response to external dsRNA (Lanford et al., 2003). Therefore, a new HCV replicon system needs to be developed using other human cell lines possessing intact TLR3 signaling pathways. We are currently making a trial to establish an HCV replicon system using PH5CH8 or NKNT-3 cells.

On the other hand, it has been recently found that an HCV serine protease, NS3-4A, can block the phosphorylation and effector action of IRF3 (Foy et al., 2003). This finding using HuH-7 cells suggests that NS3-4A mediates the proteolysis of cellular proteins within an antiviral signaling pathway upstream of IRF3, leading to persistent viral infection. The recently identified TRIF (Li et al., 2005a, 2005b) and RIG-I (Foy et al., 2005) are possible candidates for these cellular proteins. Therefore, it was thought that IFN-β induction by NS5B through the activation of TLR3 might be suppressed by NS3-4A in PH5CH8 cells. In fact, our recent study showed that NS3-4A, in a serine protease activity-dependent manner, suppressed NS5B's activation of the IFN system (Dansako et al., 2005). However, the synergistic induction of IFN-β in PH5CH8 cells co-expressing Core and NS5B was only partially suppressed by NS3-4A, whereas the induction of IFN-β by NS5B only was drastically suppressed by NS3-4A (Dansako et al., 2005). We speculate that a biological implication of this phenomenon is that HCV proteins contribute to the maintenance of a low steady state of the virus by controlling the expression level of IFN-β in the infected cells.

In addition to the delay of cell cycle progression through the S phase, enhanced susceptibility to DNA-damaging reagents was found in NS5B-expressing PH5CH8 cells. This phenomenon was attributed to IFN-β induced by NS5B. Further characterization of this phenomenon may contribute to the understanding of IFN-β's biological effects on hepatocytes and effects on the pathogenesis of hepatocellular carcinoma caused by HCV. Furthermore, the findings of the present study may contribute to an understanding of the mechanisms underlying the TLR3 activation involved in innate immunity against viral infection. In addition, our findings suggest that an antiviral state in uninfected cells may be induced by the expression of a viral protein, NS5B.

Materials and methods

Cell culture and cell cycle analysis

The non-neoplastic immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3 cells, were maintained as described previously (Ikeda et al., 1998; Kobayashi et al., 2000). Human hepatoma cell line HuH-7 cells, human cervical carcinoma HeLa cells, and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

To synchronize the cells at the G1/S transition, growing cells were treated with thymidine (Sigma, St. Louis, MO) (2.5 mM) for 19 h, washed in PBS, and released into fresh medium for 11 h. The cells were then treated with aphidicolin (Sigma) (5 μM) for 13 h, washed in PBS, and released into fresh medium. The cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdUrd; Sigma) for 1 h, fixed with 70% ethanol at indicated time points, stained with fluorescein-isothiocyanate (FITC)-conjugated mouse monoclonal antibody to BrdUrd (BD Pharmingen, San Diego, CA), and counterstained with propidium iodide (PI) (Sigma). The cellular content of DNA was determined by flow cytometry with FACScalibur instrument, and data were analyzed with CELL Quest software (BD Biosciences, San Jose, CA) (Naka et al., 2004). To determine the population of G2-M phase reached cells, the cells were treated with Nocodazole (Noc; Sigma) (200 ng/ml) at 5 h after release into the S phase. Then, after 7 h (post release from 12 h), the cell population that had accumulated in the G2-M phase was analyzed by flow cytometry. To examine the effects of IFN-β, PH5CH8 and NKNT-3 cells were treated with or without IFN-β (500 IU/ml) at 12 h prior to release, and cell cycle progression was analyzed. To assess the effect of anti-IFN-β neutralizing antibody, NS5B-expressing cells were treated with anti-IFN-β antibody (70 U/ml, OBT0377, Oxford Biotechnology, Oxfordshire, UK) during cell cycle synchronization and after release from the G1/S boundary.

Vector construction and retrovirus infection

Retroviral vectors pCXbsr (Akagi et al., 2000) and pCX4bsr (Akagi et al., 2003), which contain the resistance gene for blasticidin, were used in this study. The DNA fragments encoding the influenza hemagglutinin tagged (HA)-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, and NS5B were amplified from pMILE (HCV 1B-1 strain belonging to genotype 1b; accession no. AB080299) by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The obtained DNA fragments were subcloned into the *Eco*RI (*Bam*HI for NS5B) and *Not*I sites of pCXbsr or pCX4bsr. The DNA fragment encoding myc-tagged NS5A was also amplified from pMILE by PCR. The obtained DNA fragment was subcloned into the *Eco*RI and *Not*I sites of pCXpur (Akagi et al., 2000), which contains the resistance gene for puromycin. The DNA fragments encoding TLR3 (accession no. NM_003265), Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF or TICAM-1, accession no. NM_182919), and RIG-IC, a dominant negative inhibitor of retinoic acid-inducible gene-I (RIG-I) (Yoneyama et al., 2004), were amplified from cDNAs obtained from PH5CH8 cells. The primer sequences containing the *Sph*I (for forward) or *Not*I (for reverse) recognition sites for TLR3, TRIF, and RIG-IC were designed to enable expression of the TLR3, TRIF, and RIG-IC open-reading frames, respectively. The obtained DNA fragments were subcloned into the *Sph*I and *Not*I sites of pCXpur/myc, which can express myc-tagged protein. The *IFN-β* gene promoter region (−125 to +19) described previously (Fujita et al., 1988) was amplified using genomic DNA derived from PH5CH8 cells and a primer set of 5′-ACGGGGTACC-GAGTTTTAGAAACTACTAAAATG-3′ containing the *Kpn*I recognition site (underlined) and 5′-AGGAAGATCTTC-GAAAGGTTGCAGTTAGAATG-3′ containing the *Bgl*II recognition site (underlined). The obtained DNA fragment was

subcloned into the *Kpn*I and *Bgl*II sites of pGL3-Basic (Promega) and was termed pIFN-β(-125)-Luc. Retrovirus infections were performed as described previously (Naganuma et al., 2004).

RT-PCR and RNA interference

RT-PCR was carried out as described previously (Dansako et al., 2003). The sequences of sense and antisense primers for IFN-β (accession no. V00547) were 5′-CCCTGAGGAGATTAAGCAGCTGC-3′ and 5′-AGTTCCTTAGGATTTCCACTCTGAC-3′. The sequences of primer set for ISG56 (accession no. X03557) were 5′-AGAAGCAGGCAATCACAGAAAAGCTG-3′ and 5′-CCAGGGCTTCATTCATATTTCCTTCC-3′. Small-interference RNA (siRNA) duplexes targeting the coding regions of human TLR3, TLR4, and luciferase GL2 (Elbashir et al., 2001) as a control were chemically synthesized (Greiner, Tokyo, Japan). The sequences of the human TLR3 oligonucleotides were: 5′-CCUCCAGCACAAUGAGCUATT-3′ and 5′-UAGCUCAUUGUGCUGGAGGTT-3′. The sequences of the human TLR4 oligonucleotides were: 5′-CCUCCCCUUCUCAACCAAGTT-3′ and 5′-CUUGGUUGAGAAGGGGAGGTT-3′. The cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen, Carlsbad, CA). Total RNAs were extracted after 3 days, and RT-PCR was performed using primer sets for TLR3 (Kadowaki et al., 2001), TLR4 (Kadowaki et al., 2001), and GAPDH (Dansako et al., 2003).

Western blot analysis

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed according to standard procedures using primary antibodies, rat monoclonal anti-HA (3F10; Roche Molecular Biochemicals, Mannheim, Germany), mouse monoclonal anti-NS3 (clone MMM33; Novacastra Laboratories, Newcastle upon Tyne, UK), anti-NS5B (a gift from Dr. M. Kohara), anti-myc (PL14; Medical and biological laboratories, Nagoya, Japan), anti-β-actin (AC-15, Sigma), anti-STAT1 (clone 42; BD Transduction Laboratories, San Diego, CA), rabbit polyclonal anti-phospho-STAT1(Y701) (Cell Signaling Technology, Beverly, MA), and anti-IRF7 (H-246, Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish-peroxidase-conjugated secondary antibodies. The immune complex was visualized using the ECL Western blot detection system (Amersham Bioscience, Piscataway, NJ).

Reporter assay

The luciferase activity was measured by dual-luciferase assay system (Promega, Madison, WI) as previously described (Dansako et al., 2003). Briefly, cells were transfected with pISRE-Luc (Stratagene, LaJolla, CA) or pIFN-β(-125)-Luc reporter plasmid together with phRL-CMV (Promega) as an internal control reporter plasmid by FuGENE6 (Roche). After 48 h of transfection, cell lysates were then prepared and assayed for luciferase activities; transfection efficiency was normalized by renilla luciferase activity (internal control) derived from phRL-CMV. Three independent triplicate transfection experiments were conducted in order to verify the reproducibly of the results.

Immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 125 mM NaCl, 0.1%(v/v) Nonidet P-40 (NP-40; Sigma), 5 mM EDTA, 0.1 M NaF, and a mixture of protease inhibitors (Complete; Roche). Pre-cleared cell lysates were subjected to immunoprecipitation using agarose-conjugated anti-myc antibody (PL14, MBL). Bound proteins were eluted from beads by boiling in SDS sample buffer, and immunoblotting analysis was performed using anti-myc or anti-NS5B antibody, and HRP-conjugated anti-mouse IgG TrueBlot (eBioscience, San Diego, CA).

Immunofluorescence analysis

To examine the intracellular protein localization, 2×10^4 cells were cultured and treated on chamber slides then fixed and probed with polyclonal rabbit anti-IRF3 antibody (FL-425, Santa Cruz Biotechnology) and FITC-conjugated donkey anti-rabbit secondary antibody according to a method described previously (Foy et al., 2003). PH5CH8 cells treated with poly (IC) (2.5 μg/ml for 6 h; Amersham Biosciences) were used as a positive control for the activation of IRF3.

Evaluation of sensitivity to DNA damage

Cells in an exponential growth phase were plated onto 10-cm plates (5×10^3 cells/plate) and cultured for 4 days. The cells were treated with hydrogen peroxide (H_2O_2; Wako Pure Chemical, Osaka, Japan), methylmethane sulfonate (MMS; Sigma), Adriamycin (ADR; doxorubicin; Sigma), and neocarzinostatin chromophore (NCS; generously provided by Kayaku, Tokyo, Japan) for 2 h at 37 °C. For UV-B treatment (UV-B radiation at 302 nm), the medium was aspirated prior to exposure, the cells were washed twice with PBS, and then a fresh culture medium was added. Ten days later, the cells were fixed and stained with Coomassie brilliant blue as described previously (Naganuma et al., 2004). Only colonies containing >50 cells were scored as being derived from viable clonogenic cells.

Acknowledgments

We thank Drs. M. Kohara and T. Akagi for the anti-NS5B monoclonal antibody and pCX retroviral vectors, respectively, and A. Nozaki for suggestions and comments. We also thank T. Nakamura, A. Morishita, and H. Tawara for their technical assistance. This work was supported by grants-in-aid for research on hepatitis from the Ministry of Health, Labour, and Welfare of Japan and by the program for promotion of fundamental studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

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Journal of Hepatology 44 (2006) 869–878

Journal of Hepatology

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Epigenetic silencing of interferon-inducible genes is implicated in interferon resistance of hepatitis C virus replicon-harboring cells

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***Background/Aims*: We previously established hepatitis C virus (HCV) replicon-harboring cell lines possessing two interferon (IFN)-resistant phenotypes: a partially resistant phenotype (αR series) and a severely resistant phenotype (βR series). We recently found that the severe IFN resistance of the βR-series cells is caused by the functional disruption of type I IFN receptors. Here, we aimed to clarify the mechanism(s) underlying the partial IFN resistance of the αR-series cells.**

***Methods*: αR-series cells were pre-treated with 5-azacytidine to evaluate the effects of DNA demethylation on IFN resistance. cDNA microarray analysis was carried out in order to compare 1αR cells, which belong to the αR series, treated with both 5-azacytidine and IFN-α with cells treated with 5-azacytidine or IFN-α alone.**

***Results*: We found that the IFN-resistant phenotype of αR-series cells was impaired by treatment with 5-azacytidine. cDNA microarray analysis identified seven IFN-stimulated genes, which were up-regulated by 5-azacytidine treatment. We demonstrated here that the ectopic expression of each of these seven genes in 1αR cells frequently weakened the IFN resistance of these cells.**

***Conclusions*: The present results suggest that the epigenetic silencing of IFN-stimulated genes is implicated in the acquisition of a partially IFN-resistant phenotype of HCV replicon-harboring cells.**

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Keywords: **IFN resistance; HCV replicon; DNA methylation; cDNA microarray; Epigenetic silencing**

1. Introduction

Persistent infection by the hepatitis C virus (HCV) is a major cause of chronic hepatitis (CH) [1,2], which can progress to liver cirrhosis and hepatocellular carcinoma [3]. HCV is an enveloped RNA virus belonging to the family Flaviviridae, the genome of which consists of a positive-stranded 9.6-kb RNA encoding at least 10 structural and non-structural proteins [4]. Since, at least 170 million people are currently infected with HCV worldwide, this type of infection constitutes a global health problem [5]. Interferon (IFN)-α/ribavirin combination therapy is currently the standard clinical therapy for patients with CH C; however, the effectiveness of IFN is limited to approximately half of these patients [6]. This clinical finding suggests that HCV is resistant to the antiviral effects of IFN, and that HCV proteins directly or indirectly attenuate those effects [7].

Although HCV replicon system harboring autonomously replicating HCV subgenomic RNA containing the non-structural region [8] provides a powerful tool for various HCV studies, all of the HCV replicons established to date

Received 8 August 2005; received in revised form 30 December 2005; accepted 20 January 2006; available online 28 February 2006

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Abbreviations: aa, amino acid; 5-azaC, 5-azacytidine; CH, chronic hepatitis; HCV, hepatitis C virus; IFN, interferon; IFNAR, IFN receptor; IRF-1, IFN regulatory factor 1; ISG, IFN-stimulated gene; RT-PCR, reverse transcription-polymerase chain reaction.

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doi:10.1016/j.jhep.2006.01.030

have been highly sensitive to IFN [9–12]. Based on our assumption that prolonged IFN treatment might change HCV replicons from the IFN-sensitive phenotype into an IFN-resistant phenotype, we established nine HCV replicon-harboring cell lines possessing two IFN-resistant phenotypes: a partially resistant phenotype (αR series; 1αR, 3αR, 4αR, 5αR, and αRmix) and a severely resistant phenotype (βR series; 1βR, 3βR, 4βR, and 5βR) obtained by IFN-α and IFN-β treatment, respectively [13]. αR- and βR-series cells were derived from clones 1, 3, 4, and 5, and 1αR, 3αR, 4αR, and 5αR cells were counterparts of 1βR, 3βR, 4βR, and 5βR cells, respectively [13]. Although genetic analysis of these replicons identified one common amino acid (aa) substitution and several cell line-specific aa substitutions, we failed to obtain the evidence of the direct involvement of these aa substitutions to IFN resistance [14]. However, we found frequent non-sense mutations and deletions in type I IFN receptor (IFNAR) genes (IFNAR1 and IFNAR2c) in the βR-series cells, but such mutations were rarely observed in the αR-series cells [14]. Since we demonstrated that the ectopic expression of wild-type IFNAR in the βR-series cells restored IFN signaling, we determined that the functional disruption of type I IFNAR was responsible for this type of resistance [14]. However, the mechanism underlying the partial IFN-resistance of αR-series cells remains unclear. Since, the expression levels of IFNARs, Tyk2, and Jak1 were not lower in the αR-series cells [13,14], the functional degeneration of other cellular factor(s) involved in IFN signaling or IFN-stimulated gene(s) (ISG) was thought to contribute to the acquisition of IFN resistance.

As one potential mechanism for the partial resistance, we assumed that the epigenetic silencing of some ISGs, which are known to be involved in the anti-HCV activity of IFN, by DNA methylation around the promoter region might convert HCV replicon-harboring cells from the IFN-sensitive phenotype to the IFN-resistant phenotype. To evaluate our hypothesis, we examined whether or not pre-treatment of αR-series cells with 5-azacytidine (5-azaC), an inhibitor of DNA methyltransferase and an inducer of gene suppressed by DNA methylation, could alter the IFN sensitivity of the cells.

Here, we report that the epigenetic silencing of ISGs is implicated in the IFN-resistance of αR-series cells. We have also identified several ISGs that are up-regulated by 5-azaC treatment and weaken the IFN resistance of αR-series cells.

2. Materials and methods

2.1. Cell cultures

HCV replicon-harboring cells and cured 6Mc cells [14], from which the HCV replicons had been eliminated by IFN-γ treatment, were maintained as described previously [13].

2.2. Analysis of IFN sensitivity

HCV replicon-harboring cells were treated with 5-azaC (2–10 μM) (A-2385, Sigma, St Louis, MO) for 2 weeks. Then, human IFN-α (I-2396; Sigma) was added to the cells (with or without pre-treatment with 5-azaC) as described previously [12,13]. After 3 weeks in culture, the colonies obtained on the culture dishes were stained with Coomassie brilliant blue (CBB) as described previously [15].

2.3. Quantitative analysis of HCV replicon RNA

The quantification of HCV RNA was carried out to monitor the antiviral effects of IFN-α, and was performed by real-time LightCycler polymerase chain reaction (PCR) as described previously [16,17].

2.4. Construction of replicon plasmid and RNA synthesis

The non-structural region (6.1 kb) fragment of a 1βR1 clone obtained from 1βR cells [13] was digested with SpeI and BsiWI, and the digested

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

Gene	Direction	Nucleotide sequence	Products (bp)	Cycles
IFI27	Forward	gttttgcccctggccaggattgct	252	27
	Reverse	aatggagcccaggatgaacttggt		
9-27	Forward	tcttcttgaactggtgctgtctggg	191	28
	Reverse	agagccgaataccagtgacaggat		
LMP2	Forward	atggaaccctgggaggaatgctg	145	27
	Reverse	gcaatagcgtctgtggtgaagcg		
LMP7	Forward	ctgggataagaagggtcctggac	293	27
	Reverse	tactggtgcagcaggtcactggac		
Viperin	Forward	tggagcgccacaaagaagtgtcct	240	27
	Reverse	ccagcttcagatcagccttactcc		
IFI44	Forward	tgtggctttgctcactcatgtgga	227	31
	Reverse	cagcccatagcattcgtctcagag		
IFIT2	Forward	aggccatccaccactttatagagg	272	28
	Reverse	tgggcaccacatctctattctcca		
ISG56	Forward	tagccaacatgtcctcacagac	396	32
	Reverse	tcttctaccactggtttcatgc		
GAPDH	Forward	gactcatgaccacagtccatgc	334	26
	Reverse	gaggagaccacctggtgctcag		

fragment (5.7 kb) was ligated into the plasmid pNSS1RZ2RU [12], which was predigested with SpeI and BsiWI. The obtained plasmid was linearized by XbaI and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [12].

2.5. RNA transfection and selection of G418-resistant cells

The transfection of HCV replicon RNA synthesized in vitro into 6Mc cells was performed by electroporation, and the cells were selected in the presence of G418 (300 μg/ml; Invitrogen) for 3 weeks as described previously [14].

2.6. cDNA microarray analysis

The 1αR cells (5×10^5 cells), which were cultured for 2 weeks in the absence or presence of 5-azaC (10 μM), were plated onto a 10-cm diameter dish, and were cultured for 2 days in the absence of G418. Then the cells were treated with or without IFN-α (500 IU/ml) for 8 h. Total RNAs prepared from 80% confluent cells were subjected to cDNA microarray analysis (CodeLink™, Uniset human I containing 54840 spots of 30-mer oligonucleotides; Amersham Biosciences) as described previously [18].

2.7. Reverse transcription (RT)-PCR

RT-PCR and real-time LightCycler PCR were performed as described previously [18,19] using the primer sets in Table 1.

2.8. Expression vectors and retroviral infection

Retroviral vectors pCXbsr [20] and pC4bsr(IRES) (kindly provided by Dr T. Akagi), which contain the resistance gene for blasticidin, were used in this study. Retroviral infection and selection by blasticidin were performed as described previously [15].

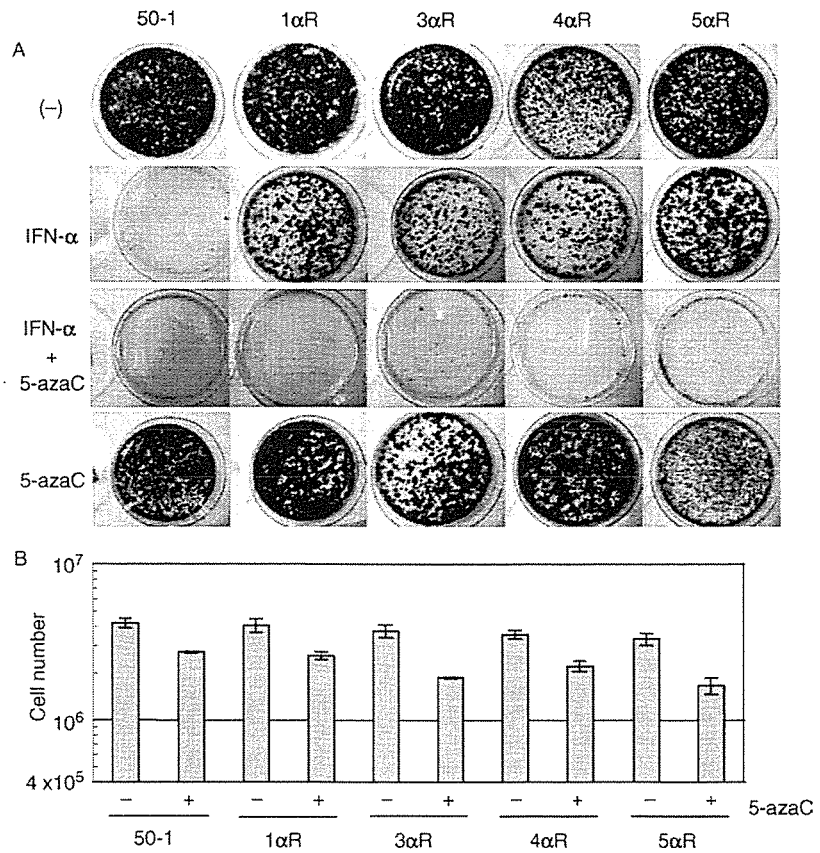

Fig. 1. 5-AzaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype. (A) IFN sensitivities of various HCV replicon-harboring cells. αR-series cells (1αR, 3αR, 4αR, and 5αR) showing a partially IFN-resistant phenotype and the parent 50-1 cells with an IFN-sensitive phenotype were used for the analysis. These cells were plated onto 10-cm diameter dishes (2×10^4 cells/dish) and were cultured for 1 day before treatment with 5-azaC. 5-AzaC was added to the cultures at a final concentration of 10 μM (4 μM for 4αR cells, and 2 μM for 5αR cells) and the cells were then cultured for 2 weeks, and were subsequently treated with or without IFN-α (400 IU/ml) for 3 weeks in the presence of G418 (300 μg/ml). The HCV replicon-harboring cells were known to possess the G418-resistant phenotype, because neomycin phosphotransferase was produced by the efficient replication of the HCV replicon in the cells. Therefore, when an HCV replicon was excluded from the cells or levels of the replicon were low, the cells did not survive in the presence of G418. The panels show G418-resistant colonies stained with CBB as described previously [15]. (B) Effect of 5-azaC on the growth of HCV replicon-harboring cells. 50-1 and αR-series cells were plated onto 10-cm diameter dishes (4×10^5 cells/dish) and were cultured for 1 day before treatment with 5-azaC. These cells were cultured in the absence or presence of 5-azaC (10 μM for 50-1, 1αR, and 3αR cells, 4 μM for 4αR cells, and 2 μM for 5αR cells) for 3 days, and then the cell number was determined by a method described previously [27]. The data indicate means ± SD of triplicates from two independent experiments. [This figure appears in colour on the web.]

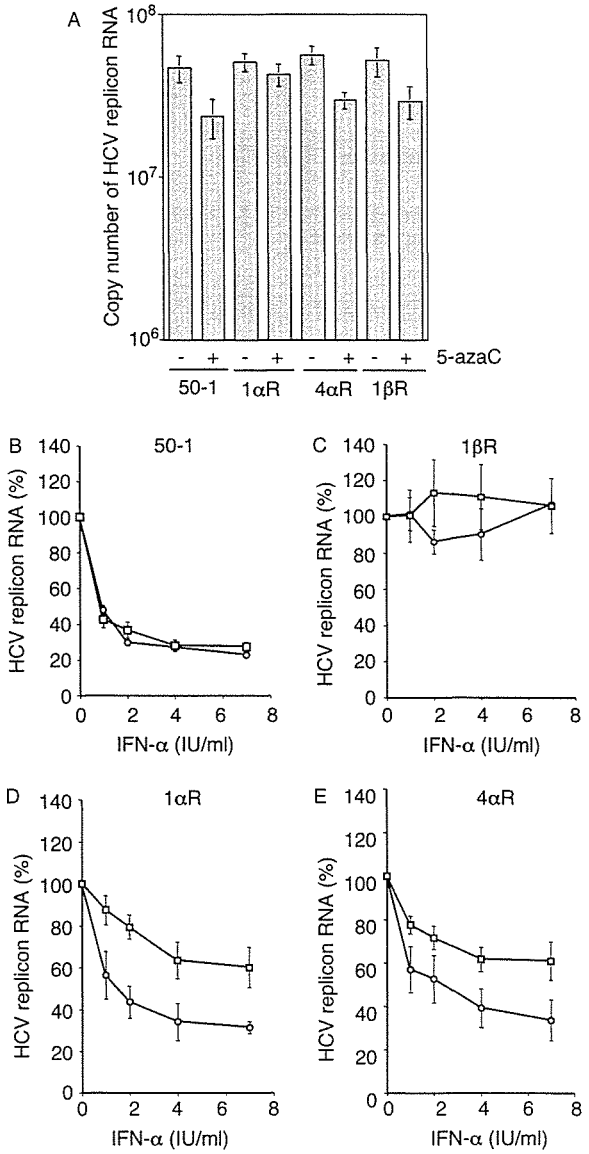

Fig. 2. IFN sensitivities of HCV replicons. (A) HCV replicon RNA levels of 50-1, 1βR, 1αR, and 4αR cells cultured in the presence or absence of 5-azaC (10 μM for 50-1, 1βR, and 1αR cells, 4 μM for 4αR cells) for 2 weeks. Real-time LightCycler PCR was carried out in order to quantitatively monitor the levels of replicon RNAs in the cells, as described previously [16,17]. The copy numbers of HCV replicon RNA per μg of total RNA determined by quantitative RT-PCR are shown. (B) 50-1, (C) 1βR, (D) 1αR, and (E) 4αR cells were cultured in the presence (open circles) or absence (open squares) of 5-azaC as described in (A), and then the cells were treated with IFN-α (0, 1, 2, 4, and 7 IU/ml each) for 3 days. Quantitative RT-PCR was carried out as described in (A). The relative level of HCV replicon RNA (%) calculated at each point, when the HCV replicon RNA level of IFN non-treated cells was assigned to be 100%, is shown here. The data indicate means ± SD of triplicates from two independent experiments.

2.9. Western blot analysis and immunoprecipitation

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed as previously described [21].

Table 2
Genes whose elevated levels in the C vs. IFN + 5-azaC experiment were more than 2.5-fold those in the C vs. IFN or C vs. 5-azaC experiment

Gene	C vs. IFN + 5-azaC	C vs. IFN	C vs. 5azaC	Accession no.
IFI27	25.21[a]	1.56	1.72	NM_005532
9-27	2703.30	600.65	−0.04	NM_003641
LMP2	23.60	6.20	1.57	NM_002800
LMP7	22.27	8.79	3.72	NM_004159
Viperin	25.26	9.07	−2.26	NM_080657
IFI44	29.60	7.05	0.03	NM_006417
IFIT2	10.67	2.96	−3.02	NM_001547

The manufacturer's protocol (Amersham Biosciences) recommended the use of a ratio of at least 2:1 as a standard for the selection of genes showing significant differences in expression.
[a] Fold.

3. Results

3.1. IFN-resistant phenotype of HCV replicon-harboring cells is impaired by co-treatment with 5-azaC and IFN-α

Based on our assumption, we examined whether or not pre-treatment of αR-series cells with 5-azaC could convert these cells from the IFN-resistant phenotype to the IFN-sensitive phenotype. Parent HCV replicon-harboring cells (50-1) [22], and αR-series cells were treated with IFN-α and/or 5-azaC, and their IFN sensitivities were compared. After IFN-α treatment, many 1αR, 3αR, 4αR, and 5αR cell colonies survived, although only a few small colonies of 50-1 cells survived. However, when 5-azaC pre-treatment following IFN-α treatment was applied, almost none of the 1αR, 3αR, 4αR, or 5αR cell colonies survived, nor did the colonies of 50-1 cells (Fig. 1(A)). Although, we observed that 5-azaC treatment slightly lowered the growth rates of these cells (Fig. 1(B)), the cell viabilities exceeded 99%, and a number of colonies were finally obtained after treatment with 5-azaC alone (Fig. 1(A)). These results suggest that 5-azaC treatment converts αR-series cells from an IFN-resistant phenotype into an IFN-sensitive phenotype.

Since, we previously observed that none of the non-sense mutations in IFNAR genes found in all clones derived from 1βR and 4βR cells were detected in 1αR and 4αR cells, which were the counterparts of 1βR and 4βR cells, respectively [14], we focused on 1αR and 4αR cells to assess the effect of 5-azaC treatment on IFN sensitivity. Quantitative RT-PCR analysis of replicon RNA in cells treated with IFN-α was performed using 1αR, 4αR, 1βR (a counterpart of 1αR), and 50-1 cells pre-treated with or without 5-azaC. We confirmed that HCV replicon RNA levels in the cells pre-treated with 5-azaC were sufficient for analysis, although HCV replicon RNA levels were a little lower by 5-azaC treatment (Fig. 2(A)). The level of replicon RNA in 50-1 cells drastically decreased after IFN-α treatment, regardless of 5-azaC pre-treatment (Fig. 2(B)). Contrary to the level observed in 50-1 cells, that of replicon

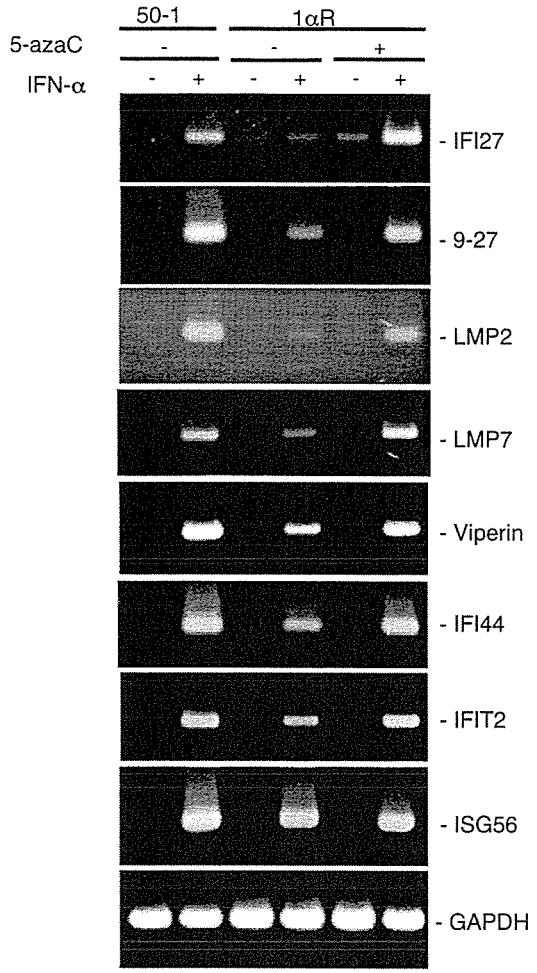

Fig. 3. RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results. 1αR cells were cultured in the presence or absence of 5-azaC (10 μM) for 2 weeks. The 1αR cells treated with 5-azaC and the 50-1 cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted from these cells were subjected to RT-PCR using the primer sets listed in Table 1. ISG56 was used as the representative ISG, which was not selected by the microarray analysis. GAPDH was used as an internal control. RT-PCR products were detected by staining the samples with ethidium bromide after 3% agarose gel electrophoresis.

RNA in 1βR cells did not decrease after IFN-α treatment, regardless of 5-azaC pre-treatment (Fig. 2(C)). However, we observed that the level of replicon RNA in 1αR cells (Fig. 2(D)) and that in 4αR cells (Fig. 2(E)) pre-treated with 5-azaC had decreased more substantially after IFN-α treatment than that in the cells lacking 5-azaC pre-treatment. In general, these results indicate that 5-azaC treatment of αR-series cells (at least 1αR and 4αR cells) may convert the cells from a partially IFN-resistant phenotype to an IFN-sensitive phenotype; moreover, the present findings suggest that some ISGs known to contribute to the anti-HCV activity of IFN are suppressed by DNA methylation in αR-series cells.

3.2. cDNA microarray analysis using 1αR cells

Based on the results presented above, we attempted to identify those ISGs that are determinative of IFN sensitivity; to this end, cDNA microarray analyses were performed by comparing the following: 1αR cells with 1αR cells treated with 5-azaC (C vs. 5-azaC), 1αR cells with 1αR cells treated with IFN-α (C vs. IFN), and 1αR cells with 1αR cells treated with IFN-α after pre-treatment with 5-azaC (C vs. IFN+5-azaC). As the first step in this analysis, we selected the only already-known genes whose expression levels were up-regulated at a ratio of more than 10 in C vs. IFN+5-azaC. Then, from among those that were selected in the first step, we performed an additional selection of genes whose elevated levels in the C vs. IFN+5-azaC experiment were more than 2.5-fold, as compared with those in the C vs. IFN or C vs. 5-azaC experiment. Finally, seven genes (IFI27, IFI44, LMP2, LMP7, 9-27, Viperin, and IFIT2) were identified as genes that were highly and selectively induced by co-treatment with IFN-α and 5-azaC (Table 2).

In order to confirm the results of our microarray selection, we conducted RT-PCR analysis and real-time LightCycler PCR to examine the mRNA levels of the identified seven genes in the 1αR cells treated with or without IFN-α after pre-treatment with or without 5-azaC. As a control, parent 50-1 cells treated with or without IFN-α were used. The results (Fig. 3 and Table 3) confirmed the results of the microarray analysis (Table 2). The findings revealed that the levels of induction of these seven genes in 1αR cells treated with IFN-α were clearly lower than those in 50-1 cells treated with IFN-α. It is noteworthy that the expression of these seven genes in 1αR cells treated with IFN-α after 5-azaC pre-treatment was remarkably elevated, whereas the IFN-α-induced level of expression of the ISG56 gene, an immediate early antiviral ISG, was slightly enhanced after 5-azaC pre-treatment (Fig. 3 and Table 3). These results suggest that the epigenetic silencing of these ISGs is involved in the acquisition of the IFN-resistant phenotype, at least in 1αR cells, and this is also likely to be the case in other αR-series cells.

3.3. Characterization of additional HCV replicon-harboring cells possessing an IFN-resistant phenotype

In order to evaluate the reproducibility of the phenomenon observed in 1αR cells, additional HCV replicon-harboring 1βR1/6Mc cells, which were recently established independently [14], were used for IFN-α treatment (Fig. 4(A)). IFN-α-treated 1βR1/6Mc cells yielded several distinct IFN-resistant colonies, which were designated as 1βR1/6McαR mixed colonies.

To assess the effects of 5-azaC treatment on IFN-sensitivity, quantitative RT-PCR analysis of replicon RNA in the cells treated with IFN-α was performed using 1βR1/6Mc cells, 1βR1/6McαR cells, and 1βR1/6McαR cells pre-treated with 5-azaC (designated as 1βR1/6McαR+AZ cells). The level of replicon RNA in 1βR1/6Mc cells

Table 3
Real-time RT-PCR analysis of mRNA expression of selected ISGs based on the microarray results

Gene	50-1		1αR				
	−		−		+		5-azaC
	−	+	−	+	−	+	IFN-α
IFI27	4.8±1.9	100	6.4±3.2	11.9±1.9	22.9±5.4	380.4±18.9	
9-27	<1.0	100	<1.0	6.8±4.6	<1.0	46.0±6.7	
LMP2	<1.0	100	<1.0	11.7±0.9	2.7±0.3	60.5±4.5	
LMP7	2.3±0.5	100	2.1±0.3	19.8±2.0	8.8±1.3	92.6±18.7	
Viperin	n.d.	100	n.d.	7.4±2.0	<1.0	35.2±5.2	
IFI44	n.d.	100	n.d.	5.9±0.5	n.d.	56.0±7.1	
IFIT2	<1.0	100	<1.0	16.8±9.1	<1.0	48.4±13.3	
ISG56	<1.0	100	<1.0	76.2±4.1	<1.0	117.1±8.7	

The experiments were performed in at least triplicate. n.d., not detected. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of each mRNA concentration to that of GAPDH. The relative level (mean±SD) of each mRNA calculated, when the level of each mRNA of 50-1 cells treated with IFN-α was assigned to be 100, is shown here.

decreased markedly after IFN-α treatment (Fig. 4(B)), as had also been observed in the 50-1 cells (Fig. 2(B)). In contrast to the 1βR1/6Mc cells, 1βR1/6McαR cells exhibited a partially IFN-resistant phenotype (Fig. 4(B)), as had been observed in the 1αR cells (Fig. 2(D)) and 4αR cells (Fig. 2(E)). However, after IFN-α treatment, the level of replicon RNA in the 1βR1/6McαR+AZ cells was lower than that in 1βR1/6McαR cells (Fig. 4(B)). This result suggests that the treatment of 1βR1/6McαR cells with 5-azaC also contributed to a weakening of the IFN-resistant phenotype in these cells.

We next considered the expression levels of the seven genes that had been identified as epigenetically suppressed genes in 1αR cells; here, IFN-α-treated 1βR1/6Mc and 1βR1/6McαR cells were compared. The results obtained by RT-PCR analysis revealed that the induction of these seven genes by IFN-α in the 1βR1/6McαR cells was weaker than

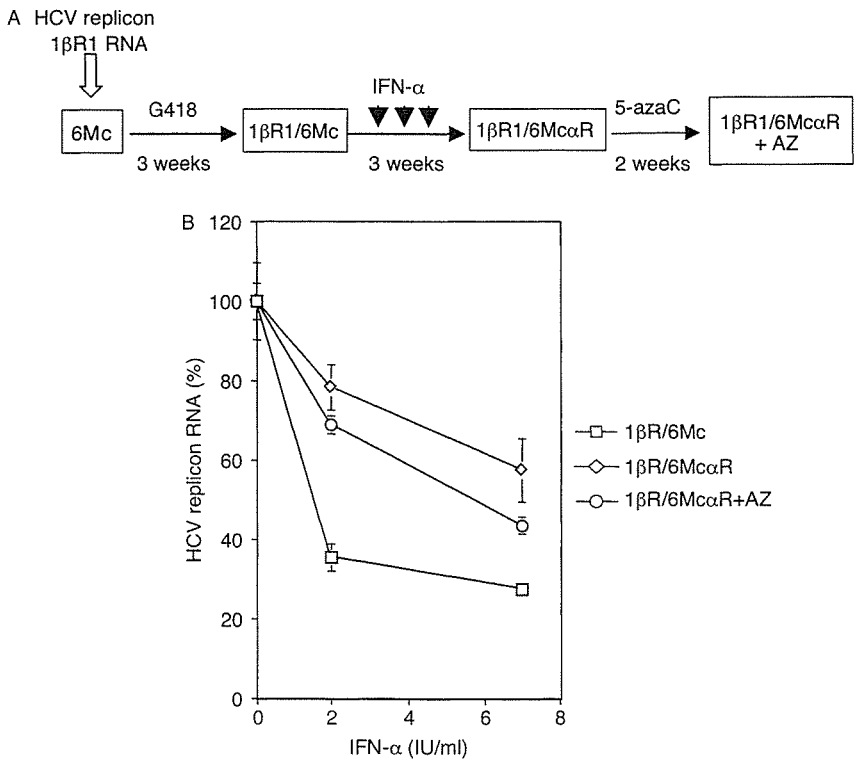

Fig. 4. Effect of 5-azaC treatment on IFN resistance in newly isolated HCV replicon-harboring cells showing a partially IFN-resistant phenotype. (A) Outline of the isolation of HCV replicon-harboring cells showing partial IFN resistance. HCV replicon-harboring cells (designated as 1βR1/6Mc cells) [14], established by the transfection of in vitro-synthesized replicon RNA (1βR1 obtained from 1βR cells) [13] into 6Mc cells, were treated with 400 IU/ml of IFN-α for 3 weeks in the presence of G418, and several colonies survived as cells with an IFN-resistant phenotype (1βR1/6McαR cells). The 1βR1/6McαR cells were treated with 5-azaC (4μM) for 2 weeks. (B) IFN sensitivities of HCV replicons. The HCV replicon-harboring cells treated with IFN-α (0, 2, and 7 IU/ml each) for 3 days were subjected to quantification of HCV replicon RNA, as described in Fig. 2.

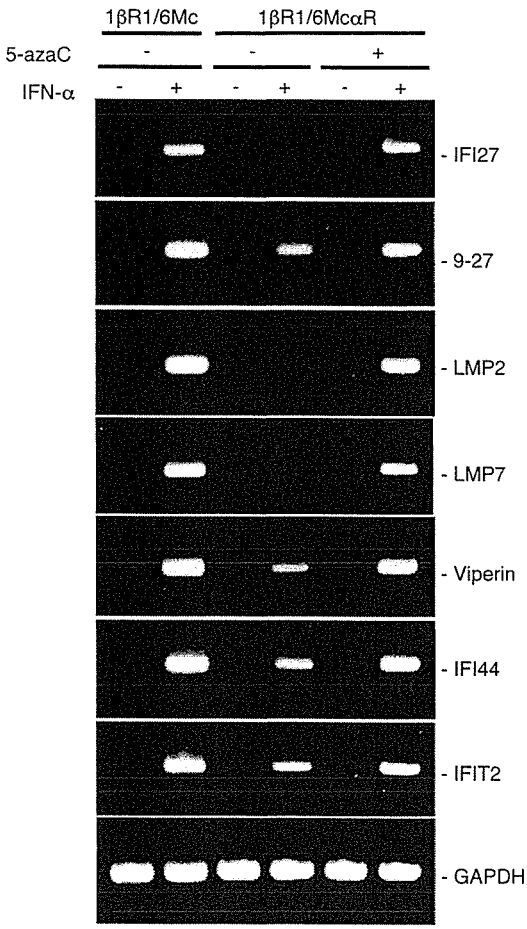

Fig. 5. RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis. 1βR1/6McαR cells were cultured in the presence or absence of 5-azaC (4 μM) for 2 weeks. The 1βR1/6McαR cells were treated with 5-azaC and the 1βR/6Mc cells were treated with or without IFN-α (500 IU/ml) for 8 h. The total RNAs extracted from the cells were subjected to RT-PCR using the primer sets listed in Table 1. GAPDH was used as an internal control. RT-PCR products were detected by staining the samples with ethidium bromide after 3% agarose gel electrophoresis.

that in the parent 1βR1/6Mc cells (Fig. 5). However, these seven genes were induced by IFN-α in 1βR1/6McαR cells pre-treated with 5-azaC (Fig. 5). These results were confirmed by real-time LightCycler PCR (Table 4), and were similar to those obtained in the study of 1αR cells (Fig. 3 and Table 3), thus demonstrating that the appearance of the IFN-resistant phenotype is reproducible. Furthermore, these results suggest that some form of epigenetic silencing such as DNA methylation is frequently involved in the acquisition of the IFN-resistant phenotype.

3.4. Ectopic expression of epigenetically suppressed genes weakens the IFN resistance of 1αR cells

To evaluate the effects of epigenetically suppressed genes on the IFN sensitivity of HCV replicons, we prepared 1αR cells stably expressing IFI27, IFI44, LMP2, LMP7, 9-27, Viperin, or IFIT2 using retroviral gene transfer system [15]. The ectopic expression of each ISG protein was confirmed by Western blot analysis or immunoprecipitation following Western blot analysis (Fig. 6(A)). The results obtained by quantitative RT-PCR analysis revealed that the level of HCV replicon RNA decreased to about half that in the cells expressing Viperin. Also, in LMP2-, LMP7-, and IFIT2-expressing cells, significant reductions of HCV replicon RNA were detected (Fig. 6(B)). These results indicate that the simple expression of these genes into the cells interferes with the level of HCV replicon RNA, and that the combination of ISGs contributes to the IFN resistance of the HCV replicon. Therefore, 1αR cells expressing each ISG protein were initially treated with IFN-α, and then the level of HCV replicon RNA was monitored by quantitative RT-PCR analysis. Interestingly, the results revealed that the level of HCV replicon RNA in the 1αR cells expressing Viperin or LMP7 was largely decreased to the level in parent 50-1 cells (Fig. 6(C)). Furthermore, we observed that the 1αR cells expressing IFIT2, 9-27, LMP2, or IFI44 also partially changed to the IFN-sensitive phenotype (Fig. 6(C)). These results suggest that the transcriptional suppression of ISGs such as LMP-7

Table 4
Real-time RT-PCR analysis of mRNA expression of seven ISGs selected by microarray analysis

Gene	1βR1/6Mc		1βR1/6McαR				
	−		−		+		5-azaC
	−	+	−	+	−	+	IFN-α
IFI27	4.0±0.8	100	13.7±2.0	18.4±3.7	60.9±10.7	383±16.4	
9-27	<1.0	100	<1.0	14.8±0.2	1.0±0.2	101.7±26.3	
LMP2	1.1±0.1	100	<1.0	6.9±0.6	3.0±0.3	112.2±38.1	
LMP7	3.5±0.4	100	<1.0	9.9±0.7	8.7±0.9	96.5±11.2	
Viperin	n.d.	100	n.d.	23.2±9.4	<1.0	104.8±14.0	
IFI44	n.d.	100	n.d.	4.5±0.9	n.d.	57.3±1.9	
IFIT2	<1.0	100	<1.0	10.2±16	<1.0	67.9±9.2	

The experiments were performed in at least triplicate. n.d., not detected. To correct the differences in RNA quality and quantity between the samples, data were normalized using the ratio of each mRNA concentration to that of GAPDH. The relative level (mean±SD) of each mRNA calculated, when the level of each mRNA of 1βR1/6Mc cells treated with IFN-α was assigned to be 100, is shown here.

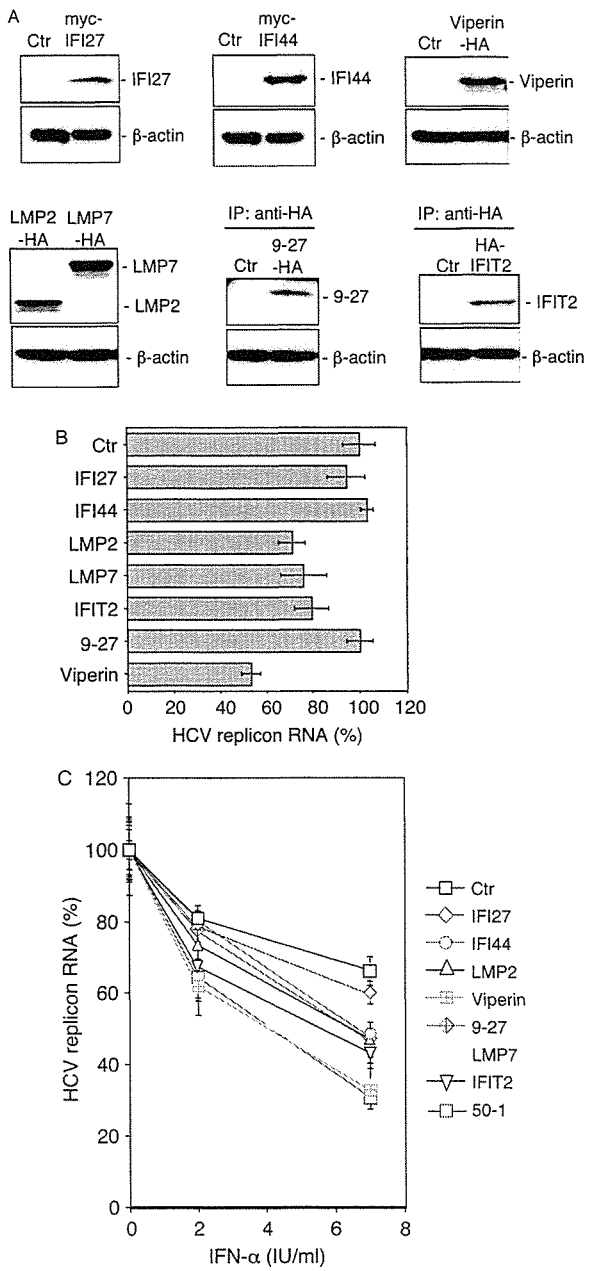

Fig. 6. IFN sensitivities of HCV replicons in 1αR cells expressing epigenetically silenced ISG. (A) Ectopic expression of epigenetically silenced ISG in 1αR cells introduced by retrovirus-mediated gene transfer. The DNA fragments encoding myc-tagged IFI27 and IFI44, and influenza hemagglutinin-tagged (HA)-LMP2, LMP7, 9-27, and IFIT2 were obtained by the PCR amplification of cDNAs derived from HCV replicon-harboring cells using primer sets designed from the nucleotide sequences (see the Accession no. in Table 2). The obtained DNA fragments were cloned into the EcoRI and NotI sites of pCXbsr, as described previously [28]. The DNA fragment encoding the HA-IFIT2 was cloned into the EcoRI and NotI sites of pC4bsr(IRES). The sequences of the plasmid inserts were confirmed by Big Dye terminator cycle sequencing on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Western blot analysis or immunoprecipitation (IP) following Western blot analysis of 1αR cells infected with pCXbsr retroviruses encoding myc-IFI27, myc-IFI44, Viperin-HA, LMP2-HA, LMP7-HA, 9-27-HA, and HA-IFIT2 was performed by anti-HA (rat monoclonal 3F10; Roche, Mannheim, Germany) or anti-myc (mouse monoclonal PL14; Medical and Biological Laboratories, Nagoya, Japan) antibodies. In order to detect 9-27-HA and HA-IFIT2, IP was performed by using anti-HA affinity matrix (3F10, Roche). pCXbsr or pC4bsr (IRES) retrovirus-infected 1αR cells were used as a control (Ctr). β-actin was detected by anti-β-actin antibody (AC-15, Sigma) as a control for the amount of protein loaded per lane. (B) The quantification of HCV replicon RNA in 1αR cells expressing epigenetically silenced ISG was performed as described in Fig. 2. pCXbsr retrovirus-infected 1αR cells were used as a control (Ctr). (C) IFN sensitivities of HCV replicon RNA in 1αR cells expressing epigenetically silenced ISG. ISG-expressed 1αR cells treated with IFN-α (0, 2, and 7 IU/ml each) for 3 days were subjected to quantification of HCV replicon RNA, as described in Fig. 2. pCXbsr retrovirus-infected 1αR cells were used as a control (Ctr). The relative level of HCV replicon RNA (%) calculated at each point, when the HCV replicon RNA level of IFN non-treated cells expressing each ISG was assigned to be 100%, is shown here. [This figure appears in colour on the web.]

and Viperin, among other ISGs, is involved in the partial IFN resistance of HCV replicon-harboring cells.

4. Discussion

Although, we identified several ISGs, which are expected to contribute to the acquisition of the IFN-resistant phenotype, the relationships among these ISGs remain unclear, in particular as regards issues such as anti-HCV activity and the mechanisms of induction of these ISGs after 5-azaC treatment. In this context, several plausible explanations can be considered: (1) all seven ISGs are independently induced by 5-azaC treatment; (2) one of the seven ISGs is induced by 5-azaC treatment, and then this ISG activates the other ISGs; (3) a certain transcriptional factor (e.g. IFN regulatory factor 1 (IRF-1), IRF-7, etc.) is induced by 5-azaC treatment, and then this factor activates the seven ISGs; (4) a certain combination of a identified ISG and an unidentified ISG in the present study exhibits anti-HCV activity; and/or (5) a certain combination of a number of ISGs identified in the present study exhibits anti-HCV activity. As regards possibilities (1) and (2), we examined the expression levels of seven ISGs in 1αR cells ectopically expressing one of these seven ISGs. We did not obtain evidence demonstrating that one of the seven ISGs activates the other ISGs (data not shown); therefore, possibility (1) was supported and possibility (2) was excluded (data not shown). As regards option (3), it was noted that ectopic IRF-1 expression suppresses the replication of the HCV replicon via the activation of ISGs [23]. However, we did not observe any significant induction of the mRNA for the IRF-1 gene in 1αR cells co-treated with IFN-α and 5-azaC (data not shown). Moreover, no induction of the IRF-7 gene was observed in 1αR cells co-treated with IFN-α and 5-azaC (data not shown). Therefore, possibility (3) also appears unlikely, although the possibility of the induction of another transcriptional factor(s) cannot be excluded. As regards

option (4), a number of ISGs that were not selected by the present microarray analysis could be considered as candidates for contributing to anti-HCV activity. However, at least with respect to the representative 2′-5′-oligoadenylate synthetase (2′-5′-OAS) and double-stranded RNA-specific adenosine deaminase (ADAR1), no significant differences in mRNA induction of 2′-5′-OAS or ADAR1 genes by IFN-α treatment were observed between 50-1 and 1αR cells, regardless of 5-azaC pre-treatment (data not shown). As regards option (5), it is likely that a combination of several ISGs is involved in the conversion to the IFN-sensitive phenotype of these cells, because the expression of LMP-2, LMP-7, or Viperin alone was associated with reductions in the level of HCV replicon RNA (Fig. 6(B)). Therefore, in order to clarify this issue, future studies involving the co-expression of these ISGs will also be necessary.

Among the genes selected in this study, LMP2 and LMP7 are of interest; these genes are known as catalytic subunits of immunoproteasome, which is induced by IFN-γ [24]. These genes have recently been identified as being suppressed in HCV replicon-harboring cells [18]. The down-regulation of these genes may contribute to persistent viral infection due to the acquisition of the IFN-resistant phenotype.

Viperin is a third gene of interest identified in this study. Viperin is induced by IFN-α/β, IFN-γ, as well as by human cytomegalovirus (HCMV) infection [25]. Stable expression of Viperin in fibroblasts inhibited HCMV infection and downregulated several HCMV proteins, although its molecular mechanism remains unclear at present [25]. Since, HCV replication complexes have been associated with inner cellular lipid membrane structures [26], viperin may contribute to the inhibition of HCV replication.

The present study using HCV replicon-harboring cell lines to investigate the mechanisms of IFN resistance may contribute to further diagnostic study of IFN sensitivity among patients with CH C. However, additional comparisons of ISG expression levels in IFN responders and non-responders with CH C will be required to understand the mechanisms underlying IFN resistance associated with HCV.

Acknowledgements

We would like to thank T. Nakamura, T. Maeta, and A. Morishita for their helpful assistance with the experiments. This work was supported by grants-in-aid for a third-term comprehensive 10-year strategy for cancer control, and for research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan, as well as by the program for the promotion of fundamental studies in Health Sciences of the Pharmaceutical and Medical Devices Agency (PMDA).

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JOURNAL OF VIROLOGY, June 2004, p. 6370–6380 Vol. 78, No. 12
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.12.6370–6380.2004
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Intramembrane Proteolysis and Endoplasmic Reticulum Retention of Hepatitis C Virus Core Protein

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Received 27 November 2003/Accepted 24 February 2004

Hepatitis C virus (HCV) core protein is suggested to localize to the endoplasmic reticulum (ER) through a C-terminal hydrophobic region that acts as a membrane anchor for core protein and as a signal sequence for E1 protein. The signal sequence of core protein is further processed by signal peptide peptidase (SPP). We examined the regions of core protein responsible for ER retention and processing by SPP. Analysis of the intracellular localization of deletion mutants of HCV core protein revealed that not only the C-terminal signal-anchor sequence but also an upstream hydrophobic region from amino acid 128 to 151 is required for ER retention of core protein. Precise mutation analyses indicated that replacement of Leu^{139}, Val^{140}, and Leu^{144} of core protein by Ala inhibited processing by SPP, but cleavage at the core-E1 junction by signal peptidase was maintained. Additionally, the processed E1 protein was translocated into the ER and glycosylated with high-mannose oligosaccharides. Core protein derived from the mutants was translocated into the nucleus in spite of the presence of the unprocessed C-terminal signal-anchor sequence. Although the direct association of core protein with a wild-type SPP was not observed, expression of a loss-of-function SPP mutant inhibited cleavage of the signal sequence by SPP and coimmunoprecipitation with unprocessed core protein. These results indicate that Leu^{139}, Val^{140}, and Leu^{144} in core protein play crucial roles in the ER retention and SPP cleavage of HCV core protein.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 19) and has been estimated to infect more than 170 million people throughout the world (15). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and finally to hepatocellular carcinoma (18, 42). HCV belongs to the genus *Hepacivirus* in the family *Flaviviridae* and possesses a viral genome consisting of a single, positive-strand RNA with a nucleotide length of about 9.4 kb (6, 48). The genome encodes a large precursor polyprotein of approximately 3,000 amino acids (6, 17). The polyprotein is processed co- and posttranslationally into at least 10 viral proteins by host and viral proteases (2, 6, 10, 45). The structural proteins of HCV are located in the N-terminal one-fourth of the polyprotein and are cleaved by host membrane proteases (10, 44). Comparison with other flaviviruses suggests that HCV core protein forms the nucleocapsid, which is surrounded by the envelope containing glycoproteins E1 and E2 (6, 48). Functional analyses suggest that HCV core protein has regulatory roles in host cellular functions. In tissue culture systems, HCV core protein regulates signaling pathways and modulates apoptosis (4, 29, 40, 41, 46, 54, 55). Moreover, transgenic mice expressing HCV core protein developed liver steatosis and thereafter hepatocellular carcinoma (34, 36). Thus, it has been suggested that HCV core protein is a multifunctional molecule that acts as a structural protein but is also involved in the pathogenesis of hepatitis C. HCV core protein has two major forms, p23 and p21 (16, 25, 31, 43, 53). HCV core protein p23 represents a 191-amino-acid product in which the C-terminal hydrophobic region also acts as a signal sequence for E1. HCV polyprotein is cleaved between residues 191 and 192 by host signal peptidase to generate C-terminal and N-terminal polypeptides encompassing the core and E1 proteins, respectively. For the full maturation of HCV core protein, the C-terminal signal-anchor sequence was thought to be further processed by an unidentified microsomal protease (25, 30, 31, 43, 53), and the 21-kDa isoform of core protein is predominantly detected both in cultured cells by transfection with expression plasmid and in viral particles obtained from sera of patients with hepatitis C (53). These results suggest that p21 is the mature form of HCV core protein (53). Immunostaining revealed that most HCV core protein is distributed diffusely throughout the cell, probably in the endoplasmic reticulum (ER) (31, 53). However, a minor population was observed in the nucleus (53).

Recently, a presenilin-related aspartic protease, signal peptide peptidase (SPP), was identified (50). SPP is located in the ER membrane and promotes intramembrane proteolysis of signal peptides. The chemical compound $(Z\text{-}LL)_2$-keton inhibits processing of signal peptides by SPP, and it was shown to suppress intramembrane proteolysis of major histocompatibility complex class I molecules, preprolactin, HCV core protein, and others (21, 30, 51). Replacement of Asp^{265} with Ala in SPP resulted in a loss of catalytic function, although this mutant could bind to TBL_4K, a derivative of $(Z\text{-}LL)_2$-keton (50). HLA-A was processed into yeast microsomes following the addition of wild-type SPP but not mutant SPP, suggesting that SPP interacts with HLA-A (50). Processing of the signal sequence of HCV core protein by SPP was inhibited by the

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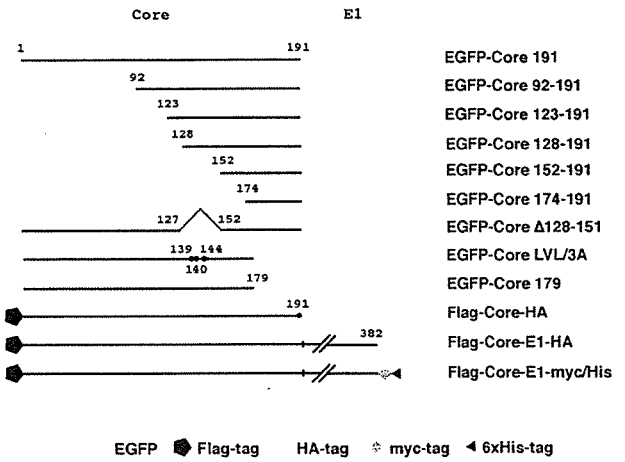

FIG. 1. Expression plasmids used in this study. The genes encoding HCV proteins and their mutants were cloned into pcDNA3.1FlagHA, pcDNA3.1/*myc*-His C, or pEGFP-C3 as described in Materials and Methods. Other plasmids are described in the text or in the other figure legends.

addition of $(Z\text{-}LL)_2$-keton, and Ser^{183} and Cys^{184} in the signal sequence of core protein were demonstrated to be important for flexibility and intramembrane proteolysis by SPP (23). Signal sequences generally have a tripartite structure, including a central hydrophobic H region and hydrophilic N- and C-terminal flanking regions (28). SPP recognizes the N- and C-terminal regions and cleaves in the middle of the H region (28). Mutational analyses suggested that the flexibility of signal peptides is generally required for substrate recognition of SPP (23). SPP contains the aspartic protease motifs YD and LGLGD, which are located in the predicted transmembrane region, and it is thought to cleave type II (N terminus in the cytosol and C terminus in the lumen)-oriented substrates (50). However, the effect of the cytoplasmic region of type II membrane substrates on intramembrane proteolysis by SPP is not known. In this study, we examined the regions of HCV core protein that are essential for ER retention and intramembrane cleavage by SPP.

MATERIALS AND METHODS

Plasmids. For expression of enhanced green fluorescence protein (EGFP)-fused HCV core proteins in culture cells, the core protein-coding region was amplified by PCR from cDNA encoding full-length HCV polyprotein type 1b (1). The PCR products were subcloned into SalI and BamHI sites 3′ of the EGFP-coding region of pEGFP-C3 (Clontech, Palo Alto, Calif.). The cDNA fragments encoding amino acids 1 to 191, 1 to 179, 92 to 191, 123 to 191, 128 to 191, 152 to 191, and 174 to 191 of HCV core proteins were amplified by PCR and then introduced into pEGFP-C3; these constructs are designated EGFP-Core 191, EGFP-Core 179, EGFP-Core 123-191, EGFP-Core 128-191, EGFP-Core 152-191, and EGFP-Core 174-191, respectively. The genes encoding core proteins with the region between amino acids 128 and 151 deleted and replacement of Leu^{139}, Val^{140}, and Leu^{144} with Ala were generated by the method of splicing by overlap extension (11, 14, 49) and introduced into pEGFP-C3; these constructs are designated EGFP-Core Δ128-151 and EGFP-Core LVL/3A, respectively (Fig. 1).

Fragments encoding Flag and hemagglutinin (HA) tags were inserted at both ends of the multicloning site of pcDNA3.1 (pcDNA3.1FlagHA). PCR products encoding either HCV core protein alone, core protein followed by E1 (Core-E1), or their mutants were cloned into pcDNA3.1FlagHA, resulting in plasmids encoding recombinant proteins sharing Flag and HA tags at the N and C termini, respectively (Fig. 1). In Flag-Core-HA and its derived mutants, Ala^{191} was replaced by Arg to avoid processing by signal peptidase for determination of cleavage by SPP, as previously shown for the processing of the E1-E2 junction (7). In addition, the region encoding Flag-Core-E1 or its mutants was cleaved from pcDNA3.1FlagHA constructs and then introduced between the SacI and XhoI sites of pcDNA3.1/*myc*-His C (Invitrogen Corp., Carlsbad, Calif.). The resulting plasmids encode HCV proteins sharing Flag and myc/His epitopes at the N and C termini, respectively (Fig. 1). Genes encoding core protein with a single amino acid (Leu^{139}, Val^{140}, or Leu^{144}), double amino acids (Leu^{139} and Val^{140}, Leu^{139} and Leu^{144}, or Val^{140} and Leu^{144}), or triple amino acids (Leu^{139}, Val^{140}, and Leu^{144}) replaced with Ala were generated by splicing by overlap extension and introduced into pcDNA3.1FlagHA and pcDNA3.1/*myc*-His C (Fig. 1; see Fig. 4).

The genes encoding the ER-targeting and ER retrieval sequences of calreticulin fused with DsRed at the N and C termini, respectively (8, 37, 39), were inserted between the EcoRV and XbaI sites of pcDNA3.1 (pcDNA ER-DsRed) to visualize the ER in culture cells. This recombinant protein is designated ER-DsRed in this study.

Cloning of SPP. The cDNA encoding SPP was amplified from human liver mRNA (Clontech) by reverse transcription-PCR and cloned into T-vector prepared from pBluescript II SK(−) (27). The gene encoding SPP with an attached HA tag and ER retrieval signal, KEKK, at the C terminus (SPP-HAER) was cloned into pcDNA3.1 to eliminate the possibility that the HA tag suppresses the endogenous ER retrieval signal of SPP. SPP-HAER was colocalized with ER-DsRed on the ER membrane and glycosylated upon transfection into cells (data not shown).

Subcellular localization of wild-type and mutant HCV core proteins. HeLa cells were maintained in the Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. HeLa cells were seeded on an eight-well chamber slide at 2×10^4 cells per well 24 h before transfection. The cells were transfected with the various plasmids by lipofection with Lipofectamine 2000 (Invitrogen). To determine protein subcellular localizations, transfected cells were fixed with phosphate-buffered saline (PBS) containing 3% paraformaldehyde at 18 h post-transfection and then observed with a confocal laser-scanning microscope (Bio-Rad, Tokyo, Japan). To confirm subcellular localization of the core proteins, transfected cells were fractionated with a subcellular proteome extraction kit (Calbiochem, Darmstadt, Germany). Stepwise extraction resulted in four distinct fractions, which contain mainly cytosolic, membrane-organelle, nuclear, and cytoskelton proteins, respectively. Each fraction was precipitated with trichloroacetic acid and analyzed by immunoblotting, and the densities of the bands were measured with Multi Gauge version 2.2 (Fujifilm, Tokyo, Japan).

Immunoblotting. After transfection, 293T cells were harvested, washed twice with PBS, and lysed in 20 mM Tris-HCl (pH 7.4) containing 135 mM NaCl, 1% Triton X-100, and 10% glycerol (lysis buffer) supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, and 1 mM Na_2VO_3. The lysate was centrifuged at 6,500 × *g* for 5 min at 4°C. The resulting supernatants were subjected to sodium dodecyl sulfate (SDS)–13.5% polyacrylamide gel electrophoresis. The separated proteins were electroblotted onto a Hybond-P polyvinylidene difluoride membrane (Amersham Bioscience, Piscataway, N.J.). These membranes were blocked with PBS containing 5% skim milk and 0.05% Tween 20 (Sigma, St. Louis, Mo.) and incubated with mouse monoclonal anti-Flag M2 (Sigma), anti-HA 16B12 (HA.11; BabCO, Richmond, Calif.), or monoclonal mouse anti-His_6-AD1.1.10 (Genzyme/Techne, Tokyo, Japan) immunoglobulin G (IgG) at room temperature for 30 min and then with horseradish peroxidase-conjugated anti-mouse IgG antibody at room temperature for 30 min. Immunoreactive bands were visualized by using the enhanced chemiluminescence Super Signal West Femto substrate (Pierce, Rockford, Ill.).

Immunoprecipitation. Immunoprecipitation analysis was carried out as described previously (32). Plasmids were transfected into 293T cells by lipofection. Transfected cells were harvested at 18 h posttransfection and lysed in lysis buffer with 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxypropanesulfonic acid (CHAPSO) (Dojindo, Kumamoto, Japan). Cell lysates were incubated with monoclonal anti-HA, anti-Glu-Glu (anti-EE) (BabCO), or anti-Flag antibody at 4°C for 1.5 h and then with protein G–Sepharose CL-4B (Amersham Bioscience) at 4°C for 1.5 h. After centrifugation at 6,500 × *g* for 3 min at 4°C, the pellets were washed five times with lysis buffer. Immunoprecipitates were subjected to immunoblotting.

Deglycosylation. Plasmids encoding core and E1 proteins were transfected into 293T cells by lipofection, and cell lysates were immunoprecipitated with anti-HA antibody at 18 h posttransfection. Immunoprecipitates were eluted from protein G–Sepharose CL-4B in 0.5% SDS and 1% 2-mercaptoethanol and digested with endo-β-*N*-acetylglucosaminidase H (Endo H) or peptide-*N*-glycosidase F

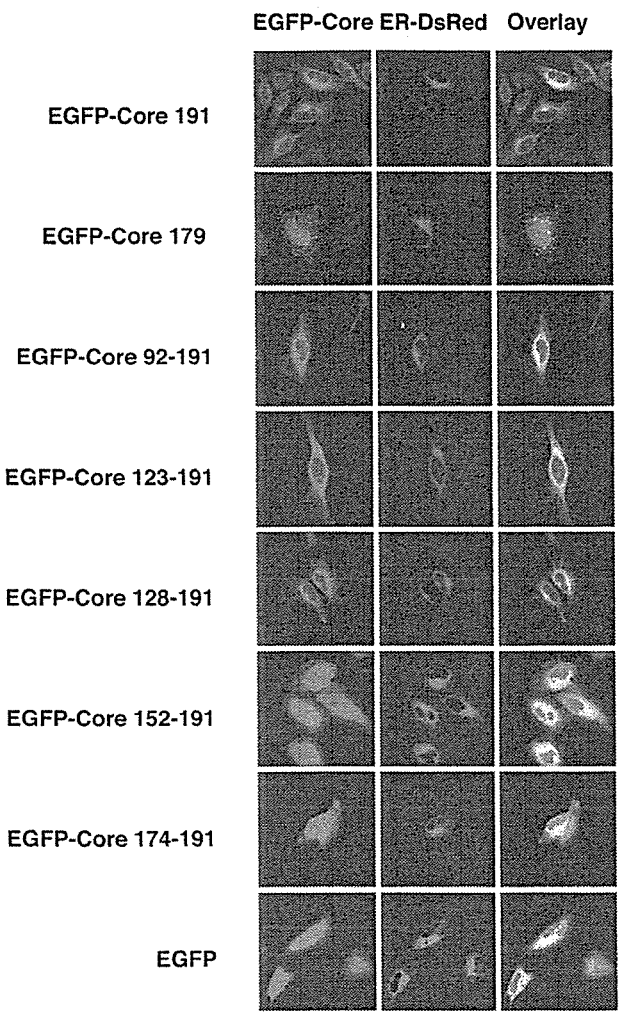

FIG. 2. Intracellular localization of EGFP-Core mutants. EGFP-Core and its deletion mutants were coexpressed with ER-DsRed in HeLa cells, and the localization of core proteins was examined by confocal microscopy.

(PNGase F) according to the protocol of the manufacturer (Roche, Mannheim, Germany). The resulting mixtures were subjected to immunoblotting.

RESULTS

Region required for ER retention of HCV core protein. To determine the regions within HCV core protein that are responsible for ER retention, EGFP-fused, N-terminally truncated HCV core protein (Fig. 1) was coexpressed with the ER marker ER-DsRed. EGFP-Core 191 colocalized with ER-DsRed to the ER (Fig. 2), whereas EGFP-Core 179 was localized primarily to the nucleus as reported previously (3, 33, 43, 47), suggesting that the C-terminal signal sequence is essential for anchoring HCV core protein to the ER membrane. However, EGFP-Core 174-191 exhibited diffuse staining similar to that of EGFP, suggesting that the signal sequence alone is not sufficient for ER localization. EGFP-Core 92-191, EGFP-Core 123-191, and EGFP-Core 128-191 were colocalized with ER-DsRed in the ER, but EGFP-Core 152-191 stained similarly to EGFP-Core 174-191 and EGFP. These data suggest that not only the C-terminal signal sequence but also the region from amino acids 128 to 151 is required for ER retention of HCV core protein.

Region essential for processing of the signal sequence of HCV core protein by SPP and signal peptidase. Based on hydrophobicity and a cluster of basic amino acids, HCV core protein was proposed to possess three regions (domains 1 to 3) (Fig. 3A, upper panel) by Hope and McLauchlan (12). To assess the involvement of the region encompassing amino acids 128 to 151 in proteolysis of the signal sequence of HCV core protein by signal peptidase and SPP, three hydrophobic amino acids, Leu^{139}, Val^{140}, and Leu^{144}, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, and Ala^{191} was replaced with Arg to eliminate processing by signal peptidase (Fig. 3A, lower panel). When a wild-type Flag-Core-HA construct was expressed in 293T cells, a single band of 23 kDa was detected by blotting with anti-Flag, but not with anti-HA, suggesting that the HA-fused signal sequence was properly processed by SPP and that Flag-core protein of 23 kDa was generated (Fig. 3B, lanes 2 and 11). In cells expressing the substitution mutants, 25- and 23-kDa bands were detected by the anti-Flag antibody (Fig. 3B, upper panel, lanes 3 to 9) and 25-kDa bands were detected by the anti-HA antibody (Fig. 3B, lower panel, lanes 3 to 9), indicating that the 25- and 23-kDa bands correspond to core proteins that are unprocessed and processed by SPP, respectively. Cleavability of the signal sequence of mutant core proteins by SPP was suppressed in accordance with the number of substitutions, and almost no processing of the signal sequence was observed in cells expressing Flag-Core LVL/3A-HA, which has three amino acid substitutions (Fig. 3B, lane 9). These results indicate that Leu^{139}, Val^{140}, and Leu^{144} play crucial roles in the processing of the signal-anchor of HCV core protein by SPP. Furthermore, deletion of the hydrophobic region including amino acids 128 to 151 from HCV core protein completely eliminated processing by SPP, and this species was seen only as a single band of 23.5 kDa which was detected by both the anti-Flag and anti-HA antibodies (Fig. 3B, lane 10). Taken together with the observation that Ala^{180}, Ser^{183}, and Cys^{184} in the signal sequence of HCV core protein of the type 1a Glasgow strain were demonstrated to be essential for SPP proteolysis (13, 23), these results indicate that the hydrophobic region from amino acid 139 to 144 in domain 2 of HCV core protein also participates in the processing of the signal sequence by SPP.

To examine the role of the region from amino acid 139 to 144 in the cleavage of the HCV core protein signal sequence by signal peptidase and SPP in more detail, substitutions of Leu^{139}, Val^{140}, and/or Leu^{144} with Ala were introduced into the Flag-Core-E1-HA polyprotein (Fig. 1). Flag-Core-E1-HA protein was cleaved to the expected molecular mass of 23 kDa of Flag-Core protein by signal peptidase and SPP (Fig. 3C, lanes 2 and 11), whereas slightly larger bands corresponding to a core protein unprocessed by SPP were detected in cells expressing polyproteins possessing mutations within amino acids 139 to 144 (Fig. 3C, lanes 3 to 9). A lack of processing by SPP was detected mainly in core proteins containing double amino acid changes of Leu^{139}, Val^{140}, and/or Leu^{144} to Ala

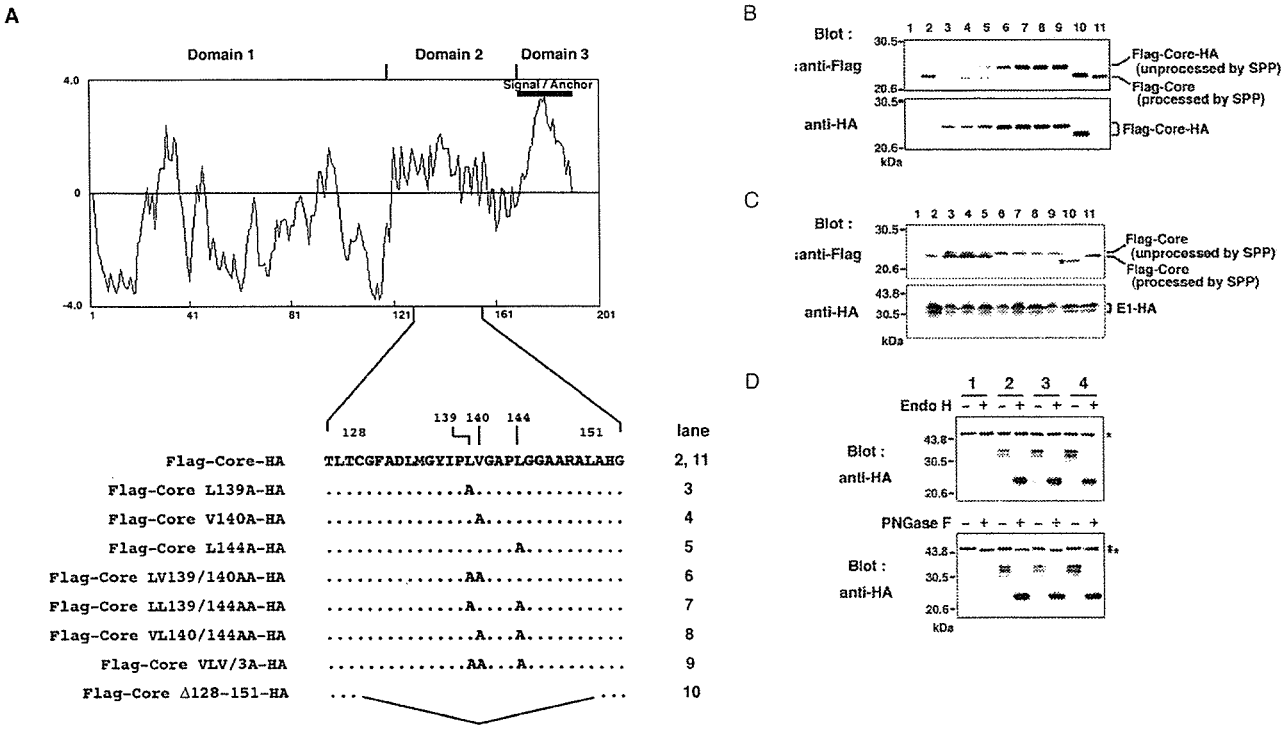

FIG. 3. Identification of the region responsible for processing of the signal sequence of HCV core protein by SPP and signal peptidase. (A) The hydrophobicity profile of HCV core protein was predicted by the method of Kyte and Doolittle (20). Hope and McLauchlan separated the HCV core protein into three regions, domains 1 to 3 (12). Two hydrophobic regions are predicted in the regions from amino acid 128 to 151 and from amino acid 164 to 186 in the C terminus of the HCV core protein. Mutations and deletions in the region from amino acid 128 to 151 of Flag-Core-HA and Flag-Core-E1-HA constructs are indicated. Dots indicate unchanged amino acids. (B) Expression of Flag-Core-HA polyproteins with changes of Ala^{191} to Arg in 293T cells. Flag-Core-HA (lanes 2 and 11), Flag-Core L139A-HA (lane 3), Flag-Core V140A-HA (lane 4), Flag-Core L144A-HA (lane 5), Flag-Core LV139/140AA-HA (lane 6), Flag-Core LL139/144AA-HA (lane 7), Flag-Core VL140/144AA-HA (lane 8), Flag-Core LVL/3A-HA (lane 9), and Flag-Core Δ128-151-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. Cells transfected with an empty plasmid were used as a negative control (lane 1). (C) Expression of Flag-Core-E1-HA mutants in 293T cells. Flag-Core-E1-HA (lanes 2 and 11), Flag-Core L139A-E1-HA (lane 3), Flag-Core V140A-E1-HA (lane 4), Flag-Core L144A-E1-HA (lane 5), Flag-Core LV139/140AA-E1-HA (lane 6), Flag-Core LL139/144AA-E1-HA (lane 7), Flag-Core VL140/144AA-E1-HA (lane 8), Flag-Core LVL/3A-E1-HA (lane 9), and Flag-Core Δ128-151-E1-HA (lane 10) were analyzed by immunoblotting with anti-Flag (upper panel) or anti-HA (lower panel) antibody. The asterisk indicates unprocessed Flag-Core Δ128-151. Cells transfected with an empty plasmid were used as a negative control (lane 1). (D) The deglycosylation procedure is described in Materials and Methods. After transfection, cell lysates were immunoprecipitated with anti-HA antibody and immunoprecipitates were digested with Endo H (upper panel) or PNGase F (lower panel). Following digestion, proteins were separated by SDS-polyacrylamide gel electrophoresis, and material from cells transfected with vector (lane 1), Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), and Flag-Core Δ128-151-E1-HA (lane 4) was detected by blotting with anti-HA. Nontreated and Endo H- or PNGase F-treated samples are indicated by − and +, respectively. Asterisks indicate mouse IgG heavy chains.

(Fig. 3C, lanes 6 to 8), and only an unprocessed band was detected in a triple amino acid substitution mutant (Fig. 3C, lane 9) and a deletion mutant lacking amino acids 128 to 151 (Fig. 3C, lane 10). In contrast to the processing of core protein, E1 protein processed from the mutant polyproteins exhibited the same molecular mass of 32 to 35 kDa and the same deglycosylation patterns following digestion with Endo H or PNGase F (Fig. 3D). These results indicate that the internal hydrophobic region from amino acid 139 to 144 of HCV core protein is essential for processing by SPP but not for cleavage of the core-E1 junction by signal peptidase and the subsequent translocation of E1 protein into the ER. It was suggested that signal peptides must be liberated from the precursor protein by cleavage with signal peptidase in order for them to become substrates for SPP (23). Our data indicate that processing by SPP is not a prerequisite for cleavage of the core-E1 junction by signal peptidase.

Amino acid sequence essential for SPP cleavage of the signal sequences of HCV core proteins of genotypes 1a and 1b. Martoglio and colleagues reported that HCV core protein is processed by SPP after cleavage by host signal peptidase and that Ala^{180}, Ser^{183}, and Cys^{184} residues in the signal sequence of HCV core protein of type 1a Glasgow strain are essential for SPP proteolysis, as they maintain the structure of the breaking α-helix (23, 30). To determine the amino acids essential for SPP cleavage of the signal sequence of type 1b HCV core protein, Flag-Core-E1-HA and its substitution mutants were expressed in 293T cells (Fig. 4). Mutation of one, two, or three amino acids, except for Flag-Core IF176/177AL-E1-HA (Fig. 4B, lane 9), did not affect the processing of the core protein signal sequence. Flag-Core IF176/177AL-E1-HA exhibited the same molecular size as Flag-Core LVL/3A-E1-HA (Fig. 4B, lane 2), suggesting that Ile^{176} and Phe^{177} in the signal sequence of core protein are essential for cleavage by SPP in our system.

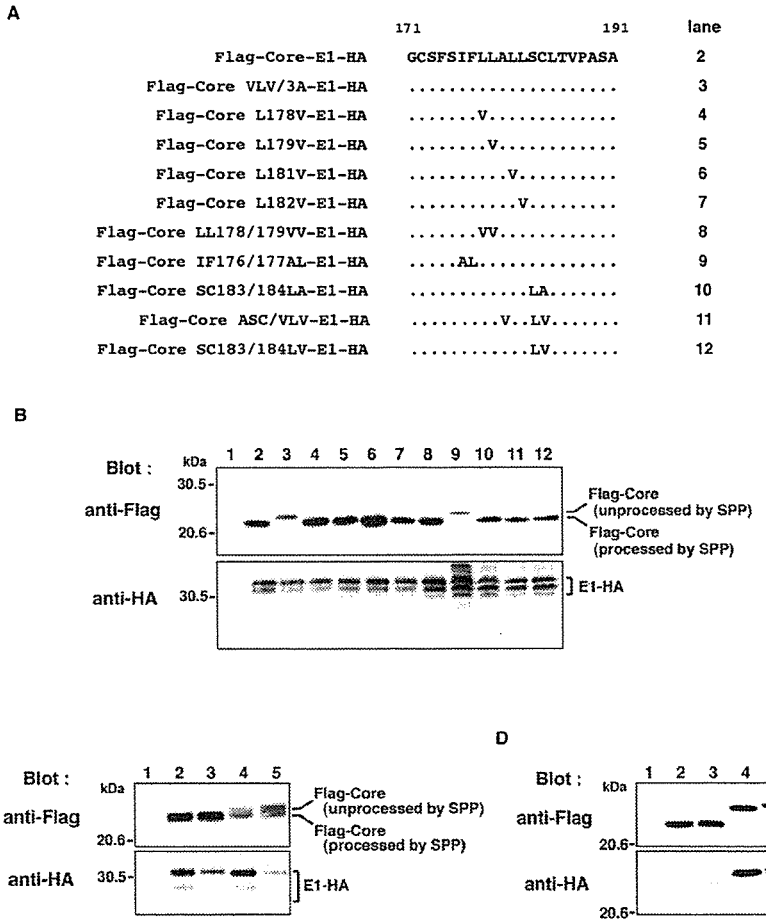

FIG. 4. Amino acid residues essential for SPP cleavage of the HCV core protein signal sequence of genotype 1a and 1b strains. (A) Mutations in the amino acid residues in the signal sequence of Flag-Core-E1-HA are indicated. Dots indicate unchanged amino acids. (B) Flag-Core-E1-HA (lane 2), Flag-Core LVL/3A-E1-HA (lane 3), Flag-Core L178V-E1-HA (lane 4), Flag-Core L179V-E1-HA (lane 5), Flag-Core L181V-E1-HA (lane 6), Flag-Core L182V-E1-HA (lane 7), Flag-Core LL178/179VV-E1-HA (lane 8), Flag-Core IF176/177AL-E1-HA (lane 9), Flag-Core SC183/184LA-E1-HA (lane 10), Flag-Core ASC/VLV-E1-HA (lane 11), or Flag-Core SC183/184LV-E1-HA (lane 12) was expressed in 293T cells. (C) The gene encoding core and E1 polyprotein of the genotype 1a H77c strain of HCV was introduced into pcDNA3.1FlagHA. Flag-H77c Core-E1-HA (lane 2), Flag-H77c Core ASC/VLV-E1-HA (lane 3), Flag-H77c Core LVL/3A-E1-HA (lane 4), or Flag-H77c Core IF176/177AL-E1-HA (lane 5) was expressed in BHK cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. (D) Expression of Flag-Core 191-HA mutants in 293T cells. The gene encoding core protein with a change of Ala^{191} to Arg was introduced into pcDNA3.1FlagHA. Flag-Core-HA (lane 2), Flag-Core ASC/VLV-HA (lane 3), Flag-Core LVL/3A-HA (lane 4), and Flag-Core IF176/177AL-HA (lane 5) were analyzed by immunoblotting with anti-Flag (upper panel) and anti-HA (lower panel) antibodies. Cells transfected with an empty plasmid were used as a negative control (lanes 1 in panels B, C, and D).

However, the triple amino acid substitution (Ala^{180}, Ser^{183}, and Cys^{184}) in the type 1b J1strain (Flag-Core ASC/LVL-E1-HA) (Fig. 4B, lane 11), which is the same as the spmt mutant of the type 1a Glasgow strain (23, 30), did not affect the processing of the signal sequence of HCV core protein by SPP. All derived E1 proteins exhibited a molecular mass of 32 to 35 kDa irrespective of the presence of mutations, and deglycosylation by digestion with endoglycosidases generated uniform 22-kDa bands of E1 proteins (data not shown). These results indicate that Ile^{176} and Phe^{177}, but not Ala^{180}, Ser^{183}, and Cys^{184}, in the signal sequence of type 1b HCV core protein are essential for processing by SPP and confirm that processing of signal sequence by SPP is not required for cleavage by signal peptidase and translocation of E1 protein into the ER. To determine whether the difference in cleavage of signal sequence depends on the genotype of HCV, Ala^{180}, Ser^{183}, and Cys^{184} in the HCV core protein of the genotype 1a H77c strain were replaced with Val, Leu, and Val, respectively. The spmt construct of the type 1a H77c strain did not affect the processing of core and E1 proteins in BHK cells (Fig. 4C, lane 3) and 293T cells (data not shown). In contrast, replacement of Leu^{139}, Val^{140}, and Leu^{144} by Ala and of Ile^{176} and Phe^{177} by Ala and Leu suppressed the processing of the core protein signal sequence of the type 1a H77c strain in BHK cells (Fig. 4C, lanes 4 and 5). These results indicate that three hydrophobic amino acids Leu^{139}, Val^{140}, and Leu^{144} in the hydrophobic peak in domain 2 and the two amino acids Ile^{176} and Phe^{177} in the transmembrane domain play important roles in the in-

tramembrane proteolysis of HCV core protein signal sequence of genotypes 1a and 1b by SPP.

To further examine the cleavage of the signal sequence of HCV core proteins by SPP, we prepared IF176/177AL and the spmt mutant core proteins carrying a substitution of Ala^{191} to Arg to avoid processing by signal peptidase as described above. In cells expressing a wild-type or LVL/3A mutant core protein, a 23-kDa processed or a 25-kDa unprocessed core protein was detected, as seen in Fig. 3B (Fig. 4D, lanes 2 and 4). The IF176/177AL mutant exhibited a 26-kDa unprocessed band which was detected by anti-HA antibody (Fig. 4D, lane 5). In contrast, the spmt core protein exhibited a major band at 23 kDa and a faint 24-kDa band after blotting with the anti-Flag antibody (Fig. 4D, lane 3). Detection of a small amount of the 24-kDa unprocessed band by the anti-HA antibody indicates that most of the spmt mutant core protein was processed by SPP. The unprocessed core proteins of spmt, LVL/3A and IF176/177AL exhibited different electrophoretic mobilities, estimated to be 24, 25, and 26 kDa, respectively (Fig. 4D, lower panel, lanes 3 to 5). Lemberg and Martoglio pointed out that the mobility of a protein does not necessarily correlate with its molecular mass when analyzed in a Tris-glycine gel system due to the unexpected electrophoretic mobility of the proteins (22). However, detection of HA-tagged unprocessed signal sequence in the core mutants clearly demonstrated that LVL/3A and IF176/177AL mutants substituted with Leu^{139}, Val^{140}, and Leu^{144} in domain 2 and with Ile^{176} and Phe^{177} in the transmembrane domain, respectively, have lost the ability to be cleaved by SPP.

Effect of a loss-of-function mutant of SPP on the processing of the signal sequence of HCV core protein. Although there are two reports suggesting that SPP is involved in the processing of the signal sequence of HCV core protein by using the SPP inhibitor $(Z\text{-}LL)_2$-keton (23, 30), a direct interaction of HCV core protein with SPP has not been demonstrated. To determine the direct involvement of SPP in the processing of HCV core protein signal sequence, the C-terminal HA tag in the Flag-Core-E1-HA constructs used in the experiments described above was replaced with a myc/His tag and coexpressed with wild-type SPP (SPP-HAER) or with a mutant SPP with amino acid substitutions in the putative protease active sites, i.e., Asp^{219} (SPP D219A-HAER) or Asp^{265} (SPP D265A-HAER) to Ala. The signal sequence of HCV core protein was processed in cells coexpressing Flag-Core-E1-myc/His and SPP-HAER (Fig. 5, anti-Flag, lane 3), whereas two bands corresponding to processed and unprocessed (the same size as Flag-Core LVL/3A-E1-myc/His [lane 6]) core proteins were detected in cells coexpressing Flag-Core-E1-myc/His and the mutant SPP constructs (Fig. 5, anti-Flag, lanes 4 and 5). Proper cleavage and glycosylation of E1 proteins in cells coexpressing Flag-Core-E1-myc/His and the SPP mutants (Fig. 5, anti-His, lanes 4 and 5) and those expressing Flag-Core LVL/3A-E1-myc/His (Fig. 5, anti-His, lane 6) indicates that processing of signal sequence by SPP is not required for the cleavage of the core-E1 junction by signal peptidase and translocation of E1 protein into the ER. These results indicate that loss-of-function mutants of SPP inhibit the intramembrane proteolysis of HCV core protein signal sequence and further confirm that the slightly larger bands detected in cells expressing Flag-Core LVL/3A-E1-HA or Flag-Core IF176/177AL-E1-HA are immature core proteins unprocessed by SPP (Fig. 4B, lanes 3 and 9).

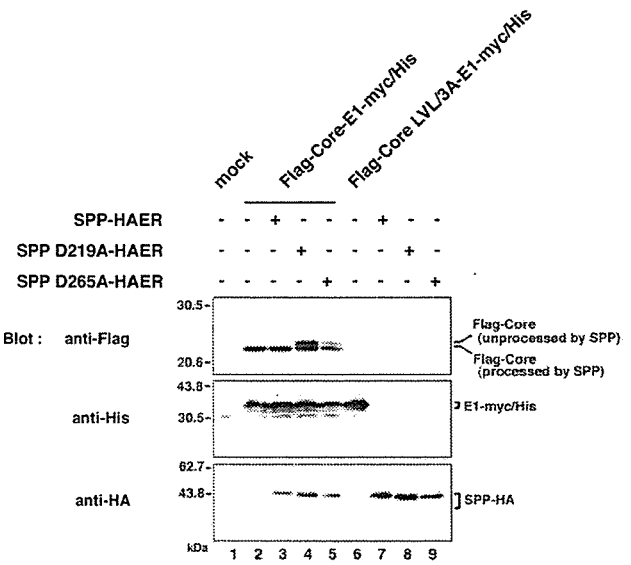

FIG. 5. Effect of loss-of-function mutants of SPP on the processing of the signal sequence of HCV core protein. SPP-HAER, SPPD219A-HAER, or SPPD265A-HAER was coexpressed with Flag-Core-E1-myc/His or Flag-Core LVL/3A-E1-myc/His in 293T cells. Cell lysates were analyzed by immunoblotting with anti-Flag (upper panel), anti-His_6 (middle panel), or anti-HA (lower panel) antibody. + and −, presence or absence of each plasmid, respectively. Lane 1, mock; lanes 2, 6, 7, 8, and 9, single expression of Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, SPP-HAER, SPPD219A-HAER, and SPPD265A-HAER, respectively; lanes 3 to 5, coexpression of Flag-Core-E1-myc/His with SPP-HAER, SPPD219A-HAER, and SPPD265A-HAER, respectively.

Interaction of HCV core protein with SPP. To examine the specific interaction of HCV core protein with SPP, Flag-Core-E1-myc/His, Flag-Core LVL/3A-E1-myc/His, or Flag-Core IF176/177AL-E1-myc/His was coexpressed with SPP-HAER or SPP D219A-HAER in 293T cells and immunoprecipitated with anti-Flag or anti-HA antibody. In cells coexpressing the loss-of-function mutant, SPP D219A-HAER, and one of the three HCV polyprotein substrates, nonspecific bands were detected by immunoblotting with the anti-HA and anti-Flag antibodies in the immunoprecipitates (Fig. 6A, upper and second panels, lanes 3 to 5). Therefore, lysates immunoprecipitated with anti-Flag and anti-HA antibodies were evaluated by comparison with those precipitated with anti-EE. Three bands corresponding to SPP D219A-HAER were coimmunoprecipitated with core proteins by anti-Flag immunoprecipitation (Fig. 6A, upper panel, lanes 8 to 10). SPP has two glycosylation sites (50), and therefore the upper, middle, and lower bands seem to correspond to SPP possessing two glycans, one glycan, and no glycan, respectively. Deglycosylation by PNGase F treatment reduced the molecular sizes of all bands to that of the lowest band (data not shown). Only unprocessed core protein was coimmunoprecipitated with SPP D219A-HAER by anti-HA (Fig. 6A, second panel, lanes 8 to 10). Coexpression of Flag-Core LVL/3A-E1-myc/His or Flag-Core-IF176/177AL-E1-myc/His reduced the expression of SPP D219A-HAER (Fig. 6A, third panel, lanes 4 and 5), suggesting that the