hahn T, Lindenbach BD, Boullier A, Quehenberger O, Paulson M, Rice CM, et al. Oxidized low-density lipoprotein inhibits hepatitis C virus cell entry in human hepatoma cells. HEPATOLOGY 2006;43:932-942.
21. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
22. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
23. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
24. Kapadia SB, Barth H, Baumert T, McKeating JA, Chisari FV. Initiation of hepatitis C virus Infection is dependent on cholesterol and cooperativity between CD81 and scavenger receptor B type I. J Virol 2007;81:374-383.
25. Grove J, Huby T, Stamataki Z, Vanwolleghem T, Meuleman P, Farquhar M, et al. Scavenger receptor BI and BII expression levels modulate hepatitis C virus infectivity. J Virol 2007;81:3162-3169.
26. Wellnitz S, Klumpp B, Barth H, Ito S, Depla E, Dubuisson J, et al. Binding of hepatitis C virus-like particles derived from infectious clone H77C to defined human cell lines. J Virol 2002;76:1181-1193.
27. Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 2002;76: 13001-13014.
28. Codran A, Royer C, Jaeck D, Bastien-Valle M, Baumert TF, Kieny MP, et al. Entry of hepatitis C virus pseudotypes into primary human hepatocytes by clathrin-dependent endocytosis. J Gen Virol 2006;87:2583-2593.
29. Schoonjans K, Annicotte JS, Huby T, Botrugno OA, Fayard E, Ueda Y, et al. Liver receptor homolog 1 controls the expression of the scavenger receptor class B type I. EMBO Rep 2002;3:1181-1187.
30. Pietschmann T, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, et al. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc Natl Acad Sci U S A 2006;103:7408-7413.
31. Koutsoudakis G, Kaul A, Steinmann E, Kallis S, Lohmann V, Pietschmann T, et al. Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses. J Virol 2006;80:5308-5320.
32. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003;125:1808-1817.
33. Nauck M, Winkler K, Marz W, Wieland H. Quantitative determination of high-, low-, and very-low-density lipoproteins and lipoprotein(a) by agarose gel electrophoresis and enzymatic cholesterol staining. Clin Chem 1995;41:1761-1767.
34. Takeuchi T, Katsume A, Tanaka T, Abe A, Inoue K, Tsukiyama-Kohara K, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636-642.
35. Dreux M, Pietschmann T, Granier C, Voisset C, Ricard-Blum S, Mangeot PE, et al. High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. J Biol Chem 2006;281:18285-18295.
36. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science 1996;271:518-520.
37. Glass C, Pittman RC, Civen M, Steinberg D. Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro. J Biol Chem 1985;260:744-750.
38. Glass C, Pittman RC, Weinstein DB, Steinberg D. Dissociation of tissue uptake of cholesterol ester from that of apoprotein A-I of rat plasma high density lipoprotein: selective delivery of cholesterol ester to liver, adrenal, and gonad. Proc Natl Acad Sci U S A 1983;80:5435-5439.
39. Reaven E, Chen YD, Spicher M, Azhar S. Morphological evidence that high density lipoproteins are not internalized by steroid-producing cells during in situ organ perfusion. J Clin Invest 1984;74:1384-1397.
40. Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34-47.
41. Gu X, Trigatti B, Xu S, Acton S, Babitt J, Krieger M. The efficient cellular uptake of high density lipoprotein lipids via scavenger receptor class B type I requires not only receptor-mediated surface binding but also receptor-specific lipid transfer mediated by its extracellular domain. J Biol Chem 1998;273:26338-26348.
42. Connelly MA, Klein SM, Azhar S, Abumrad NA, Williams DL. Comparison of class B scavenger receptors, CD36 and scavenger receptor BI (SR-BI), shows that both receptors mediate high density lipoprotein-cholesteryl ester selective uptake but SR-BI exhibits a unique enhancement of cholesteryl ester uptake. J Biol Chem 1999;274:41-47.
43. Bertaux C, Dragic T. Different domains of CD81 mediate distinct stages of hepatitis C virus pseudoparticle entry. J Virol 2006;80:4940-4948.
44. Eckhardt ER, Cai L, Shetty S, Zhao Z, Szanto A, Webb NR, et al. High density lipoprotein endocytosis by scavenger receptor SR-BII is clathrin-dependent and requires a carboxyl-terminal dileucine motif. J Biol Chem 2006;281:4348-4353.
45. Webb NR, de Villiers WJ, Connell PM, de Beer FC, van der Westhuyzen DR. Alternative forms of the scavenger receptor BI (SR-BI). J Lipid Res 1997;38:1490-1495.
46. Eckhardt ER, Cai L, Sun B, Webb NR, van der Westhuyzen DR. High density lipoprotein uptake by scavenger receptor SR-BII. J Biol Chem 2004;279:14372-14381.