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Interleukin-1 and Tumor Necrosis Factor-α Trigger Restriction of Hepatitis B Virus Infection via a Cytidine Deaminase Activation-induced Cytidine Deaminase (AID)*

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Background: Cytokines and host factors triggering innate immunity against hepatitis B virus (HBV) are not well understood.
Results: IL-1 and TNFα induced cytidine deaminase AID, an anti-HBV host factor, and reduced HBV infection into hepatocytes.
Conclusion: IL-1/TNFα reduced host susceptibility to HBV infection through AID up-regulation.
Significance: Proinflammatory cytokines modulate HBV infection through a novel innate immune pathway involving AID.

Virus infection is restricted by intracellular immune responses in host cells, and this is typically modulated by stimulation of cytokines. The cytokines and host factors that determine the host cell restriction against hepatitis B virus (HBV) infection are not well understood. We screened 36 cytokines and chemokines to determine which were able to reduce the susceptibility of HepaRG cells to HBV infection. Here, we found that pretreatment with IL-1β and TNFα remarkably reduced the host cell susceptibility to HBV infection. This effect was mediated by activation of the NF-κB signaling pathway. A cytidine deaminase, activation-induced cytidine deaminase (AID), was up-regulated by both IL-1β and TNFα in a variety of hepatocyte cell lines and primary human hepatocytes. Another deaminase APOBEC3G was not induced by these proinflammatory cytokines. Knockdown of AID expression impaired the anti-HBV effect of IL-1β, and overexpression of AID antagonized HBV infection, suggesting that AID was one of the responsible factors for the anti-HBV activity of IL-1/TNFα. Although AID induced hypermutation of HBV DNA, this activity was dispensable for the anti-HBV activity. The antiviral effect of IL-1/TNFα was also observed on different HBV genotypes but not on hepatitis C virus. These results demonstrate that proinflammatory cytokines IL-1/TNFα trigger a novel antiviral mechanism involving AID to regulate host cell permissiveness to HBV infection.

The intracellular immune response can eliminate pathogens from a host, and host cells possess different mechanisms to counteract viral infection depending on the virus type. Human immunodeficiency virus (HIV) infection is restricted by cellular proteins designated as restriction factors, including APOBEC3G (A3G),[3] TRIM5α, tetherin/BST-2, and SAMHD1 (1, 2). All of these factors can be induced by stimulation with interferon (IFN). Hepatitis C virus (HCV) is eliminated by type I and III IFNs derived from dendritic cells or infected hepatocytes (3–6). In hepatocytes, this process involves a series of antiviral factors that are downstream genes of IFN, IFN-stimulated genes (ISGs). Influenza virus spread and virulence is inhibited by cytokines such as IFNs and TNFα. Responsive genes for these mechanisms include IFN-induced cellular Mx proteins that are dynamin-like GTPases (7, 8). However, these cytokine-induced antiviral immune responses are poorly understood in hepatitis B virus (HBV) infection.

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[3] The abbreviations used are: A3G, APOBEC3G; AID, activation-induced cytidine deaminase; HBV, hepatitis B virus; HCV, hepatitis C virus; ISG, IFN-stimulated gene; QNZ, 6-amino-4-(4-phenoxyphenylethylamino)quinazoline; GEq, genome equivalent; PHH, primary human hepatocyte; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ISRE, interferon sensitivity-responsive element; cccDNA, covalently closed circular DNA.

HBV infection is a worldwide health problem affecting more than 350 million people and is a major cause of the development of liver cirrhosis and hepatocellular carcinoma (9–11). During the course of infection, a number of cytokines and chemokines are up-regulated in HBV-infected patients, including IFNα/γ/λ, TNFα, IL-1, IL-6, IL-10, IL-12, IL-15, and IL-8 (12–15). Some of these cytokines are reported to suppress HBV replication (3, 16–21). In particular, type I, II, and III IFNs suppress the replication of HBV *in vitro* and *in vivo* (19, 20, 22–26). Although one of the downstream genes of IFN, *A3G*, has the potential to reduce HBV replication (27–34), it is still under discussion whether this protein is responsible for the anti-HBV activity of type I IFN, because it has been previously reported by Trono and co-workers (28, 35) that the induction of A3G does not explain the IFN-induced inhibition of HBV replication. Moreover, these studies were carried out using an HBV transgene that only reproduces a portion of the whole HBV life cycle, mainly focusing on intracellular HBV replication.

Here, we screened for cytokines and chemokines that affected HBV infection in HepaRG cells, a human hepatocyte cell line susceptible to HBV infection and reproducing the whole HBV life cycle (36, 37). IL-1 and TNFα decreased the host cell permissiveness to HBV infection, and this effect was at least partly mediated by the induction of activation-induced cytidine deaminase (AID). The anti-HBV activity of IL-1/TNFα was mechanistically different from that of IFNα. This study presents the activity of IL-1/TNFα to suppress HBV infection into hepatocytes independent of the effect on immune cells and the physiological role of AID in this machinery. Moreover, as far as we know, this is the first report to show the AID function to inhibit the infection of human pathogenic virus.

EXPERIMENTAL PROCEDURES

Reagents—All cytokines were purchased from PeproTech or R & D Systems. Heparin was obtained from Mochida Pharmaceutical. Lamivudine, PD98059, SP600125, SB203580, and Bay11-7082 were obtained from Sigma. Entecavir was obtained from Santa Cruz Biotechnology. BMS-345541 and 6-amino-4-(4-phenoxyphenylethylamino)quinazoline (QNZ) were purchased from Merck.

Cell Culture—HepaRG cells (Biopredic) were cultured with Williams' medium E (Invitrogen) supplemented with 2 mM L-glutamine, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 5 μg/ml insulin (Wako), 20 ng/ml EGF (PeproTech), 50 μM hydrocortisone (Sigma), and 2% DMSO (Sigma). HepG2, HepAD38 (kindly provided by Dr. Seeger at Fox Chase Cancer Center) (38), and HepG2.2.15 cells (a kind gift from Dr. Urban at Heidelberg University) (39) were cultured with DMEM/F-12 + GlutaMAX (Invitrogen) supplemented with 10 mM HEPES (Invitrogen), 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 50 μM hydrocortisone, and 5 μg/ml insulin in the presence (HepAD38 and HepG2.2.15) or absence (HepG2) of 400 μg/ml G418 (Nacalai Tesque). HepAD38 cells were cultured with 0.3 μg/ml tetracycline when terminating HBV induction. Huh-7.5.1 cells (kindly provided from Dr. Chisari at Scripps Research Institute) were cultured as described previously (40). Primary human hepatocytes (PHH) isolated from urokinase-type plasminogen activator transgenic/SCID mice inoculated with PHH (PhoenixBio) or purchased from Lonza were cultured with DMEM supplemented with 20 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% FBS, and 44 mM $NaHCO_3$ or with 1 mM pyruvate, nonessential amino acids, 20 mM HEPES, 200 units/ml penicillin, 200 μg/ml streptomycin, 10% FBS, 0.25 μg/ml insulin (Wako), 5 ng/ml EGF, and 50 nM dexamethasone.

HBV Preparation and Infection—HBV used in this study was mainly derived from HepAD38 cells, which is classified as genotype D (38). Media from HepAD38 cells at days 7–31 post-induction of HBV by depletion of tetracycline were recovered every 3 days. Media were cleared through a 0.45-μm filter and precipitated with 10% PEG8000 and 2.3% NaCl. The precipitates were washed and resuspended with medium at ~200-fold concentration. The HBV DNA was quantified by real time PCR. HBV genotype A and C in Fig. 7*B* was recovered from the media of HepG2 cells transfected with the plasmid pHBV/Aeus and pHBV/C-AT (41).

HepaRG cells were infected with HBV at 2000 (Fig. 7*B*) or 6000 (other figures) genome equivalent (GEq)/cell in the presence of 4% PEG8000 for 16 h as described previously (36). Urban and co-workers (42) reported that more than 10^3 GEq/cell amount of HBV derived from HepAD38 or HepG2.2.15 cells (*i.e.* $1.25–40 \times 10^4$ GEq/cell) as inoculum was required for efficient infection into HepaRG cells. The anti-HBV effect of IL-1/TNFα shown in this study was also observed when inoculated with HBV at 300 GEq/cell (data not shown).

Extraction of DNA and RNA—HBV DNA was extracted from the cells or from the medium using a DNA kit (Qiagen) according to the manufacturer's protocol. Total RNA was recovered with RNeasy mini kit (Qiagen) according to the manufacturer's protocol.

Real Time PCR and RT-PCR—HBV DNA was quantified by real time PCR analysis using the primer set 5′-ACTCACCAACCTCCTGTCCT-3′ and 5′-GACAAACGGGCAACATACCT-3′ and probe 5′-carboxyfluorescein (FAM)-TATCGCTGGATGTGTCTGCGGCGT-carboxytetramethylrhodamine (TAMRA)-3′ (43). The PCR was performed at 50 °C for 2 min, 94 °C for 10 min, and 50 cycles of 94 °C for 15 s and 60 °C for 1 min. Detection of cccDNA was achieved using 5′-CGTCTGTGCCTTCTCATCTGC-3′ and 5′-GCACAGCTTGGAGGCTTGAA-3′ as primers and 5′-CTGTAGGCATAAATTGGT (MGB)-3′ as a probe (44). This primer-probe set theoretically detected neither relaxed circular DNA nor HBV DNA integrated into host genome but can capture cccDNA as described previously (44). For quantification of cellular mRNA, cDNA was synthesized from extracted RNA using SuperScriptIII (Invitrogen), followed by PCR with TaqMan Gene Expression Master Mix (Applied Biosystems) and primer-probe set (TaqMan Gene Expression Assay, Applied Biosystems) or with Power SYBR Green PCR Master Mix (Applied Biosystems) and 5′-AAATGTCCGCTGGGCTAAGG-3′ and 5′-GGAGGAAGAGCAATTCCACGT-3′ as primers for *AID*.

RT-PCR was performed as described previously (45) using a one-step RNA PCR kit (Takara). Primers for amplifying each gene were as follows: 5′-CTCTGAGGTTTAGCATTTCA-3′ and 5′-CTCCAGGTCCAAAATGAATA-3′ for *cIAP*; 5′-GCA-

GATTTATCAACGGCTTT-3′ and 5′-CAGTTTTCCACCACAACAAA-3′ for *XIAP*; 5′-TAGCCAACATGTCCTCACAGAC-3′ and 5′-TCTTCTACCACTGGTTTCATGC-3′ for *ISG56*; 5′-GCCTTTTCATCCAAATGGAATTC-3′ and 5′-GAAATCTGTTCTGGGCTCATG-3′ for *PKR*; and 5′-CCATGGAGAAGGCTGGGG-3′ and 5′-CAAAGTTGTCATGGATGACC-3′ for *GAPDH*, respectively.

ELISA—HBs protein was quantified by ELISA using plates incubated at 4 °C overnight with a sheep anti-HBs antibody at 1:5000 dilution (Maxisorp nunc-immuno plate, Nunc catalog no. 439454) followed by coating with 0.2% BSA, 0.02% NaN_3, 1× PBS at 4 °C until use. Samples were incubated with the plates for 2 h and after washing with TBST four times, horseradish peroxidase-labeled rabbit anti-HBs antibody was added for 2 h. The substrate solution (HCV core ELISA kit: Ortho) was reacted for 30 min before the A_{450} values were measured.

Indirect Immunofluorescence Analysis—Indirect immunofluorescence analysis was performed essentially as described previously (45). After fixation with 4% paraformaldehyde and permeabilization with 0.3% Triton X-100, an anti-HBc antibody (DAKO, catalog no. B0586) was used as the primary antibody.

MTT Assay—The MTT assay was performed as described previously (46).

Immunoblot Analysis—Immunoblot analysis was performed as described previously (47). The polyclonal antibody against AID was generated using a peptide derived from AID protein as an immunogen as described previously for preparation of the anti-AID antibody 1 (48). The specificity of the antibody was described previously (48, 49).

Lentiviral Vector-mediated Gene Transduction—Lentivirus carrying shRNAs was prepared with 293T cells transfected with expression plasmids for HIV-1 Gag-Pol, VSV G, and shRNAs (sh-control, sh-cyclophilin A, sh-AID(1), sh-AID(2); Mission shRNA) (Sigma) with Lipofectamine 2000 (Invitrogen). Recovered lentiviral vector was transduced into HepaRG cells followed by selection with 1.5 μg/ml puromycin. Lentivirus overexpressing AID, AID mutant, A3G, or the control lentivirus was recovered using expression plasmids for HIV-1 Gag-Pol, Rev, VSV G, and the corresponding expression vector as described previously (50).

Southern Blot Analysis—Southern blot was performed as described previously (41). After digestion of free nucleic acids with DNase I and RNase A, cell lysates were digested with proteinase K, and HBV DNA in the core particles was extracted with phenol/chloroform, followed by isopropyl alcohol precipitation. Probe was prepared by cutting pHBV/D-IND60 (41) with SacII and BspHI to generate a full-length HBV DNA probe and labeled with AlkPhos direct labeling reagents (GE Healthcare). Labeled bands were visualized with CDP-star detection reagent (GE Healthcare).

Quantification of Nucleocapsid-associated HBV RNA—After digestion of free nucleic acids with DNase I and RNase A, nucleocapsid was precipitated with PEG8000 (41). Total RNA was then extracted from the resuspended precipitates. HBV RNA was quantified by real time RT-PCR with 5′-TCCCTCGCCTCGCAGACG-3′ and 5′-GTTTCCCACCTTATGAGTC-3′ as primers with Power SYBR Green PCR Master Mix (Applied Biosystems).

Co-immunoprecipitation Assay—Co-immunoprecipitation assay was essentially performed as described (45).

Differential DNA Denaturation PCR—Differential DNA denaturation PCR was performed as described previously (51).

Reporter Assay—DNA transfection was performed with pNF-κB-luc or pISRE-TA-luc (Stratagene) and pRL-TK (Promega), which express firefly luciferase driven by NF-κB or ISRE and *Renilla* luciferase by herpes simplex virus thymidine kinase promoter, respectively, and Polyethylenimine Max (Polysciences Inc., catalog no. 24765). After compound or cytokine treatment, cells were lysed, and luciferase activities were measured as described previously (52). A reporter carrying HBV core promoter was constructed by inserting the DNA fragment (1413–1788 nucleotide number) of HBV DNA (D-IND60) into pGL4.28 vector (Promega) (41). In the reporter assay using this construct (Fig. 1*H*), HX531, a retinoid X receptor antagonist was used as a positive control as retinoid X receptor was involved in the transcription from the core promoter (53).

RESULTS

IL-1 Reduced Host Cell Susceptibility to HBV Infection—To evaluate the effect of cytokines and chemokines on susceptibility to HBV infection, we treated HepaRG cells (36) with cytokines for 3 h prior to and 16 h during HBV infection, followed by culture without stimuli for an additional 12 days (Fig. 1*A*, *lower scheme*). Heparin, a competitive inhibitor of HBV attachment (54), was used as a positive control and decreased secretion of the viral envelope surface protein (HBs) from HBV-infected cells (Fig. 1*A*, *upper graph, lane 38*), which suggests a successful HBV infection in this experiment. Examination of 36 cytokines and chemokines revealed that IL-1β drastically decreased protein secretion from HBs (Fig. 1*A*, *upper graph, lane 8*). Although IFNs had a strong anti-HBV effect by a continuous treatment after HBV infection (Fig. 3*C*, *panel b*, and data not shown), they had only a limited effect in this screening where cytokines were only pretreated and cotreated with HBV (Fig. 1*A*, *lanes 2–7*). HBc protein expression (Fig. 1*B*) and HBV DNA (Fig. 1*C*) in the cells and medium (Fig. 1*D*) were significantly decreased by treatment with IL-1β without cytotoxicity (Fig. 1*G*). HBV cccDNA and HBV RNA was also decreased in infected cells treated with IL-1β (Fig. 1, *E* and *F*). IL-1β did not decrease HBV core promoter activity at least in HepG2 cells (Fig. 1*H*). These results suggest that IL-1β suppressed HBV infection to HepaRG cells. IL-1β did not decrease the expression of sodium taurocholate cotransporting polypeptide (*NTCP*), a recently reported HBV entry receptor (data not shown) (55). Similar results were obtained using primary human hepatocytes (Fig. 1*I*).

NF-κB Signaling Was Critical for Anti-HBV Activity—As shown in Fig. 2*A*, IL-1β suppressed HBV infection in a dose-dependent manner. This anti-HBV effect was reversed by cotreatment with a neutralizing antibody for the IL-1 receptor, IL-1RI (Fig. 2*B*), suggesting that receptor engagement was required for anti-HBV activity. IL-1Ra is a natural antagonist that associates with IL-1RI but does not trigger downstream signal transduc-

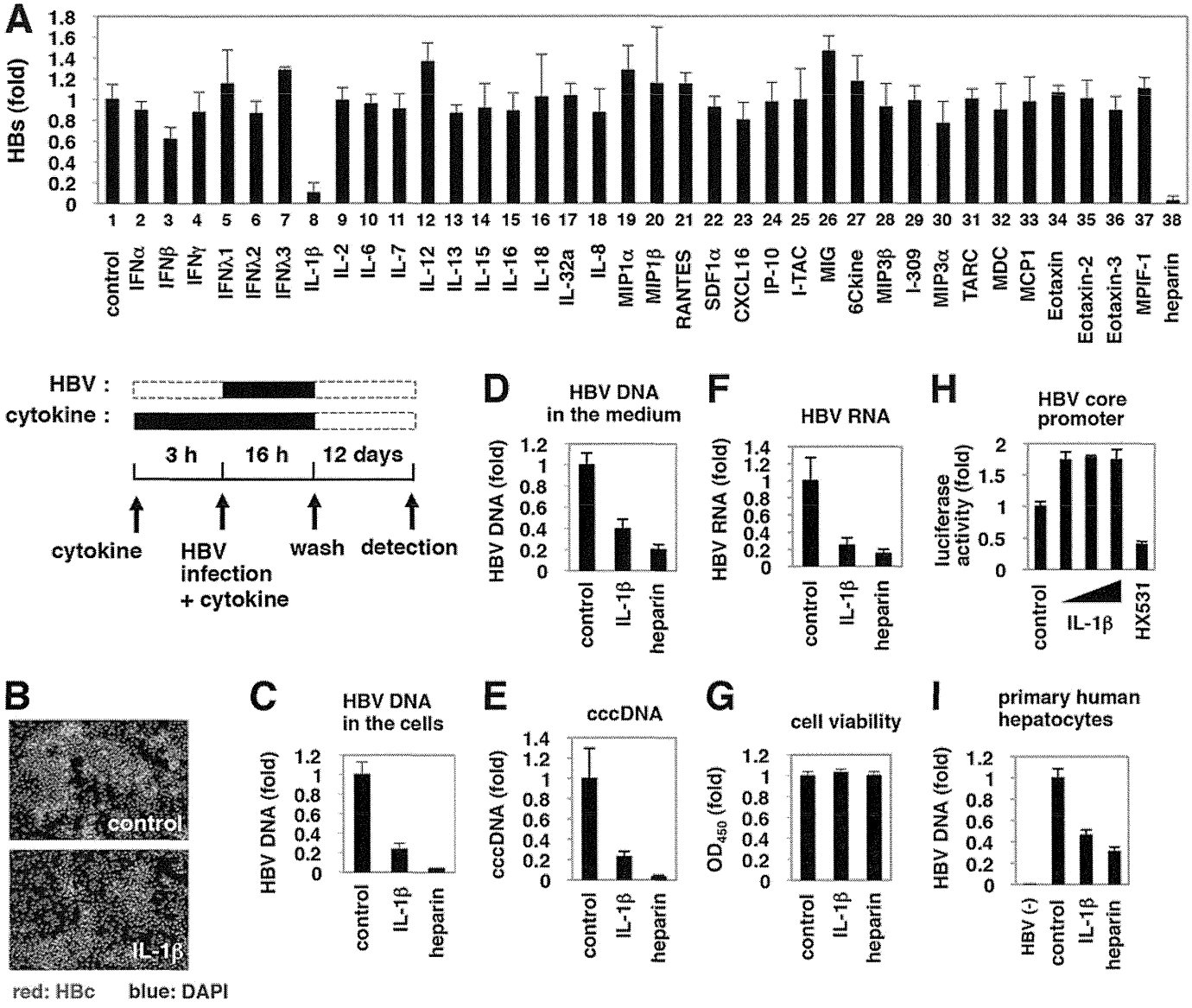

FIGURE 1. **Suppression of HBV infection by IL-1β.** *A, upper graph,* HepaRG cells were pretreated with cytokines at 100 ng/ml (except for IFNα and IFNβ at 100 IU/ml) or heparin at 25 units/ml as a positive control or were left untreated (*control*) for 3 h and then infected with HBV in the presence of each stimuli for 16 h. After washing, cells were cultured in normal growth medium for 12 days. HBs protein secreted into the medium was quantified by ELISA. *Lower scheme* indicates the treatment procedure for HepaRG cells. *Black* and *dashed line boxes* indicate the periods with and without treatment, respectively. *B–G* and *I,* HepaRG cells (*B–G*) or PHH (*I*) were treated as shown in *A* with or without 100 ng/ml IL-1β or 25 units/ml heparin as a positive control. HBc protein in the cells (*red*) was detected by indirect immunofluorescence analysis, and the nucleus was stained with DAPI (*blue*) at 12 days post-infection (*B*). HBV DNA (*C* and *I*), cccDNA (*E*), and HBV RNA (*F*) in the cells as well as HBV DNA in the medium (*D*) were detected. Cell viability was quantified by MTT assay (*G*). *HBV(−)* in *I* indicates uninfected cells. All of the data, except in *I*, are based on the average of three independent experiments. *I* shows the average results from one representative experiment, but the reproducibility of the data were confirmed in three independent experiments. *H,* reporter plasmid carrying the HBV core promoter was transfected with HepG2 cells and then treated with or without IL-1β (1, 10, and 100 ng/ml) and an retinoid X receptor antagonist HX531 as a positive control for 6 h. Luciferase activity was measured.

tion (56). Treatment with IL-1Ra did not decrease HBV infectivity (Fig. 2*C*), suggesting that signal transduction triggered by IL-1 was required for anti-HBV activity.

To identify the signal transduction pathway essential for anti-HBV activity, we treated HepaRG cells with PD98059, SP600125, SB203580, and Bay11-7082, which are inhibitors for MEK, JNK, p38, and NF-κB, respectively (57). As shown in Fig. 2*D*, only cotreatment with Bay11-7082 significantly removed the anti-HBV effect of IL-1β. Luciferase assay and RT-PCR analysis indicated that Bay11-7082, but not other inhibitors, blocked the transactivation of NF-κB (Fig. 2*E, upper panels*) and NF-κB downstream genes, *cIAP* and *XIAP* (Fig. 2*E, lower panels*). Additional NF-kB inhibitors, BMS-345541 and QNZ (Fig. 2*G*), also reversed the anti-HBV effect of IL-1β (Fig. 2*F*). These data suggest a critical role for NF-κB activation in the anti-HBV activity. Additionally, IL-1β did not augment the activity of interferon sensitivity-responsive element (ISRE) and mRNAs for ISGs, *ISG56*, and double-stranded RNA-dependent protein kinase (*PKR*) in HepaRG cells (Fig. 2*H*), suggesting that the anti-HBV activity is independent of ISG up-regulation. TNFα, another cytokine that activates NF-κB signaling (Fig. 2*E, lower panels*), also inhibited HBV infection (Fig. 2*I*). Thus, NF-κB activation in host hepatocytes was critical for the anti-HBV activity of proinflammatory cytokines.

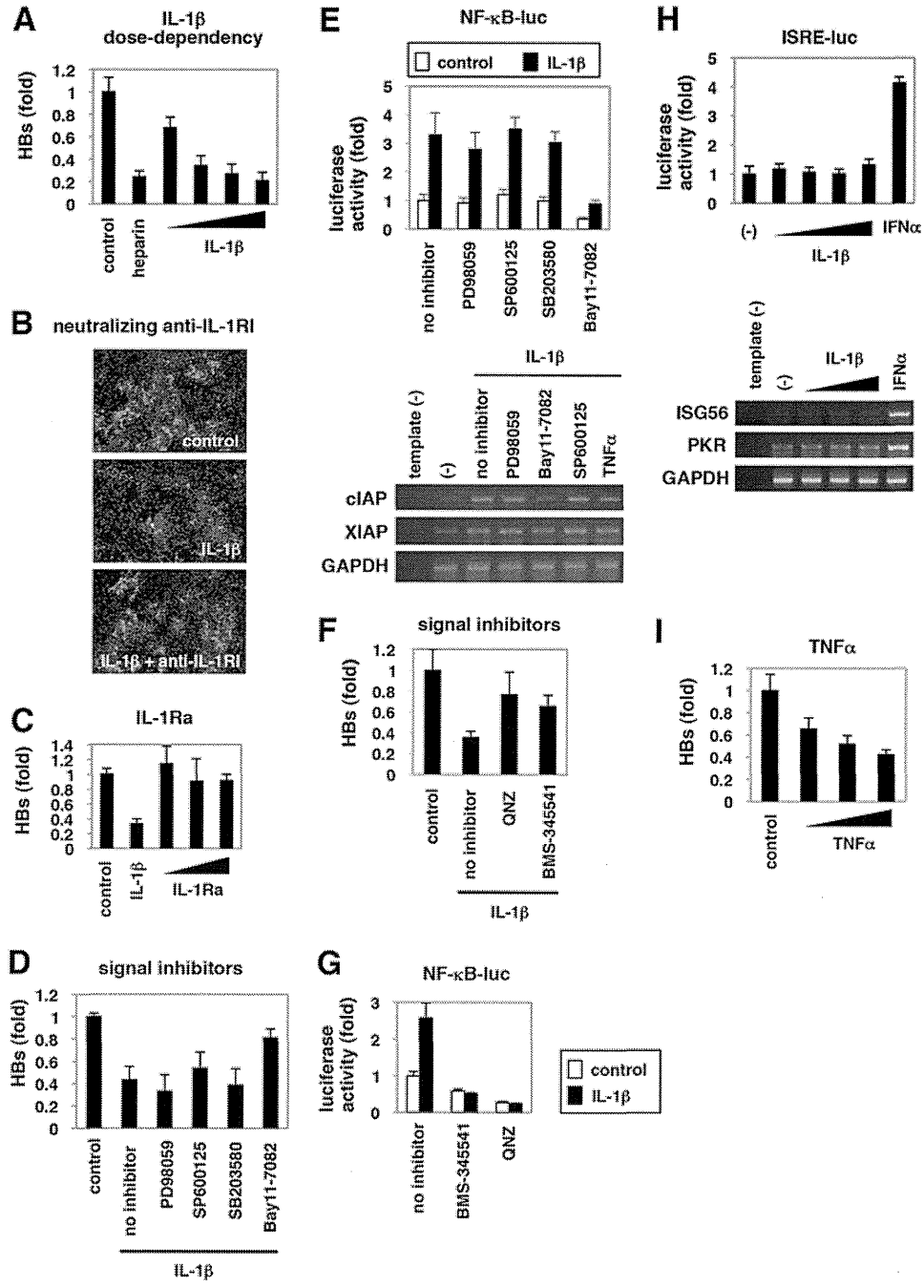

FIGURE 2. **NF-κB activation triggered by IL-1 and TNFα was critical for anti-HBV activity.** *A–D, F,* and *I,* HepaRG cells were left untreated (*control*) or treated with varying concentrations of IL-1β (1, 10, 30, and 100 ng/ml) or 25 units/ml heparin (*A*), with 30 ng/ml IL-1β together with or without a neutralizing anti-IL-1RI antibody at 20 μg/ml (*B*), with 10 ng/ml IL-1β or varying concentrations of IL-1Ra (10, 30, and 100 ng/ml) (*C*), with 3 ng/ml IL-1β together with or without PD98059, SP600125, SB203580, or Bay11-7082 (*D*), or QNZ or BMS-345541 (*F*), or with TNFα (10, 100, and 300 ng/ml) (*I*) according to the treatment schedule shown in Fig. 1*A*. HBV infection was monitored by HBs protein secretion into the medium in *A, C, D, F,* and *I* and with HBc protein in the cells in *B. E, G,* and *H,* NF-κB (*E* and *G*) and ISRE activity (*H*) were measured by reporter assay in the cells transfected with the reporter plasmid expressing luciferase driven from five tandem repeats of NF-κB elements (*E, upper graph,* and *G*) or ISRE (*H, upper graph*) or by RT-PCR in the cells (*E* and *H, lower panels*) upon signaling inhibitors used in *D* and *F* together with or without IL-1β (*E* and *G*), or upon IL-1β (10, 30, and 100 ng/ml) or IFNα 100 IU/ml as a positive control (*H*) for 6 h. The *white* and *black bars* in the *upper graph* of *E* and *G* show the data in the absence or presence of IL-1β, respectively. Bands for mRNA for *cIAP, XIAP,* and *GAPDH* (*E*) or *ISG56, PKR,* or *GAPDH* (*H*) are presented in the *lower panels*. All of the data are based on averages of three independent experiments.

Early Phase of HBV Infection as Well as HBV Replication Were Impaired by IL-1 Treatment—Although heparin, an attachment inhibitor, could block HBV infection only if added together with the HBV inoculum, pretreatment with IL-1β before HBV infection was sufficient to show anti-HBV activity (Fig. 3*A, panel b*). This activity was amplified by a prolonged treatment time of up to 12 h (Fig. 3*B*). Intriguingly, HBV cellular DNA was also reduced by IL-1β treatment following HBV infection (Fig. 3*C, panel b*). In contrast, IFNα was not effective by pretreatment (Figs. 3*C, panel a,* and 1*A*), although it did decrease HBV DNA by treatment after HBV infection (Fig. 3*C, panel b*), consistent with previous reports that IFNα can sup-

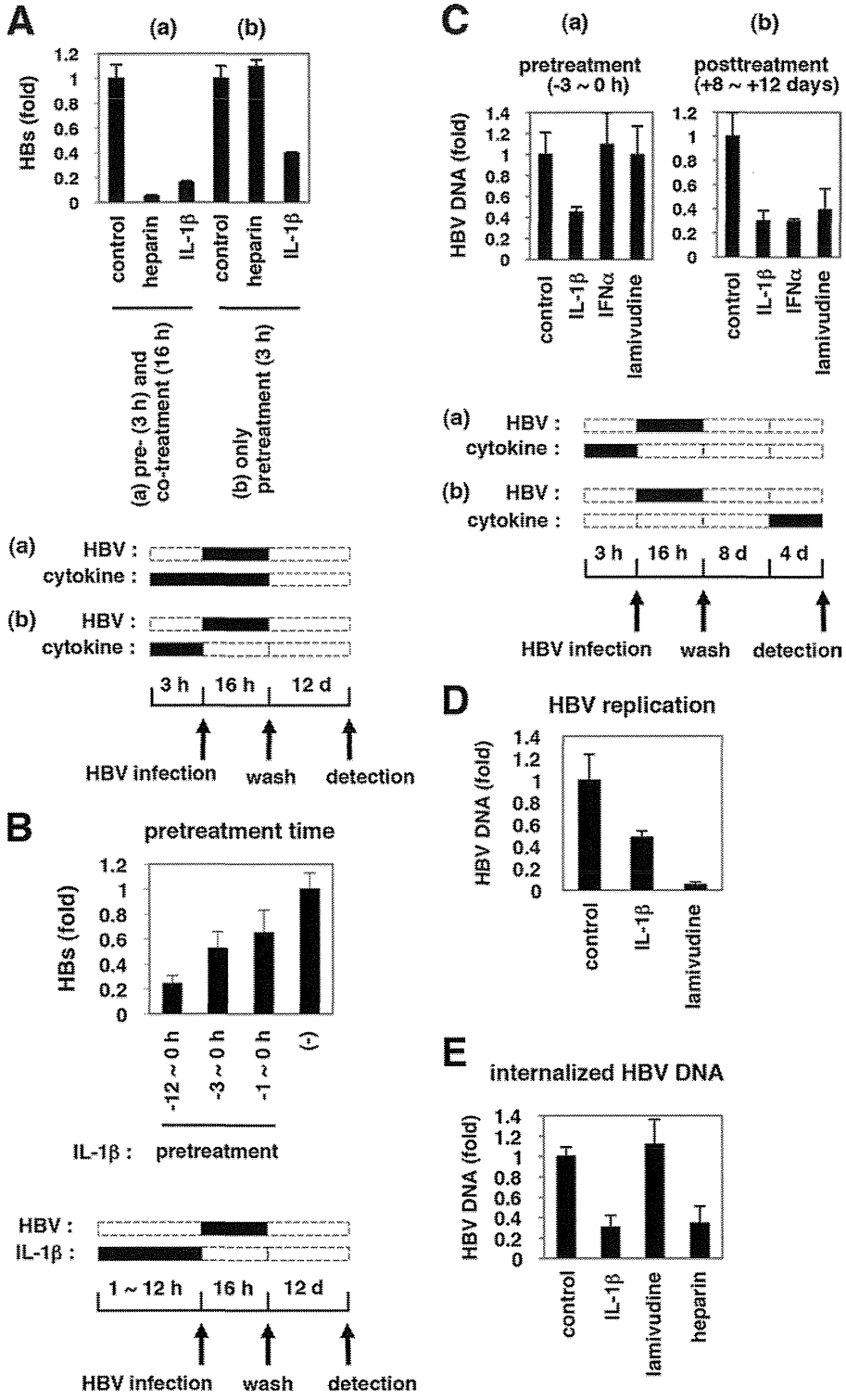

FIGURE 3. **Defining the steps of the HBV life cycle targeted by IL-1β.** *A*, HepaRG cells were pretreated with IL-1β or heparin for 3 h and then infected with HBV in the presence (*A, panel a*) or absence (*A, panel b*) of IL-1β or heparin for 16 h. HBV infection was monitored with HBs protein secretion from the infected cells. Only pretreatment with IL-1β and not heparin could inhibit HBV infectivity. *d*, day. *B*, HepaRG cells were pretreated with IL-1β or left untreated (−) for the indicated time (*h*) and infected with HBV without IL-1β. Anti-HBV activity was amplified by a prolonged treatment time. *C, panel a*, HepaRG cells were pretreated with 10 ng/ml IL-1β, 100 IU/ml IFNα, or 1 μM lamivudine for 3 h, followed by infection with HBV for 16 h in the absence of cytokines (*pretreatment*). *C, panel b*, HepaRG cells were infected with HBV for 16 h without pretreatment. After washing out the input virus, cells were cultured in normal medium for the first 8 days and then cultured with IL-1β, IFNα, or lamivudine for the following 4 days (*post-treatment*). HBV DNA in the cells was measured by real time PCR. IL-1β showed an anti-HBV activity in both pretreatment and post-treatment, although an anti-HBV effect of IFNα was seen only with post-treatment. *D*, HepAD38 cells were treated with 100 ng/ml IL-1β or 1 μM lamivudine, or left untreated for 6 days in the absence of tetracycline. HBV replication was evaluated by measurement of HBV DNA in the medium. *E*, HepaRG cells were pretreated with IL-1β, lamivudine, or heparin for 3 h or left untreated and infected with HBV for 16 h in the presence or absence of each compound. After trypsinization and extensive washing of the cells, cellular DNA was immediately recovered to detect HBV DNA. HBV DNA at 16 h post-infection was decreased by treatment with IL-1β but not lamivudine.

press HBV replication (19, 20, 26). Thus, the anti-HBV activity of IL-1β is likely to be mechanistically different from that of IFNα.

The HBV life cycle can be divided into at least two phases as follows: 1) the early phase of infection that includes attachment, entry, nuclear import, and cccDNA formation; and 2) the late phase representing HBV replication, including transcription, assembly, reverse transcription, DNA synthesis, and viral release (58). The early phase of HBV infection is not supported, but HBV DNAs persistently replicate in HepAD38 cells in the presence of tetracycline (38). IL-1β decreased the HBV DNA levels in HepAD38 cells (Fig. 3*D*), suggesting suppression of HBV replication. In addition, to examine the early phase preceding HBV replication, we infected HepaRG cells with HBV in the presence of IL-1β for 16 h and then immediately recovered cellular DNA in the trypsinized cells for quantification of HBV DNA (Fig. 3*E*). This procedure likely detected HBV DNA that had been internalized and evaded the host restriction before initiation of HBV replication because lamivudine showed no effect on the amount of DNA detected (Fig. 3*E*). In this experiment, IL-1β significantly decreased HBV DNA (Fig. 3*E*). cccDNA was also decreased by IL-1β, suggesting that the early phase of HBV infection before cccDNA formation was also interrupted by IL-1β.

IL-1 and TNFα Induced the Expression of AID—The innate immune pathway against HBV infection remains largely unknown. Recently, accumulating evidence suggested that several APOBEC family proteins, especially A3G, suppressed HBV replication when overexpressed (27–33). In contrast, there was no report available suggesting the anti-HBV function of other restriction factors against HIV, TRIM5α, tetherin/BST-2, and SAMHD1. We then investigated APOBEC family proteins as a candidate for an anti-HBV effector. The APOBEC family includes APOBEC1 (A1), A2, A3s, A4, and AID (59). Because some of these proteins are reported to be up-regulated in cytokine-stimulated hepatocytes (27, 28, 60, 61), we examined the expression of these genes in cells treated with IL-1β, TNFα, and IFNα as a control for 12 h. The mRNA levels of *A1*, *A2*, and *A3A* were below the detection threshold. *A3G* and *A3F* mRNA were significantly expressed in HepaRG cells, and their expression levels were remarkably increased by IFNα treatment (Fig. 4*A*), as observed in other reports (27, 28, 61). IL-1β and TNFα did not significantly up-regulate A3s, and only AID was up-regulated 6–10-fold by both cytokines (Fig. 4*A*). Induction of A3s by both IL-1β and TNFα was not observed at any time point examined until 12 h (data not shown). In contrast, induction of *AID* mRNA by IL-1β and TNFα was conserved in human hepatocyte cell lines, such as HepG2 and FLC4 cells, and in primary human hepatocytes (Fig. 4*B*). AID protein production was also increased in primary human hepatocytes by treatment with IL-1β and TNFα (Fig. 4*C*). This AID induction by IL-1β was suggested to be NF-κB-dependent, because the up-regulation of *AID* mRNA was canceled by addition of NF-κB inhibitors, Bay11-7082 or QNZ (Fig. 4*D*).

AID Played a Significant Role in the IL-1-mediated restriction of HBV—To examine the function of AID during HBV infection, we transduced AID ectopically into HepaRG cells using a lentiviral vector (Fig. 5*A*, *left panel*). The susceptibility of these AID-overexpressing cells to HBV was decreased by approximately one-third compared with the parental or empty vector-transduced HepaRG cells (Fig. 5*A*, *right panel*), suggesting that AID can restrict HBV infection. An AID mutant AID(M139V), with reported diminished activity to support class switching (48), also decreased the susceptibility to HBV infection, although the reduction in HBV susceptibility was moderate compared with the case of the wild type AID (Fig. 5*B*).

To examine the relevance of endogenous AID in the anti-HBV activity of IL-1, we transduced a lentiviral vector carrying a short hairpin RNA (shRNA) against AID (sh-AID) or a non-relevant protein cyclophilin A (Fig. 5*C*), and we observed the anti-HBV activity of IL-1β in these cells. IL-1β decreased HBV infection in the control and sh-cyclophilin A -transduced cells by ~3.0-fold as determined by HBs secretion (Fig. 5*D*, *lanes 1* and *2*, *black bars*). In contrast, anti-HBV activity of IL-1β was limited to only 1.6–1.7-fold in the cells transduced with sh-AIDs (Fig. 5*D*, *lanes 3* and *4*, *black bars*). Such relieved anti-HBV activity following AID knockdown was not observed in the case for heparin treatment (Fig. 5*D*, *lanes 1–4*, *gray bars*). Similar results were obtained by monitoring intracellular HBV DNA after infection (data not shown). Although the anti-HBV effect of IL-1β was not completely blunted, these data suggest that AID plays a significant role in mediating the anti-HBV effect of IL-1β.

Similar observations were obtained in HBV-replicating cells overexpressing AID (Fig. 5, *E* and *F*). Core particle-associated HBV DNA in HepG2 cells transfected with an HBV-encoding plasmid was decreased by overexpression with AID as well as with A3G (Fig. 5*E*, *lanes 1* and *3*). Intriguingly, HBV DNA in core particles was also decreased by expression of an AID mutant AID(H56Y), which contains a mutation in the cytidine deaminase motif and is derived from a class switch deficiency patient (Fig. 5*E*, *lane 2*) (48). Southern blot also showed that the HBV rcDNA level in HepG2.2.15 cells was reduced by transduction with AID and another mutant AID(M139V), with diminished activity to support class switching (Fig. 5*F*) (48). These data suggest that AID could suppress HBV replication, and this restriction activity can be still observed with reduced enzymatic activity. In addition, AID was shown to interact with HBV core protein by coimmunoprecipitation assay (Fig. 5*G*). Moreover, overexpression of AID reduced the levels for nucleocapsid-associated HBV RNA (Fig. 5*H*). These results further suggest an antiviral activity of AID against HBV replication.

AID Could Induce Hypermutation of HBV DNA—Major enzymatic activity for APOBEC family proteins is the introduction of hypermutation in target DNA/RNA, and hypermutation accounts for antiviral activity for A3G against HIV-1 to some extent (2). Several groups reported that APOBEC family proteins could induce hypermutation in HBV DNA (27, 30, 32, 34). Next we asked whether AID could induce hypermutations in HBV DNA. In differential DNA denaturation PCR analysis, a high content of A/T bases introduced by hypermutation decreased denaturation temperatures (51). As shown in Fig. 6*A*, ectopic expression of AID decreased the denaturation temperature of HBV DNA as shown by that of A3G. Sequence analyses of the HBV DNA X region amplified at 83 °C by differential DNA denaturation PCR indicated a massive accumulation of

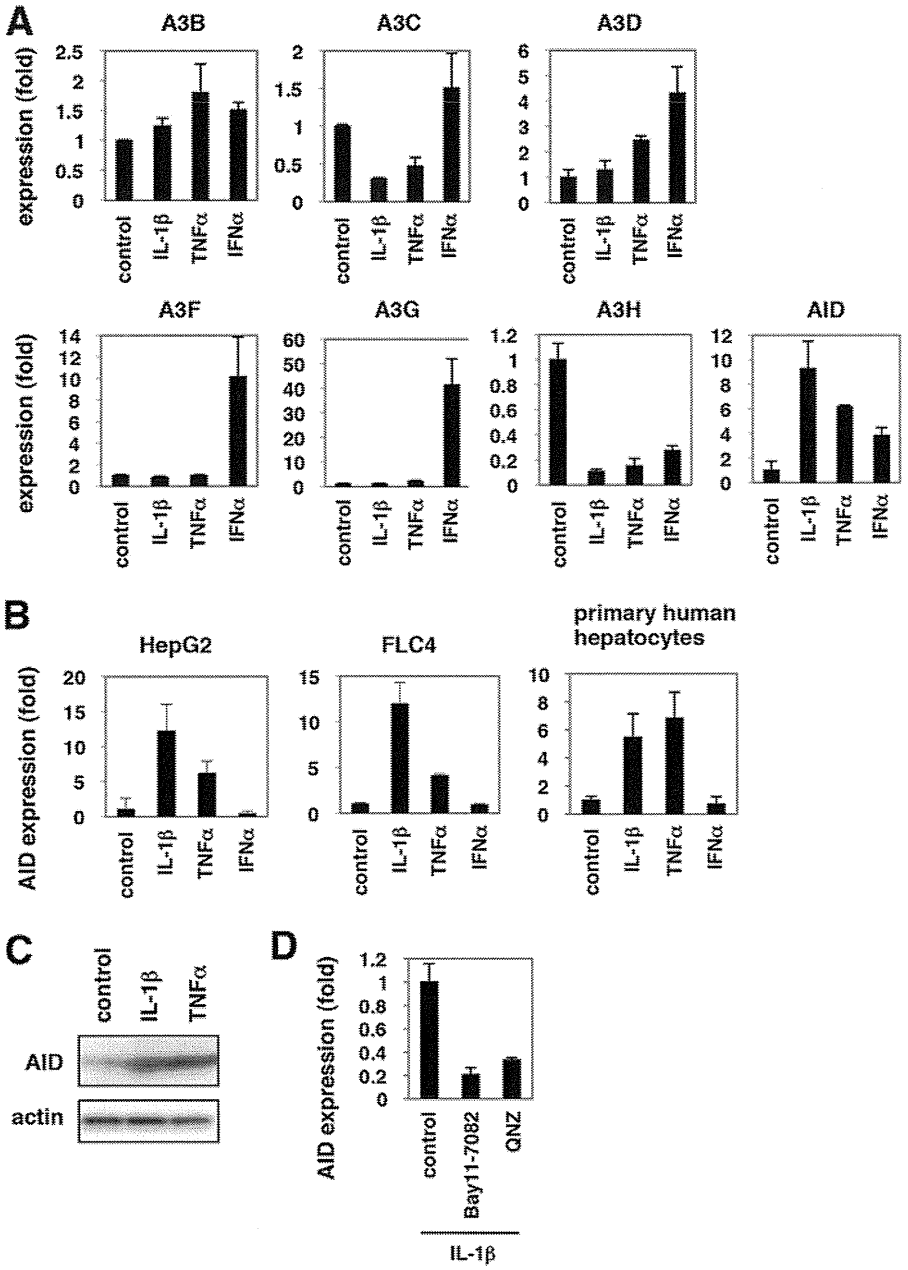

FIGURE 4. **AID expression was induced by IL-1β and TNFα.** *A*, mRNAs for *A3B, -C, -D, -F, -G, -H* and *AID* were quantified by real time RT-PCR analysis in HepaRG cells treated with 100 ng/ml IL-1β, 100 ng/ml TNFα, or 100 IU/ml IFNα for 12 h or left untreated. *Graphs* show the relative expression levels compared with the controls set at 1. *B, AID* mRNA was detected in HepG2, FLC4 cells, and PHH treated with IL-1β, TNFα, or IFNα or left untreated. Induction of AID by IL-1β and TNFα was observed in HepG2 and FLC4 cells and primary human hepatocytes. *C*, AID protein (*upper panel*) and actin levels as an internal control (*lower panel*) were examined by immunoblot of primary human hepatocytes treated with IL-1β or TNFα or left untreated. *D*, AID mRNA was detected in PHH treated with 100 ng/ml IL-1β in the presence or absence of NF-κB inhibitors, Bay11-7082, or QNZ for 12 h.

G-to-A mutations by AID (Fig. 6*B*). The frequency of G-to-A mutations was augmented by AID expression (Fig. 6*C*). In this experiment, AID(JP8Bdel), a hyper-active mutant of AID (62), further promoted the accumulation of the G-to-A and C-to-T mutations, although AID(H56Y) showed mutations in HBV DNA equivalent with mock GFP control sample (Fig. 6*C*). Thus, AID had the potential to introduce hypermutation in nucleocapsid-associated HBV DNA.

IL-1 Suppressed the Infection of Different HBV Genotypes but Not That of HCV—We examined whether the antiviral activity of IL-1β and TNFα could be generalized to other viruses or was specific to HBV. As shown in Fig. 7*A*, the production of infectious HCV and HCV core proteins in the medium was not significantly altered by treatment with these cytokines in HCV-infected cells, compared to when IFNα was used as a positive control (Fig. 7*A*). In contrast, IL-1 suppressed the infection of HBV genotype A and C into HepaRG cells (Fig. 7*B*) as well as genotype D (Fig. 1*C*). These data suggest that the antiviral activity of proinflammatory cytokines IL-1 and TNFα is specific to HBV.

DISCUSSION

In this study, cytokine screening revealed that IL-1 and TNFα decreased the host cell susceptibility to HBV infection.

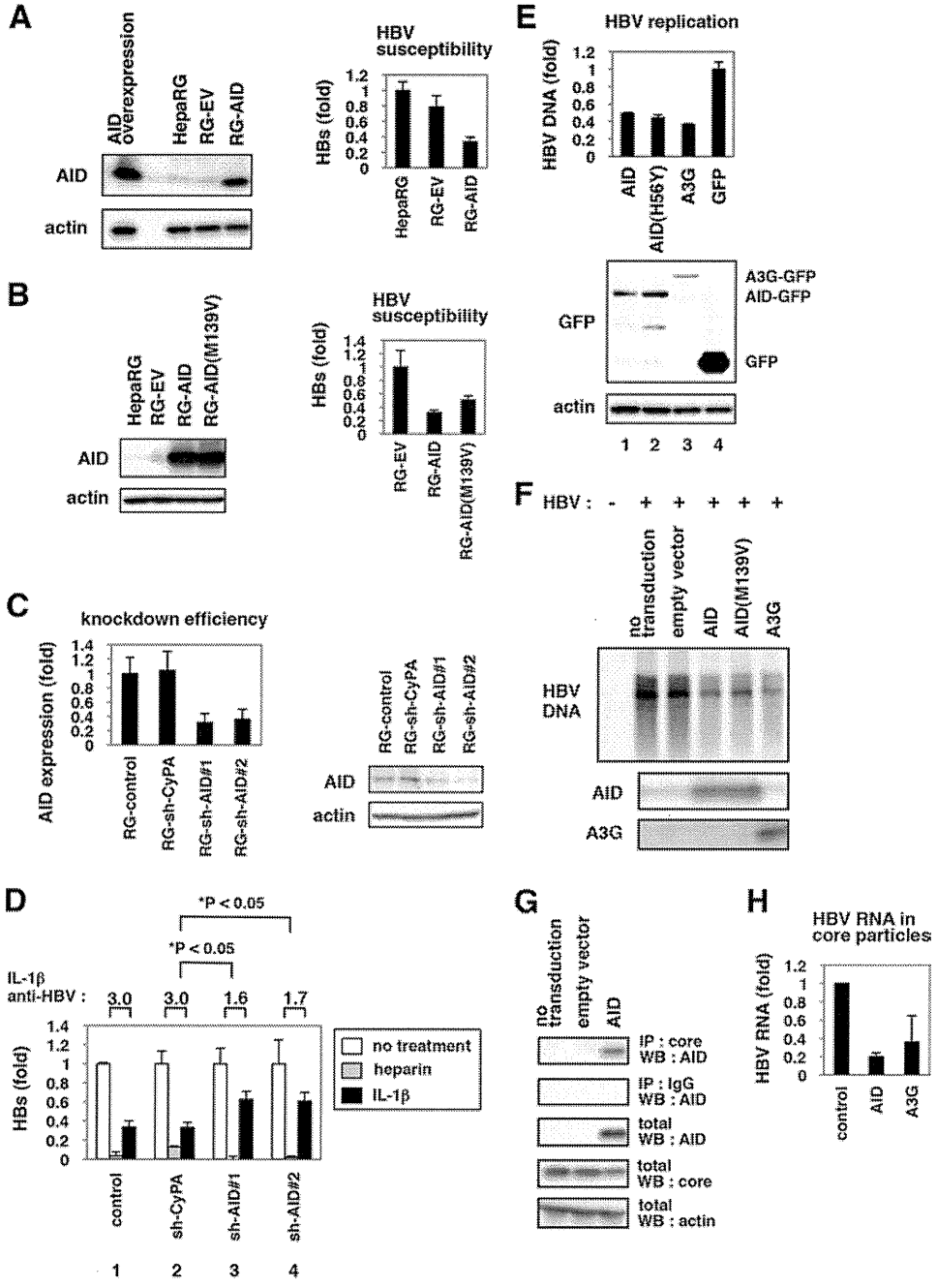

FIGURE 5. **AID played a significant role in IL-1-mediated anti-HBV activity.** *A* and *B, left panels,* HepaRG cells were transduced with a lentiviral vector carrying the expression plasmid for AID (*RG-AID*), AID(M139V) mutant (*RG-AID(M139V)*) (*B*), or the control vector (*RG-EV*). Protein expression for AID (*upper panel*) and actin (*lower panel*) in these cells, the parental HepaRG cells (*HepaRG*), and those transiently transfected with AID expression plasmid (*AID overexpression*) (*A*) was examined by immunoblot. *Right panels,* these cells were infected with HBV followed by detection of secreted HBs protein as Fig. 1*A*. AID-transduced cells were less susceptible to HBV infection. *C,* HepaRG cells were transduced with lentiviral vector carrying shRNAs for AID (*RG-shAID#1* and *RG-shAID#2*) or for cyclophilin A (*RG-shCyPA*) as a control. AID mRNA (*left panel*) and protein (*right panel*) were quantified by real time RT-PCR and immunoblot analysis. *D,* cells produced in *C* were infected with HBV in the absence or presence of IL-1β or heparin, and HBs was detected in the medium as in Fig. 1*A* to examine the anti-HBV effect of IL-1β and heparin. The fold reduction of HBV infection by IL-1β treatment is shown as *IL-1β anti-HBV* above the *graph*. The *white*, *gray*, and *black bars* indicate HBs value of the cells without treatment and with heparin and IL-1β treatment, respectively. The anti-HBV activity of IL-1β but not heparin was reduced in the AID-knockdown cells. *E,* AID and its mutant suppressed HBV replication. HepG2 cells were cotransfected with GFP-tagged AID, AID(H56Y), A3G, and GFP itself along with an HBV-encoding plasmid. Following 3 days, cytoplasmic nucleocapsid HBV DNA was quantified (*upper graph*), and the overexpressed proteins as well as actin were detected (*lower panels*). *F,* lentiviral vectors carrying AID, AID(M139V) mutant, A3G, or an empty vector (*empty vector*) were transduced or left untransduced (*no transduction*) into HepG2.2.15 cells. Nucleocapsid associated HBV DNA in these cells or in HepG2 cells (*HBV−*) was detected by Southern blot (*upper panel*). AID (*middle panel*) and A3G protein (*lower panel*) were also detected by immunoblot. *G,* HBV core interacted with AID. HepAD38 cells transduced without (*no transduction*) or with AID-expressing vector or the empty vector (*empty vector*) were lysed and treated with anti-core antibody (*1st panel*) or control normal IgG (*2nd panel*) for immunoprecipitation (*IP*). Total fraction without immunoprecipitation (*3rd* to *5th panels*) was also recovered to detect AID (*1st* to *3rd panels*), HBV core (*5th panel*), and actin (*5th panel*) by immunoblot. *WB,* Western blot. *H,* HBV RNA in core particles was extracted as shown under "Experimental Procedures" in HepG2 cells overexpressing HBV DNA together with or without AID or A3G.

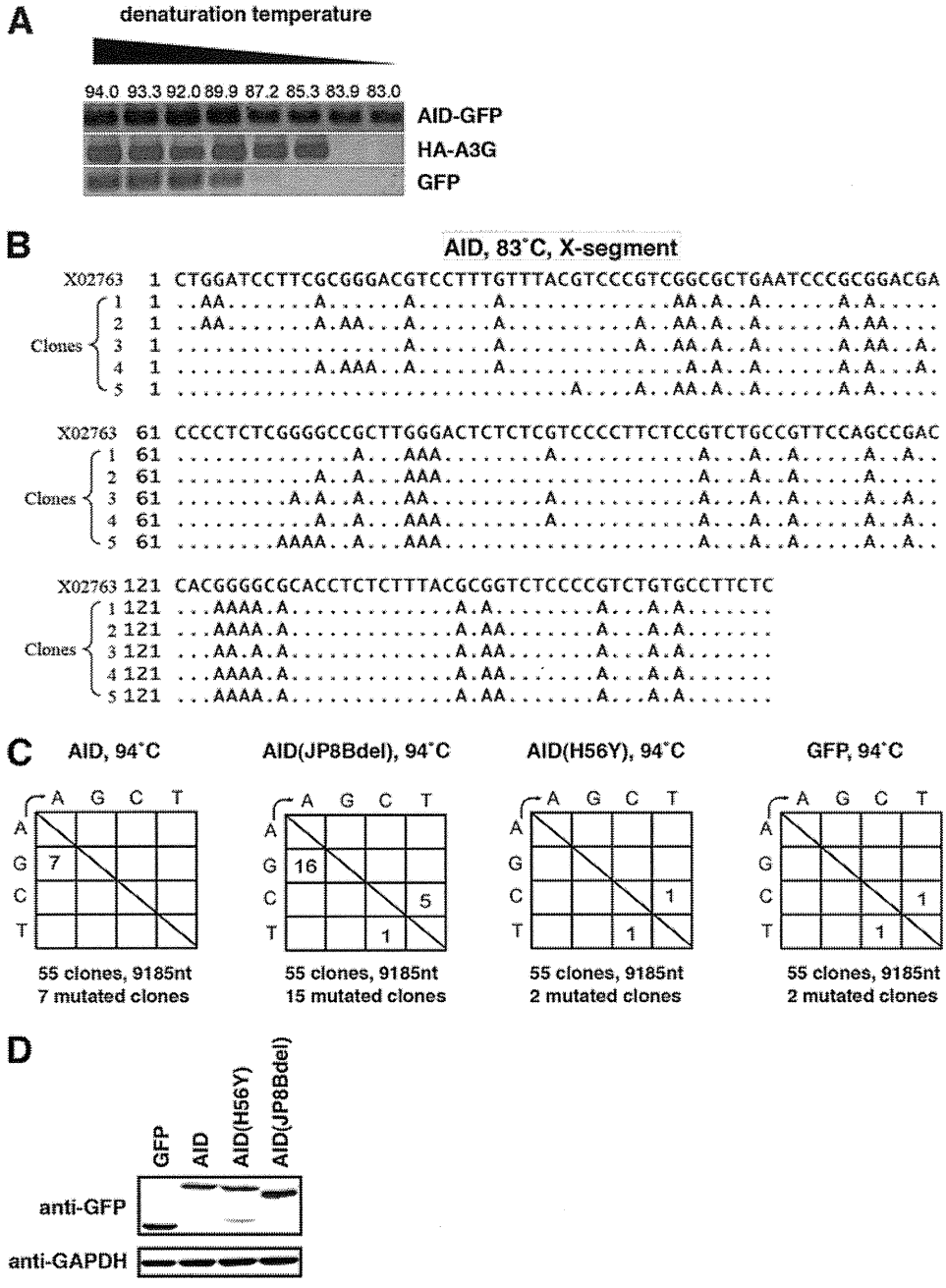

FIGURE 6. **AID could induce hypermutation of HBV DNA.** *A* and *B*, HepG2 cells were cotransfected with an expression vector for GFP-tagged AID, HA-tagged A3G, or GFP along with an HBV-encoding plasmid. 3 days after transfection, nucleocapsid-associated HBV DNA was extracted, and differential DNA denaturation PCR was performed to amplify the X gene segments. The *numbers above* the panels in *A* show denaturing temperatures. The X gene fragment amplified at 83 °C in the AID sample was cloned in to a T vector and sequenced in *B*. Alignment of independent five clones with reference sequence (X02763) is indicated. *C*, AID and its mutant (JP8Bdel) induced G-to-A and C-to-T hypermutations in HBV DNA. HepG2 cells were transfected with expression vectors of GFP-tagged AID, AID(H56Y), AID(JP8Bdel), or GFP itself together with HBV encoding plasmid. Three days after transfection, cells were harvested, and nucleocapsid-associated HBV DNA was extracted. X gene fragments were amplified at 94 °C and cloned in T vector. 55 clones were sequenced as described under "Experimental Procedures." The *numbers* indicate the clone numbers carrying the mutation. *D*, expression of GFP, GFP-tagged AID, AID(H56Y), and AID(JP8Bdel) is shown by immunoblot.

This antiviral mechanism is rather unique, given that the intracellular immune response against viruses is typically triggered by IFNs. So far, type I, II, and III IFNs are reported to suppress the replication step of the HBV life cycle (19, 20, 25, 26). In contrast, we suggest that IL-1 and TNFα inhibit the early phase of HBV infection as well as the replication. This is consistent with cumulative clinical evidence suggesting that these proinflammatory cytokines contribute to HBV elimination (63–65). IL-1 and TNFα are generally produced mainly in macrophages and also in other cell types, including T cells and endothelial cells (66). Although the main producer cells of these cytokines in hepatitis B patients are not defined, it has been reported that the secretion of IL-1 and TNFα in nonparenchymal cells were increased by HBV infection into hepatocytes (67). TNFα production in macrophages was augmented by addition of recombinant HBc (68). A number of clinical studies cumulatively

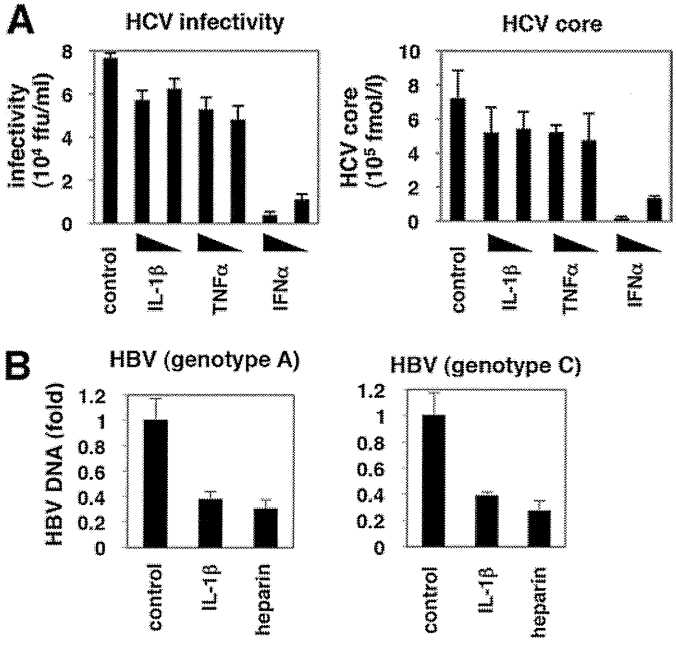

FIGURE 7. **Antiviral activity of AID was specific to HBV.** *A,* Huh-7.5.1 cells were pretreated with IL-1β, TNFα, or IFNα for 3 h or left untreated and then coincubated with HCV for 4 h. After washing HCV and cytokines and culturing the cells with normal medium for 72 h, the infectivity of HCV (*left panel*) as well as HCV core protein (*right panel*) in the medium was quantified. *B,* HepaRG cells were treated with IL-1β or heparin or left untreated for 3 h prior to and 16 h during infection of HBV genotype A (*left graph*) or C (*right graph*) as shown in Fig. 1*A*. HBV infection was monitored with cellular HBV DNA at 12 days after the infection as Fig. 1C.

show that serum levels of IL-1 and TNFα are increased in hepatitis B patients (12). Recently, it has been a significant clinical problem that HBV reactivates during the course of treatment with immunosuppressants such as anti-TNFα agents (64, 65). Taken together, it is proposed that acute or chronic HBV infection induces IL-1/TNFα from macrophages or other cells in the liver of infected patients, which can directly suppress HBV infection in hepatocytes, in addition to their immunomodulatory effects to the host immune cells. Although IL-1 level in HBV-infected patients varies between papers, Daniels *et al.* (63) reported that the peak IL-1β level in HBV-infected patients was 9–36 ng/ml under Toll-like receptor stimulation, at which concentration IL-1β showed significant anti-HBV effects in this study. In general, downstream genes of NF-κB include a number of antiviral factors such as *viperin*, *iNOS*, and *RANTES* (69). Although some of these genes may function cooperatively for IL-1- and TNFα-induced anti-HBV machinery, our data suggest that AID, at least in part, plays a role in the elimination of HBV that was potentiated by proinflammatory cytokines IL-1 and TNFα.

AID belongs to APOBEC family proteins that share enzyme activity to convert cytidine to uracil in mainly DNA, and occasionally RNA (51, 70, 71). Although AID was initially identified in B cells, chronic inflammation can trigger its expression in hepatocytes (60). The induction of AID was reportedly mediated by NF-κB (60), consistent with the results in this study. Although AID in B cells is essential for class switch recombination and somatic hypermutation of immunoglobulin genes (70, 72), the physiological role of AID in hepatocytes is unknown. Although expression of AID in hepatocytes is still lower than in B cells, AID is reportedly expressed in the liver both in cell culture and *in vivo* settings (34, 60). Our results raise the idea that AID plays a role in innate antiviral immunity. AID also has a role in virus-induced pathogenesis as it was reported to counteract oncogenesis induced by Abelson-murine leukemia virus (73). In addition, AID was reported to restrict L1 retrotransposition, which can predict the role of AID in innate immunity (74). This study is significant in that it revealed a biological function of AID in viral infection itself, linking it to the restriction of a pathogenic human virus. It will be interesting to analyze the role of AID in the infection process of other viruses in the future.

Although the mechanism for AID suppression of the HBV life cycle is the subject of future study, AID possibly targets the early phase of HBV infection, including entry as well as the replication stage, including assembly and reverse transcription (Fig. 3). It has been recently reported that chicken AID reduced cccDNA of duck HBV possibly through targeting cccDNA as well as nucleocapsid-associated HBV DNA (75). This study is likely to support the idea that AID may target cccDNA formed after HBV entry into hepatocytes, and also associates with nucleocapsid-associated HBV DNA during HBV replication, although it is not clear whether the innate immune machinery against HBV/duck HBV is conserved in human and chicken cells. A3G blocked HBV replication through the inhibition of reverse transcriptase (29), packaging of pregenomic RNA (33), and the destabilization of packaged pregenomic RNA (31) independently of its deaminase activity, and it also induced hypermutation of HBV DNA (27, 30, 32, 34). It was recently reported that AID was packaged into the HBV nucleocapsid (51). Moreover, AID induced C-to-T and G-to-A hypermutations in HBV DNA/RNA, although the anti-HBV activity has not been demonstrated so far (51). The hypermutation activity of AID was likely to be dispensable for its anti-HBV replication function (Figs. 5 and 6), as reported for APOBEC3G by several groups (29, 30, 33). Further analysis is required to elucidate the precise mechanisms for AID-mediated suppression of the HBV life cycle.

In conclusion, we have identified that host cell susceptibility to HBV infection is modulated by IL-1 and TNFα, and AID is involved in this machinery. This sheds new light on the link between proinflammatory cytokines and the development of the innate antiviral defense.

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Signal Peptidase Complex Subunit 1 Participates in the Assembly of Hepatitis C Virus through an Interaction with E2 and NS2

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Abstract

Hepatitis C virus (HCV) nonstructural protein 2 (NS2) is a hydrophobic, transmembrane protein that is required not only for NS2-NS3 cleavage, but also for infectious virus production. To identify cellular factors that interact with NS2 and are important for HCV propagation, we screened a human liver cDNA library by split-ubiquitin membrane yeast two-hybrid assay using full-length NS2 as a bait, and identified signal peptidase complex subunit 1 (SPCS1), which is a component of the microsomal signal peptidase complex. Silencing of endogenous SPCS1 resulted in markedly reduced production of infectious HCV, whereas neither processing of structural proteins, cell entry, RNA replication, nor release of virus from the cells was impaired. Propagation of Japanese encephalitis virus was not affected by knockdown of SPCS1, suggesting that SPCS1 does not widely modulate the viral lifecycles of the *Flaviviridae* family. SPCS1 was found to interact with both NS2 and E2. A complex of NS2, E2, and SPCS1 was formed in cells as demonstrated by co-immunoprecipitation assays. Knockdown of SPCS1 impaired interaction of NS2 with E2. Our findings suggest that SPCS1 plays a key role in the formation of the membrane-associated NS2-E2 complex via its interaction with NS2 and E2, which leads to a coordinating interaction between the structural and non-structural proteins and facilitates the early step of assembly of infectious particles.

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Introduction

Over 170 million people worldwide are chronically-infected with hepatitis C virus (HCV), and are at risk of developing chronic hepatitis, cirrhosis, and hepatocellular carcinoma [1]. HCV is an enveloped virus of the family *Flaviviridae*, and its genome is an uncapped 9.6-kb positive-strand RNA consisting of the 5′ untranslated region (UTR), an open reading frame encoding viral proteins, and the 3′ UTR [2]. A precursor polyprotein is further processed into structural proteins (Core, E1, and E2), followed by p7 and nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B), by cellular and viral proteases. The structural proteins (Core to E2) and p7 reside in the N-terminal region, and are processed by signal peptidase from the polyprotein. NS2, NS3, and NS4A are prerequisites for proteolytic processing of the NS proteins. NS3 to NS5B are considered to assemble into a membrane-associated HCV RNA replicase complex. NS3 also possesses activities of helicase and nucleotide triphosphatase. NS4 is a cofactor that activates the NS3 protease. NS4B induces vesicular membrane alteration. NS5A is considered to play an important but undefined role in viral RNA replication. NS5B is the RNA-dependent RNA polymerase. It is now accepted that NS proteins, such as NS2, NS3, and NS5A, contribute to the assembly or release of infectious HCV [3–9].

NS2 protein is a transmembrane protein of 21–23 kDa, with highly hydrophobic N-terminal residues forming transmembrane helices that insert into the endoplasmic reticulum (ER) membrane [5,10]. The C-terminal part of NS2 resides in the cytoplasm, enabling zinc-stimulated NS2/3 autoprotease activity together with the N-terminal domain of NS3. The crystal structure of the C-terminal region of NS2 reveals a dimeric cysteine protease containing two composite active sites [11]. Prior work showed that NS2 is not essential for RNA replication of subgenomic replicons [12]; however, the protein is required for virus assembly independently of protease activity [5,6]. Several adaptive mutations in NS2 that increase virus production have been reported [13–17]. In addition, there is increasing evidence for genetic and biochemical interaction of NS2 with other HCV proteins, including E1, E2, p7, NS3-4A, and NS5A [10,18–25]. Thus, NS2 is now suggested to act as a scaffold to coordinate interactions between the structural and NS proteins for viral assembly. However, the molecular mechanism by which NS2 is involved in virus assembly remains unclear.

In this study, we identified signal peptidase complex subunit 1 (SPCS1) as a host factor that interacts with NS2 by yeast two-hybrid screening with a split-ubiquitin system. SPCS1 is a component of the microsomal signal peptidase complex which is

Author Summary

Viruses hijack host cells and utilize host-derived proteins for viral propagation. In the case of hepatitis C virus (HCV), many host factors have been identified that are required for genome replication; however, only a little is known about cellular proteins that interact with HCV proteins and are important for the viral assembly process. The C-terminal half of nonstructural protein 2 (NS2), and the N-terminal third of NS3, form the NS2-3 protease that cleaves the NS2/3 junction. NS2 also plays a key role in the viral assembly process independently of the protease activity. We performed split-ubiquitin yeast two-hybrid screening and identified signal peptidase complex subunit 1 (SPCS1), which is a subunit of the microsomal signal peptidase complex. In this study, we provide evidence that SPCS1 interacts with both NS2 and E2, resulting in E2-SPCS1-NS2 complex formation, and has a critical role in the assembly of infectious HCV particles. To our knowledge, SPCS1 is the first NS2-interacting cellular factor that is involved in regulation of the HCV lifecycle.

responsible for the cleavage of signal peptides of many secreted or membrane-associated proteins. We show that SPCS1 is a novel host factor that participates in the assembly process of HCV through an interaction with NS2 and E2.

Results

SPCS1 is a novel host protein that interacts with HCV NS2 protein

To gain a better understanding of the functional role of NS2 in the HCV lifecycle, we screened a human liver cDNA library by employing a split-ubiquitin membrane yeast two-hybrid system with the use of NS2 as a bait. It is known that the split ubiquitin-based two-hybrid system makes it possible to study protein-protein interactions between integral membrane proteins at the natural sites of interactions in cells [26]. From the screening, several positive clones were identified from the 13 million transformants, and the nucleotide sequences of the clones were determined. A BLAST search revealed that one of the positive clones encodes a full-length coding region of signal peptidase complex subunit 1 (SPCS1). SPCS1 is a component of the microsomal signal peptidase complex which consists of five different subunit proteins in mammalian cells [27]. Although catalytic activity for SPCS1 has not been indicated to date, a yeast homolog of this subunit is involved in efficient membrane protein processing as a component of the signal peptidase complex [28].

To determine the specific interaction of NS2 with SPCS1 in mammalian cells, FLAG-tagged NS2 (FLAG-NS2; Fig. 1A) was co-expressed in 293T cells with myc-tagged SPCS1 (SPCS1-myc; Fig. 1A), followed by co-immunoprecipitation and immunoblotting. SPCS1 was shown to be co-immunoprecipitated with NS2 (Fig. 1B). Co-immunoprecipitation of SPCS1-myc with NS2 was also observed in the lysate of Huh-7 cells infected with cell culture-produced HCV (HCVcc) derived from JFH-1 isolate [29] (Fig. 1C). To determine the region of SPCS1 responsible for the interaction with NS2, deletion mutants of myc-tagged SPCS1 were constructed (Fig. 1A) and co-expressed with FLAG-tagged NS2. Since the expression of C-terminal deletion mutants, d3 and d4, was difficult to detect (Fig. 1D), N-terminal deletions (d1 and d2) as well as wild-type SPCS1 were subjected to immunoprecipitation analysis. SPCS1-myc, -d1, and -d2 were co-immunoprecipitated with NS2 (Fig. 1E), suggesting that the SPCS1 region spanning amino acids (aa) 43 to 102 is involved in its interaction with NS2. Next, to identify the NS2 region responsible for its interaction with SPCS1, deletion mutants for FLAG-NS2 (Fig. 1A) were co-expressed with SPCS1-myc-d2 in cells, followed by being immunoprecipitated with anti-myc antibody. SPCS1 was co-immunoprecipitated with the NS2 deletions, except for a mutant lacking transmembrane (TM) 2 and TM3 (dTM23) domains (Fig. 1F). These finding suggests that the TM3 region of NS2 is involved in the interaction with SPCS1.

To investigate SPCS1-NS2 interaction *in situ*, the proximity ligation assay (PLA) [30], which is based on antibodies tagged with circular DNA probes, was used. Only when the antibodies are in close proximity, the probes can be ligated together and subsequently be amplified with a polymerase. We were able to detect PLA signal predominantly in the cytoplasm of the cells expressing FLAG-NS2 and SPCS1-myc-d2 tagged with V5 at N-terminus (Fig. 1G). By contrast, the PLA signal was not observed in the context of NS2-Core co-expression. We further analyzed the SPCS1-NS2 interaction by the monomeric Kusabira-Green (mKG) system [31], which is based on fusion proteins with complementary fragments (mKG-N and mKG-C) of the monomeric coral fluorescent reporter protein. When the mKG fragments are in close proximity due to the protein-protein interaction, the mKG fragments form a beta-barrel structure and emit green fluorescence. Co-expression of SPCS1-mKG-N and NS2-mKG-C fusion proteins in cells reconstituted green cellular fluorescence as shown in Fig. 1H. Thus, these results represented structures with SPCS1 and NS2 in close proximity, and strongly suggest their physical interaction in cells.

SPCS1 participates in the propagation of infectious HCV particles

To investigate the role(s) of endogenous SPCS1 in the propagation of HCV, four small interfering RNAs (siRNAs) for SPCS1 with different target sequences or scrambled control siRNA were transfected into Huh7.5.1 cells, followed by infection with HCVcc. Among the four SPCS1-siRNAs, the highest knockdown level was observed by siRNA #2. siRNAs #3 and #4 showed moderate reductions of SPCS1 expression, and only a marginal effect was obtained from siRNA #1 (Fig. 2A). As indicated in Fig. 2B, the infectious viral titer in the culture supernatant was significantly reduced by the knockdown of SPCS1. It should be noted that the infectious titers correlated well with the expression levels of endogenous SPCS1. siRNA #2 reduced the HCV titer to ~5% of the control level in Huh7.5.1 cells. To rule out the possibility of off-target effect of SPCS1-siRNA on HCV propagation, we also used "C911" mismatch control siRNAs in which bases 9 through 11 of siRNAs are replaced with their complements but other parts of antisense- and sense-strand sequences are kept intact. These mismatch designed-control siRNAs have been shown to reduce the down-regulation of the targeted mRNA, but maintains the off-target effects of the original siRNA [32]. The C911 controls against SPCS1-siRNA #2, #3, and #4 (C911-#2, -#3, and -#4) showed little effect on knockdown of SPCS1 as well as propagation of HCV (Fig. S1A and B).

We further determined the loss- and gain-of-function of SPCS1 on HCV propagation in an SPCS1-knockdown cell line. To this end, Huh-7 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to SPCS1 and were selected with hygromycin B, resulting in clone KD#31 where little or no expression of SPCS1 was detectable (Fig. 2C). KD#31 cells and parental Huh-7 cells were transfected with an RNA polymerase I (pol)-driven full-genome HCV plasmid [33] in the presence or

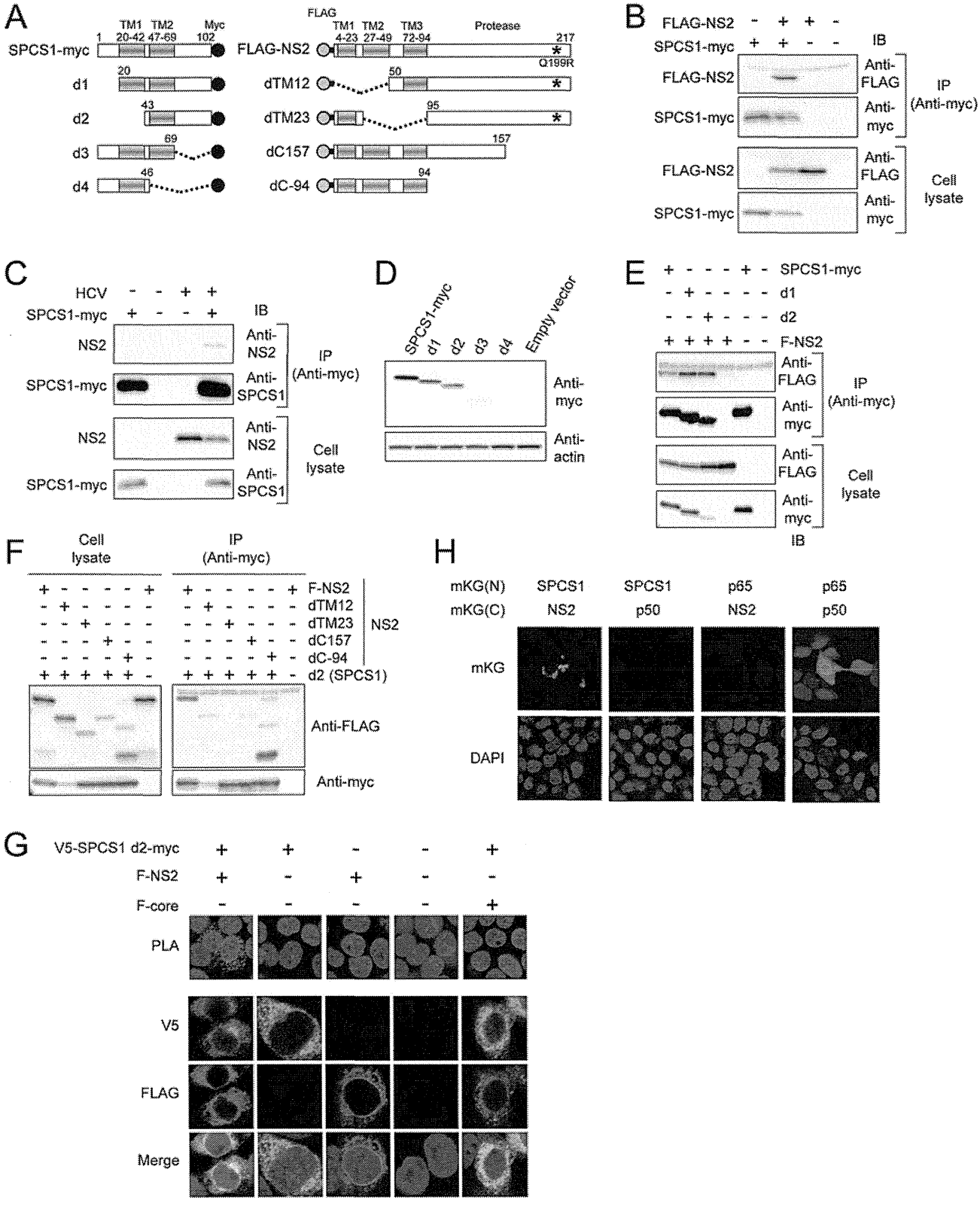

Figure 1. Interaction of HCV NS2 protein with SPCS1 in mammalian cells. (A) Expression constructs of SPCS1-myc and FLAG-NS2 used in this study. TM regions are represented as gray. Myc-tag regions are depicted by the black circles. Gray circles and bold lines indicated FLAG-tag and spacer (GGGGS) sequences, respectively. Adaptive mutations are indicated as asterisks. Positions of the aa resides are indicated above the boxes. (B) 293T cells were co-transfected with a FLAG-tagged NS2 expression plasmid in the presence of a SPCS1-myc expression plasmid. Cell lysates of the transfected cells were immunoprecipitated with anti-myc antibody. The resulting precipitates and whole cell lysates used in immunoprecipitation (IP) were examined by immunoblotting using anti-FLAG- or anti-myc antibody. An empty plasmid was used as a negative control. (C) HCVcc infected

Huh-7 cells were transfected with a SPCS1-myc expression plasmid. Cell lysates of the transfected cells were immunoprecipitated with anti-myc antibody. The resulting precipitates and whole cell lysates used in immunoprecipitation (IP) were examined by immunoblotting using anti-NS2 or anti-SPCS1 antibody. (D) Expression of SPCS1-myc and its deletion mutants. 293T cells were transfected with indicated plasmids. The cell lysates were examined by immunoblotting using anti-myc or anti-actin antibody. (E) Cells were co-transfected with indicated plasmids, and then lysates of transfected cells were immunoprecipitated with anti-myc antibody. The resulting precipitates and whole cell lysates used in IP were examined by immunoblotting using anti-FLAG- or anti-myc antibody. (F) Lysates of the transfected cells were immunoprecipitated with anti-myc antibody. The resulting precipitates (right panel) and whole cell lysates used in IP (left panel) were examined by immunoblotting using anti-FLAG or anti-myc antibody. (G) 293T cells were transfected with indicated plasmids. 2 days posttransfection, cells were fixed and permeabilized with Triton X-100, then subjected to in situ PLA (Upper) or immunofluorescence staining (Lower) using anti-FLAG and anti-V5 antibodies. (H) Detection of the SPCS1-NS2 interaction in transfected cells using the mKG system. 293T cells were transfected by indicated pair of mKG fusion constructs. Twenty-four hours after transfection, cell were fixed and stained with DAPI, and observed under a confocal microscope.
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absence of an expression plasmid for shRNA-resistant SPCS1 (SPCS1- sh^r). Western blotting confirmed the expression levels of SPCS1 in cells (Fig. 2D). As expected, viral production in the culture supernatants of the transfected cells was significantly impaired in SPCS1-knockdown cells compared with parental Huh-7 cells (Fig. 2E white bars). Expression of SPCS1- sh^r in KD#31 cells recovered virus production in the supernatant to a level similar to that in the parental cells. Expression of SPCS1-sh^r in parental Huh-7 cells did not significantly enhance virus production. Taken together, these results demonstrate that SPCS1 has an important role in HCV propagation, and that the endogenous expression level of SPCS1 is sufficient for the efficient propagation of HCV.

A typical feature of the *Flaviviridae* family is that their precursor polyprotein is processed into individual mature proteins mediated by host ER-resident peptidase(s) and viral-encoded protease(s). We therefore next examined the role of SPCS1 in the propagation of Japanese encephalitis virus (JEV), another member of the *Flaviviridae* family. SPCS1 siRNAs or control siRNA were transfected into Huh7.5.1 cells followed by infection with JEV or HCVcc. Although knockdown of SPCS1 severely impaired HCV production (Fig. 3A), the propagation of JEV was not affected under the SPCS1-knockdown condition (Fig. 3B). Expression of the viral proteins as well as knockdown of SPCS1 were confirmed (Fig. 3C). This suggests that SPCS1 is not a broadly active modulator of the flavivirus lifecycle, but rather is involved specifically in the production of certain virus(es) such as HCV.

Knockdown of SPCS1 exhibits no influence on the processing of HCV proteins and the secretion of host-cell proteins

Since SPCS1 is a component of the signal peptidase complex, which plays a role in proteolytic processing of membrane proteins at the ER, it may be that SPCS1 is involved in processing HCV

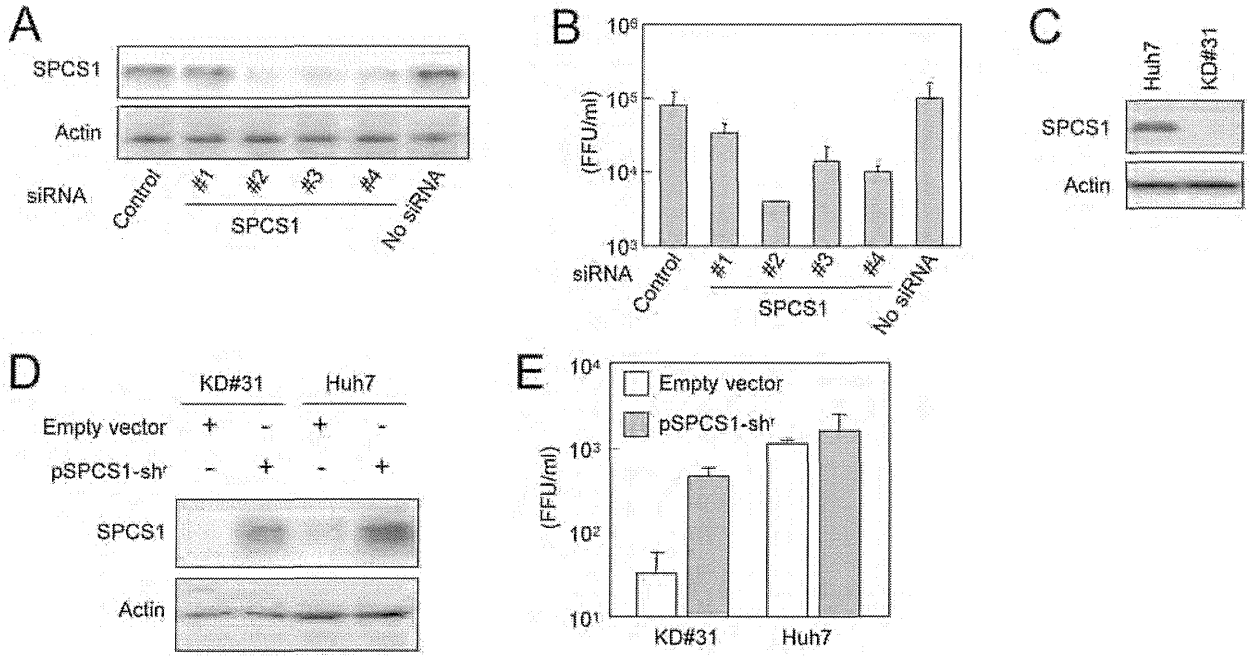

Figure 2. Effect of SPCS1 knockdown on the production of HCV. (A) Huh7.5.1 cells were transfected with four different siRNAs targeted for SPCS1 or control siRNA at a final concentration of 15 nM, and infected with HCVcc at a multiplicity of infection (MOI) of 0.05 at 24 h post-transfection. Expression levels of endogenous SPCS1 and actin in the cells were examined by immunoblotting using anti-SPCS1 and anti-actin antibodies at 3 days post-infection. (B) Infectious titers of HCVcc in the supernatant of cells infected as above were determined at 3 days postinfection. (C) Huh-7 cells were transfected with pSilencer-SPCS1, and hygromycin B-resistant cells were selected. The SPCS1-knockdown cell line established (KD#31) and parental Huh-7 cells were subjected to immunoblotting to confirm SPCS1 knockdown. (D) KD#31 cells or parental Huh-7 cells were transfected with RNA pol I-driven full-length HCV plasmid in the presence or absence of shRNA-resistant SPCS1 expression plasmid. Expression levels of SPCS1 and actin in the cells at 5 days post-transfection were examined by immunoblotting using anti-SPCS1 and anti-actin antibodies. (E) Infectious titers of HCVcc in the supernatants of transfected SPCS1-knockdown cells or parental Huh-7 cells at 5 days post-transfection were determined.
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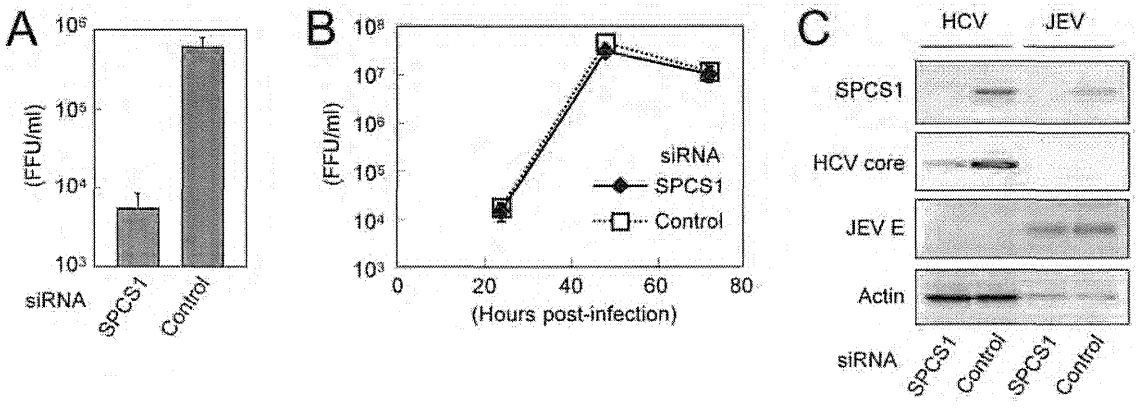

Figure 3. Effect of SPCS1 knockdown on the propagation of JEV. Huh7.5.1 cells were transfected with SPCS1 siRNA or control siRNA at a final concentration of 10 nM, and infected with JEV or HCVcc at an MOI of 0.05 at 24 h post-transfection. (A) Infectious titers of HCVcc in the supernatant at 3 days post-infection were determined. (B) Infectious titers of JEV in the supernatant at indicated time points were determined. (C) Expression levels of endogenous SPCS1 and actin as well as viral proteins in the cells were determined by immunoblotting using anti-SPCS1, anti-actin, anti-HCV core, and anti-JEV antibodies 3 days post-infection.
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proteins via interacting with ER membranes. To address this, the effect of SPCS1 knockdown on the processing of HCV precursor polyproteins in cells transiently expressing the viral Core-NS2 region was analyzed. Western blotting indicated that properly processed core and NS2 were observed in KD#31 cells as well as Huh-7 cells (Fig. 4A). No band corresponding to the unprocessed precursor polyprotein was detected in either cell line (data not shown). We also examined the effect of SPCS1 knockdown on the cleavage of the NS2/3 junction mediated by NS2/3 protease. Processed NS2 was detected in both cell lines with and without SPCS1 knockdown, which were transfected with wild-type or protease-deficient NS2-3 expression plasmids (Fig. 4B & C).

Signal peptidase plays a key role in the initial step of the protein secretion pathway by removing the signal peptide and releasing the substrate protein from the ER membrane. It is now accepted that the secretion pathways of very-low density lipoprotein or apolipoprotein E (apoE) are involved in the formation of infectious HCV particles and their release from cells [34,35]. ApoE is synthesized as a pre-apoE. After cleavage of its signal peptide in the ER, the protein is trafficked to the Golgi and trans-Golgi network before being transported to the plasma membrane and secreted. As shown in Fig. 4D, the secreted levels of apoE from Huh-7 cells with knocked-down of SPCS1 were comparable to those from control cells. In addition, the level of albumin, an abundant secreted protein from hepatocytes, in the culture supernatants of the cells was not influenced by SPCS1 knockdown (Fig. 4E). These data suggest that the knockdown of SPCS1 has no influence on the processing of viral and host secretory proteins by signal peptidase and HCV NS2/3 protease.

SPCS1 is involved in the assembly process of HCV particles but not in viral entry into cells and RNA replication

To further address the molecular mechanism(s) of the HCV lifecycle mediated by SPCS1, we examined the effect of SPCS1 knockdown on viral entry and genome replication using single-round infectious trans-complemented HCV particles (HCVtcp) [33], of which the packaged genome is a subgenomic replicon containing a luciferase reporter gene. This assay system allows us to evaluate viral entry and replication without the influence of reinfection. Despite efficient knockdown of SPCS1 (Fig. 5A), luciferase activity expressed from HCVtcp in SPCS1-knockdown cells was comparable to that in control or non-siRNA-transfected cells (Fig. 5B), suggesting that SPCS1 is not involved in viral entry into cells and subgenomic RNA replication. As a positive control, knockdown of claudin-1, a cell surface protein required for HCV entry, reduced the luciferase activity. We also examined the effect of SPCS1 knockdown on full-genome replication using HCVcc-infected cells. Despite efficient knockdown of SPCS1, expression of HCV proteins was comparable to that in control cells (Fig. 5C). By contrast, knockdown of PI4 Kinase (PI4K), which is required for replication of HCV genome, led to decrease in expression of HCV proteins. As cells that had already been infected with HCV were used, knockdown of claudin-1 had no effect on HCV protein levels. These data suggest that SPCS1 is not involved in viral entry into cells and the viral genome replication. We also observed properly processed Core, E2, NS2 and NS5B in SPCS1-knockdown cells in consistent with the result as shown in Fig. 4A, indicating no effect of SPCS1 on HCV polyprotein processing.

Next, to investigate whether SPCS1 is involved in the assembly or release of infectious particles, SPCS1-shRNA plasmid along with a pol I-driven full-genome HCV plasmid [33] were transfected into CD81-negative Huh7-25 cells, which can produce infectious HCV upon introduction of the viral genome, but are not permissive to HCV infection [36]. It is therefore possible to examine viral assembly and the release process without viral reinfection. The infectivity within the transfected cells as well as supernatants was determined 5 days post-transfection. Interestingly, both intra- and extracellular viral titers were markedly reduced by SPCS1 knockdown (Fig. 5C).

Taken together, in the HCV lifecycle, SPCS1 is most likely involved in the assembly of infectious particles rather than cell entry, RNA replication, or release from cells.

Role of SPCS1 in complex formation between NS2 and E2

It has been shown that HCV NS2 interacts with the viral structural and NS proteins in virus-producing cells [18–21], and that some of the interactions, especially the NS2-E2 interaction, are important for the assembly of infectious HCV particles. However, the functional role of NS2 in the HCV assembly process has not been fully elucidated. To test whether SPCS1 is involved in the interaction between NS2 and E2, cells were co-transfected

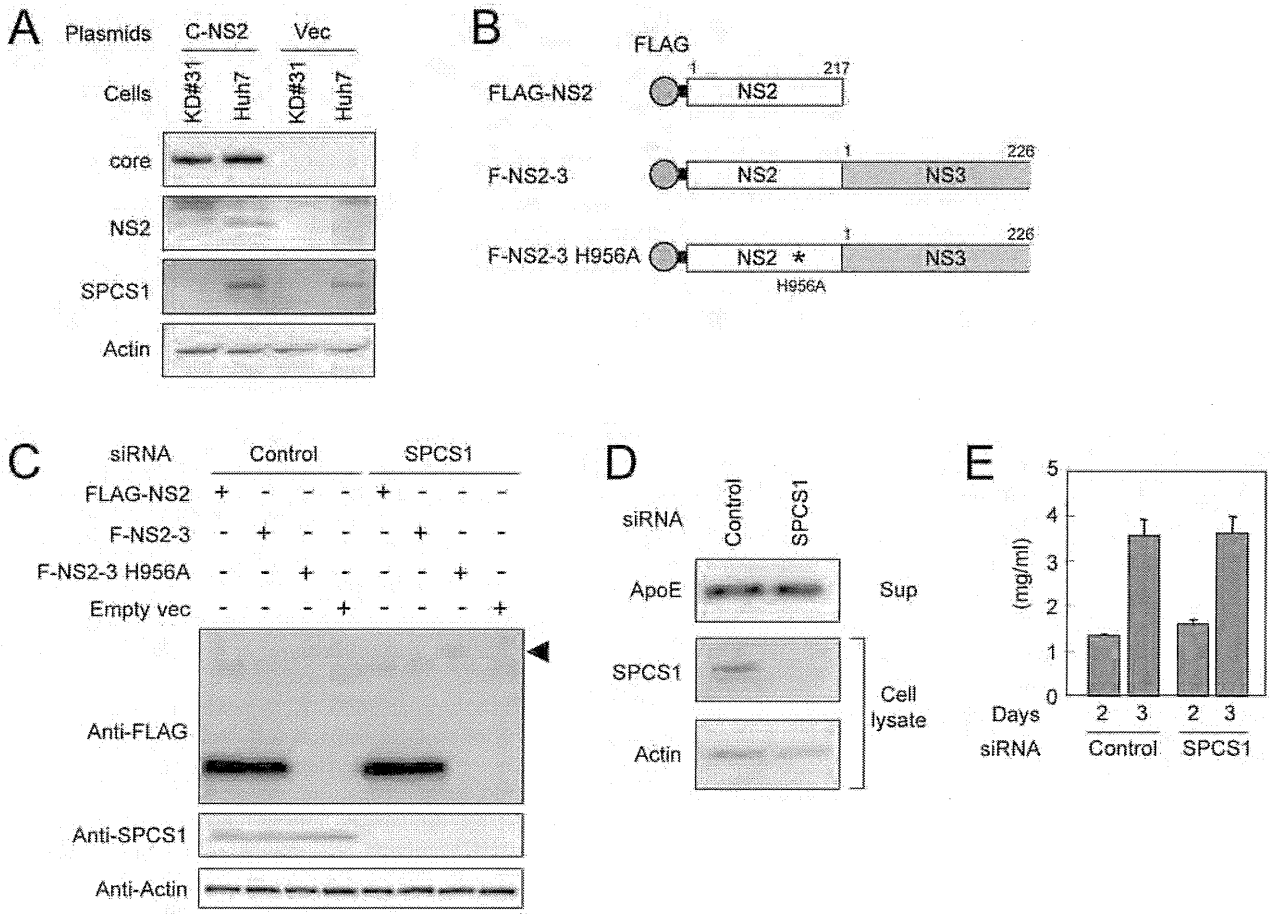

Figure 4. Effect of SPCS1 knockdown on the processing of HCV structural proteins and secretion of host proteins. (A) Core-NS2 polyprotein was expressed in KD#31 cells or parental Huh-7 cells. Core, NS2, SPCS1, and actin were detected by immunoblotting 2 days post-transfection. (B) Expression constructs of NS2 and NS2/3 proteins. His to Ala substitution mutation at aa 956 in NS2 is indicated by an asterisk. Gray circles and bold lines indicate FLAG-tag and the spacer sequences, respectively. Positions of the aa residues are indicated above the boxes. (C) Effect of SPCS1 knockdown on processing at the NS2/3 junction. Huh-7 cells were transfected with SPCS1 siRNA or control siRNA at a final concentration of 30 nM, and then transfected with plasmids for FLAG-NS2, F-NS2-3, or F-NS2-3 with a protease-inactive mutation (H956A). NS2 in cell lysates was detected by anti-FLAG antibody 2 days post-transfection. Arrowhead indicates unprocessed NS2-3 polyproteins. (D) Effect of SPCS1 knockdown on the secretion of apoE. Huh7.5.1 cells were transfected with SPCS1 siRNAs or control siRNA at a final concentration of 20 nM, and apoE in the supernatant and SPCS1 and actin in the cells were detected 3 days post-transfection. (E) Effect of SPCS1 knockdown on the secretion of albumin. Huh7.5.1 cells were transfected with SPCS1 siRNA or control siRNA, and albumin in the culture supernatants at 2 and 3 days post-transfection was measured by ELISA.
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with expression plasmids for E2, FLAG-NS2, and SPCS1-myc. E2 and NS2 were co-immunoprecipitated with SPCS1-myc, and E2 and SPCS1-myc were co-immunoprecipitated with FLAG-NS2 (Fig. 6A), suggesting the formation of an E2-NS2-SPCS1 complex in cells. To investigate the interaction of SPCS1 with E2 in the absence of NS2, HCV Core-p7 polyprotein or E2 protein were co-expressed with SPCS1-myc in cells, followed by immunoprecipitation with anti-myc antibody. As shown in Fig. 6B and Fig. S2, E2 was co-immunoprecipitated with SPCS1-myc. The interaction between SPCS1 and E2 was further analyzed *in situ* by PLA and mKG system. Specific signals indicating formation of the SPCS1-E2 complex were detected in both assays (Fig. S3), suggesting physical interaction between SPCS1 and E2 in cells.

We further determined the region of SPCS1 responsible for the interaction with E2 by co-immunoprecipitation assays. Full-length and deletion mutant d2 of SPCS1 (Fig. 1A) were similarly co-immunoprecipitated with E2, while only a limited amount of d1 mutant SPCS1 (Fig. 1A) was co-precipitated (Fig. 6C). It may be that the aa 43–102 region of SPCS1, which was identified as the region involved in the NS2 interaction (Fig. 1D), is important for its interaction with E2, and that deletion of the N-terminal cytoplasmic region leads to misfolding of the protein and subsequent inaccessibility to E2.

Finally, to understand the significance of SPCS1 in the NS2-E2 interaction, Huh7.5.1 cells with or without SPCS1 knockdown by siRNA were transfected with expression plasmids for Core-p7 and FLAG-NS2, followed by co-immunoprecipitation with anti-FLAG antibody. As shown in Fig. 6D, the NS2-E2 interaction was considerably impaired in the SPCS1-knockdown cells as compared to that in the control cells. A similar result was obtained in the stable SPCS1-knockdown cell line (Fig. 6E). In contrast, in that cell line, the interaction of NS2 with NS3 was not impaired by SPCS1 knockdown (Fig. 6E).

These results, together with the above findings, suggest that SPCS1 is required for or facilitates the formation of the membrane-associated NS2-E2 complex, which participates in the proper assembly of infectious particles.

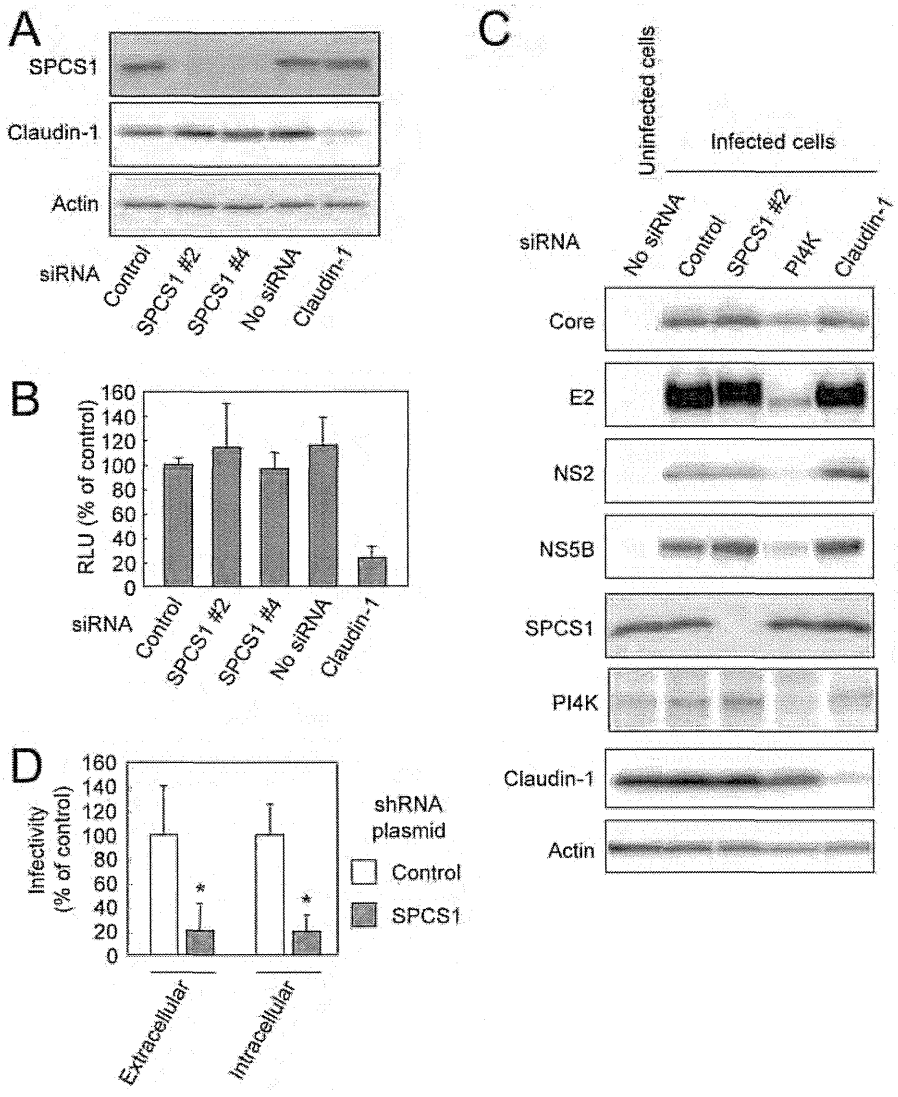

Figure 5. Effect of SPCS1 knockdown on entry into cells, genome replication, and assembly or release of infectious virus. (A) Huh7.5.1 cells were transfected with siRNA for SPCS1 or claudin1, or control siRNA at a final concentration of 30 nM. Expression levels of endogenous SPCS1, claudin-1, and actin in the cells at 2 days post-transfection were examined by immunoblotting using anti-SPCS1, anti-actin, and anti-claudin-1 antibodies. (B) Huh7.5.1 cells transfected with indicated siRNAs were infected with HCVtcp at 2 days post-transfection. Luciferase activity in the cells was subsequently determined at 2 days post-infection. Data are averages of triplicate values with error bars showing standard deviations. (C) Effect of SPCS1 knockdown on replication of HCV genome. HCV-infected Huh-7 cells transfected with siRNA for SPCS1, PI4K or claudin1, or control siRNA at a final concentration of 30 nM. Expression levels of HCV proteins as well as endogenous SPCS1, PI4K, claudin-1, and actin in the cells at 3 days post-transfection were examined by immunoblotting. (D) HCV infectivity in Huh7.5.1 cells inoculated with culture supernatant and cell lysate from Huh7-25 cells transfected with pSilencer-SPCS1 or control vector along with pHH/JFH1am at 5 days post-transfection. Statistical differences between Control and SPCS1 knockdown were evaluated using Student's t-test. *$p<0.005$ vs. Control.
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Discussion

In this study, we identified SPCS1 as a novel host factor that interacts with HCV NS2, and showed that SPCS1 participates in HCV assembly through complex formation with NS2 and E2. In general, viruses require host cell-derived factors for proceeding and regulating each step in their lifecycle. Although a number of host factors involved in genome replication and cell entry of HCV have been reported, only a few for viral assembly have been identified to date. To our knowledge, this is the first study to identify an NS2-interacting host protein that plays a role in the production of infectious HCV particles.

NS2 is a hydrophobic protein containing TM segments in the N-terminal region. The C-terminal half of NS2 and the N-terminal third of NS3 form the protease, which is a prerequisite for NS2-NS3 cleavage. In addition, it is now accepted that this protein is essential for particle production [4–6,12]. However, the mechanism of how NS2 is involved in the assembly process of HCV has been unclear.

So far, two studies have screened for HCV NS2 binding proteins by yeast two-hybrid analysis [37,38]. Erdtmann et al. reported that no specific interaction was detected by a conventional yeast hybrid screening system using full-length NS2 as a bait, probably due to hampered translocation of the bait to the