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Microbes and Infection 15 (2013) 45—55
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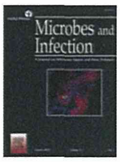

Original article

Selective estrogen receptor modulators inhibit hepatitis C virus infection at multiple steps of the virus life cycle

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Received 15 June 2012; accepted 13 October 2012

Abstract

We screened for hepatitis C virus (HCV) inhibitors using the JFH-1 viral culture system and found that selective estrogen receptor modulators (SERMs), such as tamoxifen, clomifene, raloxifene, and other estrogen receptor α (ERα) antagonists, inhibited HCV infection. Treatment with SERMs for the first 2 h and treatment 2—24 h after viral inoculation reduced the production of HCV RNA. Treating persistently JFH-1 infected cells with SERMs resulted in a preferential inhibition of extracellular HCV RNA compared to intracellular HCV RNA. When we treated two subgenomic replicon cells, which harbor HCV genome genotype 2a (JFH-1) or genotype 1b, SERMs reduced HCV genome copies and viral protein NS5A. SERMs inhibited the entry of HCV pseudo-particle (HCVpp) genotypes 1a, 1b, 2a, 2b and 4 but did not inhibit vesicular stomatitis virus (VSV) entry. Further experiment using HCVpp indicated that tamoxifen affected both viral binding to cell and post-binding events including endocytosis. Taken together, SERMs seemed to target multiple steps of HCV viral life cycle: attachment, entry, replication, and post replication events. SERMs may be potential candidates for the treatment of HCV infection.

Keywords: HCV; Tamoxifen; SERM (Selective estrogen receptor modulator)

1. Introduction

Over 170 million people in the world are infected with the hepatitis C virus (HCV). Approximately 20% of infected patients develop cirrhosis and hepatocellular carcinoma after chronic HCV infection. No HCV vaccine is available yet, and the current standard of care, which consists of a combination of interferon (IFN) and ribavirin, is only effective for approximately 50% of infected patients, and many patients have serious side effects. Because of the urgent need for novel HCV therapeutics, research is being conducted to develop new anti-HCV drugs. In addition to *in vitro* screening assays that target HCV-specific enzymes, other approaches that use replicon cells and the recently described Huh 7.5.1-JFH-1 (genotype 2a)-infection system have been developed [1]. The Huh 7.5.1-JFH-1-infection system is an excellent system to identify HCV inhibitors that interfere with individual steps of the HCV life cycle, such as viral attachment, entry, and release. This experimental system allows both viral and host components that are involved in HCV infection to be targeted. Although drugs that target the host components may be toxic, such drugs are unlikely to select for resistant viruses.

We screened chemicals using a cell-based screening system [2] and found that tamoxifen and other selective estrogen receptor modulators (SERMs) inhibited HCV infection. Tamoxifen has been successfully used for the treatment of breast cancer since it was found to be an ER antagonist over 30 years ago. Clomifene and raloxifene, which are compounds

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1286-4579/$ - see front matter
http://dx.doi.org/10.1016/j.micinf.2012.10.003

that are related to tamoxifen, have been developed and used for the treatment of breast cancer and for the treatment of anovulation and osteoporosis. Currently, these three SERMs and toremifene have been approved in Japan and the US, and next-generation SERMs are undergoing clinical evaluation.

Because tamoxifen exhibited the ability to inhibit HCV infection, we determined which SERMs could effectively inhibit HCV infection and be approved for clinical use. The first-generation SERMs—tamoxifen, clomifene, and raloxifene—were all effective against HCV as were other ERα antagonists. We examined whether SERMs could be utilized as new drugs for the treatment of HCV.

2. Materials and methods

2.1. Cells and virus

Human hepatoma cell line, Huh 7.5.1 cells and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co. St. Louis, MO, USA) with 10% fetal bovine serum (FBS). HCV-JFH-1 (HCVcc) (genotype 2a) was the culture supernatant of infected Huh 7.5.1 cells as described previously [2]. A subgenomic replicon cell line, clone #4-1, which harbors the genotype 2a (JFH-1) [3,4], and clone #5-15, that harbors the genotype 1b HCV genome [5], were also cultured in DMEM with FBS.

2.2. Chemicals

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology of Japan. Tamoxifen, diethylstilbestrol, triphenylethylene, 17β-estradiol, and brefeldin A were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Clomifene was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), and hydroxytamoxifen ((*z*)-4-hydroxytamoxifen) and raloxifene were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Chloroquine was purchased from WAKO (Osaka, Japan). Other chemicals were purchased from Tocris Bioscience (Bristol, UK).

2.3. Quantification of the viral titer in medium

Huh 7.5.1 cells were seeded in 96-well plates at a density of 2×10^4 cells per well in a volume of 120 μl. The next day, 15 μl of media that contained the test compound and 15 μl of the HCVcc virus stock solution at a moi of 0.01 were added to each well. After 5 days, 100 μl of the culture supernatant was taken from each well, and viral RNA was extracted. Total RNA was also extracted from the cells. Quantitative real-time RT-PCR was then performed with One step SYBR PrimeScript RT-PCR Kit (Takara-Bio Co., Otsu, Japan) as described previously [2]. In the case of #4-1 replicon cell, as an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured with primers 5′-CCACCCATGGCAAATTCC-3′ and 5′-TGGGATTTCCATTGAT-3′. Cell growth was monitored using the MTT assay as described previously [6].

2.4. Western blotting

Western blotting was performed as previously described [2]. Briefly, cell lysates that contained equal quantities of protein were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies against the core antigen (2H9), NS5A (Austral Biologicals, San Raman, CA, USA), or GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, the protein bands on the PVDF membranes were detected using an ECL system (GE Healthcare UK Ltd., Amersham Place, UK).

2.5. Production of and infection with pseudo-particles

HCV pseudo-particles (HCVpp) were generated using the following 3 plasmids: a Gag-Pol packaging construct (Gag-Pol 5349), a transfer vector construct (Luc 126), and a glycoprotein-expressing construct (HCV E1E2) (JFH-1, 2a). The generation of the pseudo-particles was performed according to the method described by Bartosch et al. [7]. To express the glycoproteins of other HCV genotypes, HCV E1E2 constructs of the genotypes 1a (H77), 1b (UKN1B 12.6), 2b (UKN2B 2.8), and 4 (UKN4 11.1) were generously provided by Dr. F. Cosset (INSERM, France) [8]. To produce VSVpp, a plasmid that coded the vesicular stomatitis virus (VSV) envelope, pCAG-VSV, was generously provided by Dr. Y. Matsuura (Osaka University, Japan). Gag-Pol 5349 (3.1 μg), Luc 126 (3.1 μg), and each of the individual glycoprotein-expression constructs (1.0 μg) were co-transfected into 293T cells that were seeded on a 10-cm dish (2.5×10^6 cells) using TransIT-LT1 Transfection Reagent (21.6 μl) (Mirus Bio LLC, Madison, WI, USA). The medium from the transfected cell cultures was harvested and used as the pseudo-particle stock. For the infection assay, Huh 7.5.1 cells were seeded onto a 48-well plate at a density of 4×10^4 cells per well one day prior to infection. The medium was then removed, and the cells were subsequently infected with the pseudo-particles in the presence or absence of drug. The cells were then incubated for 3 h. The VSVpp preparation was diluted (1:600) to infect with similar RLU activity compared to the HCVpp. The supernatant was then removed, fresh culture medium was added to the cells, and the cells were incubated for an additional 3 days. The luciferase assays were performed using a luciferase assay system (Promega Co. Madison WI, USA). Anti-CD81 antibody (sc-23962) was purchased from Santa Cruz Biotech.

3. Results

3.1. Tamoxifen and estrogen receptor α antagonists inhibited HCV infection

Using quantitative RT-PCR, we screened the compounds in the SCADS inhibitor kit I. Drugs and HCVcc at a moi of 0.01

were added to Huh 7.5.1 cells. Five days later, the quantity of HCV RNA in the culture supernatant was measured using quantitative real-time RT-PCR [2]. We found that tamoxifen reduced the levels of JFH-1 RNA in the culture supernatant. We also examined the effects of other SERMs and agonists and antagonists of ERα. As shown in Fig. 1, tamoxifen, clomifene, and hydroxytamoxifen, which have a triphenylethylene backbone, exhibited intense inhibitory effects (EC_{50}: approximately 0.1 μM). Triphenylethylene showed reduced inhibitory activity (data not shown). Raloxifene also inhibited viral RNA production at a similar concentration. (EC_{50}: approximately 0.1 μM) (Fig. 1a). Tamoxifen and raloxifene display both ERα antagonist and agonist properties in a dose- and tissue-dependent manner [9]. In contrast, ICI 182,780 (fulvestrant), ZK164015, and MPP (methyl-piperidino-pyrazole) are exclusively antagonistic [10–12]. These ERα antagonists also showed inhibitory activity against JFH-1, but their EC_{50} values were approximately 1 μM (Fig. 1b). As the 50% toxic concentrations (TC_{50}) for these compounds were observed to be greater than 10 μM (Fig. 1a and b), these specific indexes are over 100. In contrast, the ERα agonists 17β-estradiol, diethylstilbestrol, and PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrozole) did not inhibit HCV (Fig. 1c). As expected, the SERMs that were observed to effectively inhibit HCV RNA production also reduced the core protein levels intracellularly (Fig. 1d).

3.2. SERMs inhibited more than one step of the JFH-1 life cycle

To determine which step of the JFH-1 life cycle was inhibited by the SERMs studied, we performed time-of-addition experiments. As described previously [2], JFH-1 appears to complete one infectious life cycle in approximately 48 h. Huh 7.5.1 cells were inoculated with JFH-1-containing medium (moi 0.1) with or without drug and were then incubated for 2 h. After the medium was removed, fresh medium with or without drug was added. The cells were then incubated for another 46 h. Treatment with 10 μM tamoxifen for 48 h reduced the amount of viral RNA in the medium to 1.7% of levels observed in the control. Treatment with tamoxifen for the first 2 h after infection (0–2 h) reduced viral RNA to 2.3% of the levels observed in the control. The addition of tamoxifen to the fresh medium just after the removal of the virus (2–48 h) resulted in a reduction in the amount of viral RNA to 10.7% of the levels observed in the control. The addition of tamoxifen 24 h after viral inoculation (24–48 h) resulted in a decrease in the amount of viral RNA to 60% of the levels observed in the control (Fig. 2a). This result suggests that tamoxifen inhibits mainly viral entry and some steps during replication. 10 μM of raloxifene exhibited a similar inhibitory pattern but less inhibited by the treatment after the entry step (Fig. 2b). A pure ERα antagonist, ICI 182,780 (30 μM), also exhibited inhibition of both viral entry and the replication steps, but the inhibition of the entry step was not so marked (Fig. 2c).

To further investigate effect on HCV post replication, we infected HCV in the presence of the drugs for 72 h (moi 0.1) and examined their effects on intracellular and extracellular HCV RNA levels. Brefeldin A, an inhibitor of protein transport [13], was used as a positive control of post replication inhibition. In this experimental setting, brefeldin A showed intracellular HCV RNA accumulation suggesting post replication inhibition (Fig. 2d). SERMs generally reduced HCV RNA in cell as well as HCV RNA in medium, although the extent of reduction was different (Fig. 2d). Lower concentration of SERMs reduced extracellular HCV RNA more robustly than intracellular HCV RNA. At a concentration of 0.1 μM, tamoxifen exclusively inhibited HCV RNA in the culture supernatant but not intracellular HCV RNA levels, in a manner similar to that of brefeldin A (Fig. 2d). The results suggest that SERMs inhibit post replication step(s) such as assembly or release. Because low concentrations of tamoxifen failed to inhibit intracellular HCV RNA, SERMs potentially target post replication step(s) more efficiently than replication step. In this condition, higher concentrations (1 and 3 μM) of tamoxifen seemed to inhibit intracellular HCV RNA rather than extracellular HCV RNA, although the reason is not clear.

To determine the effect of these drugs on chronic infection, we used pre-infected Huh 7.5.1 cells. We infected the cell with HCVcc at a moi of 0.01 and incubated for 3 days. Three days after infection, the drugs were added, and the cells were further incubated for 48 h. At the time of drug addition, the cells were persistently infected, and HCVcc was continuously produced and released into the culture supernatant, which is similar condition to chronic infection. HCV RNA was extracted from the culture supernatant and the cells after 48 h and measured copy number of HCV RNA. Both HCV RNA in the culture supernatant and that in the cell were reduced by treatment with the SERMs, but the intracellular HCV RNA levels were less reduced (Fig. 2e). This suggested that the SERMs caused preferential reduction in extracellular HCV RNA through interference with some post replication step(s), such as assembly or release. Brefeldin A accumulated intracellular HCV RNA, and reduced HCV RNA level in the culture supernatant (Fig. 2e).

These data suggested that the SERMs inhibit multiple steps in the HCV life cycle: entry, viral RNA replication and some post replication step(s).

3.3. SERMs inhibited copies and NS5A protein expression in replicon cells

To confirm the effect of these drugs on viral replication, we used two subgenomic replicon cells. The subgenomic replicon cells, derived from Huh7 cells, harbor HCV viral RNA that replicates autonomously, and they express viral proteins. We treated cells that harbored a subgenomic replicon (#4-1, genotype 2a) [3,4] with the SERMs for 48 h and measured the amount of cellular replicon RNA by quantitative RT-PCR. Treatment with 10 μM of tamoxifen, raloxifene, or 3 μM of clomifene, inhibited HCV RNA compare to GAPDH RNA, although statistical significance was shown in only the inhibition of 10 μM of tamoxifen. ICI 182,780 did not show specific inhibition of HCV RNA (Fig. 3a).

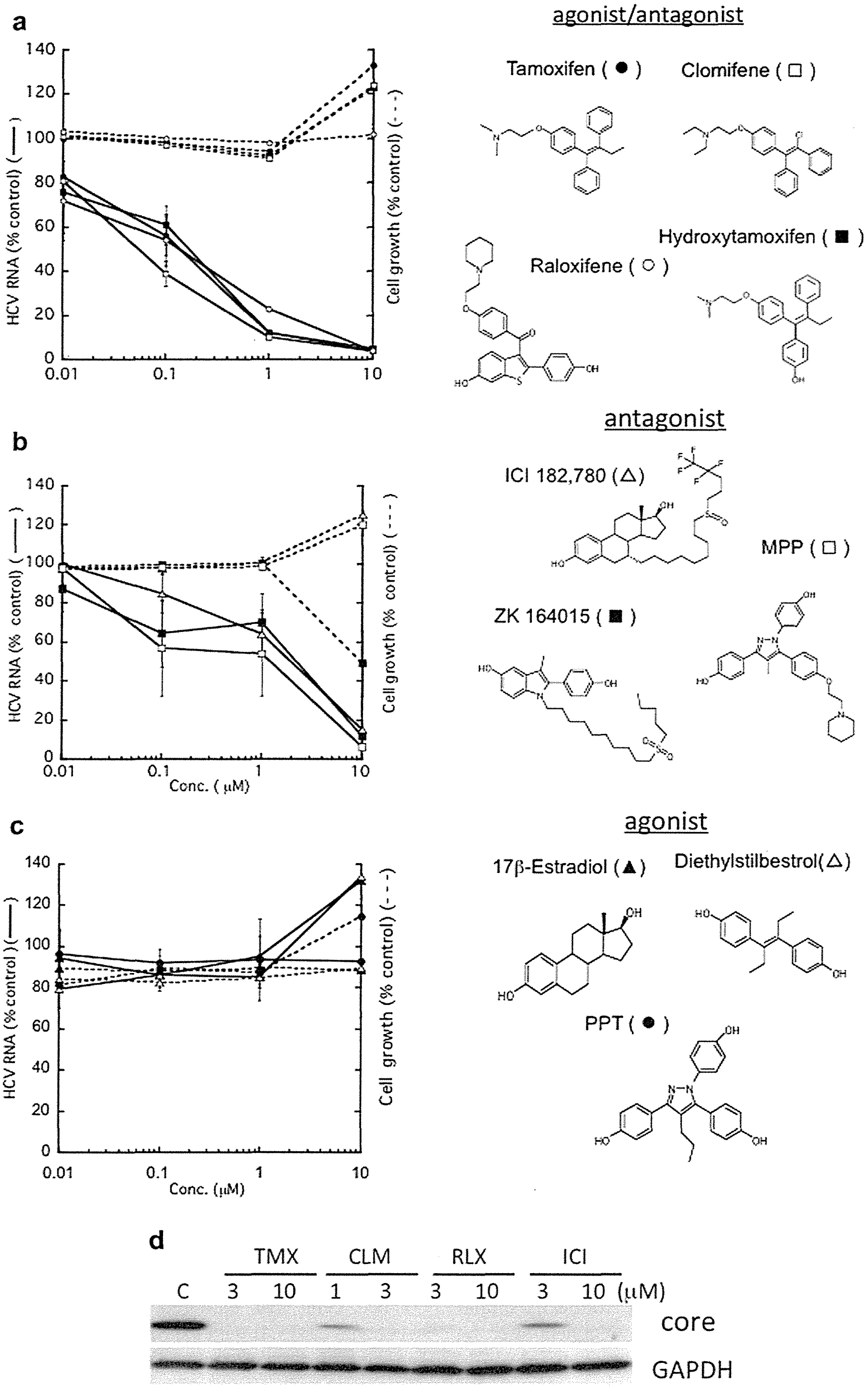

Fig. 1. Effects of SERMs on JFH-1 HCV RNA levels. a) Effects of tamoxifen, clomifene, and raloxifene. Huh 7.5.1 cells were infected with HCV JFH-1 (moi 0.01) in the presence of drugs and were incubated for 5 days. Drugs were added just before viral inoculation. HCV RNA in the medium was measured by tube-capture-RT-PCR [2]. Parallel cultures of cells without virus were analyzed using the MTT assay to detect the inhibition of cell growth due to drug exposure. Tamoxifen (closed circles), clomifene (open rectangles), hydroxytamoxifen (closed rectangles), and raloxifene (open circles). The percentages to control HCV RNA and

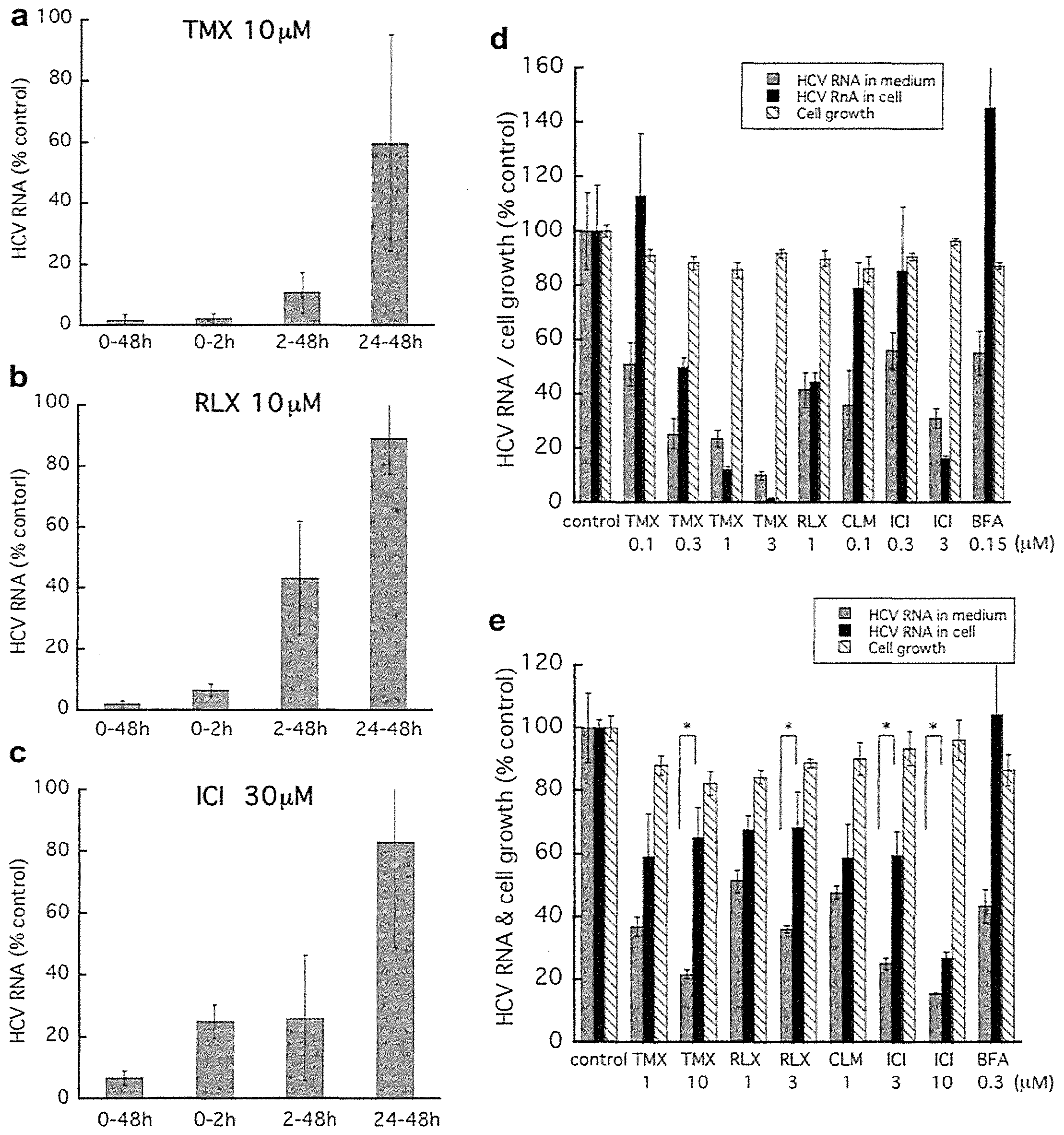

Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μM, a), raloxifene (RLX) (10 μM, b), or ICI 182,780 (ICI) (30 μM, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. *P-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

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control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ERα antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ERα agonists: 17β-estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10 μM of clomifene. Clomifene at concentrations less than 10 μM and tamoxifen, raloxifene and ICI 182,780 at 10 μM concentration or lower did not inhibit cell growth (Fig. 3c).

3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10 μM, ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

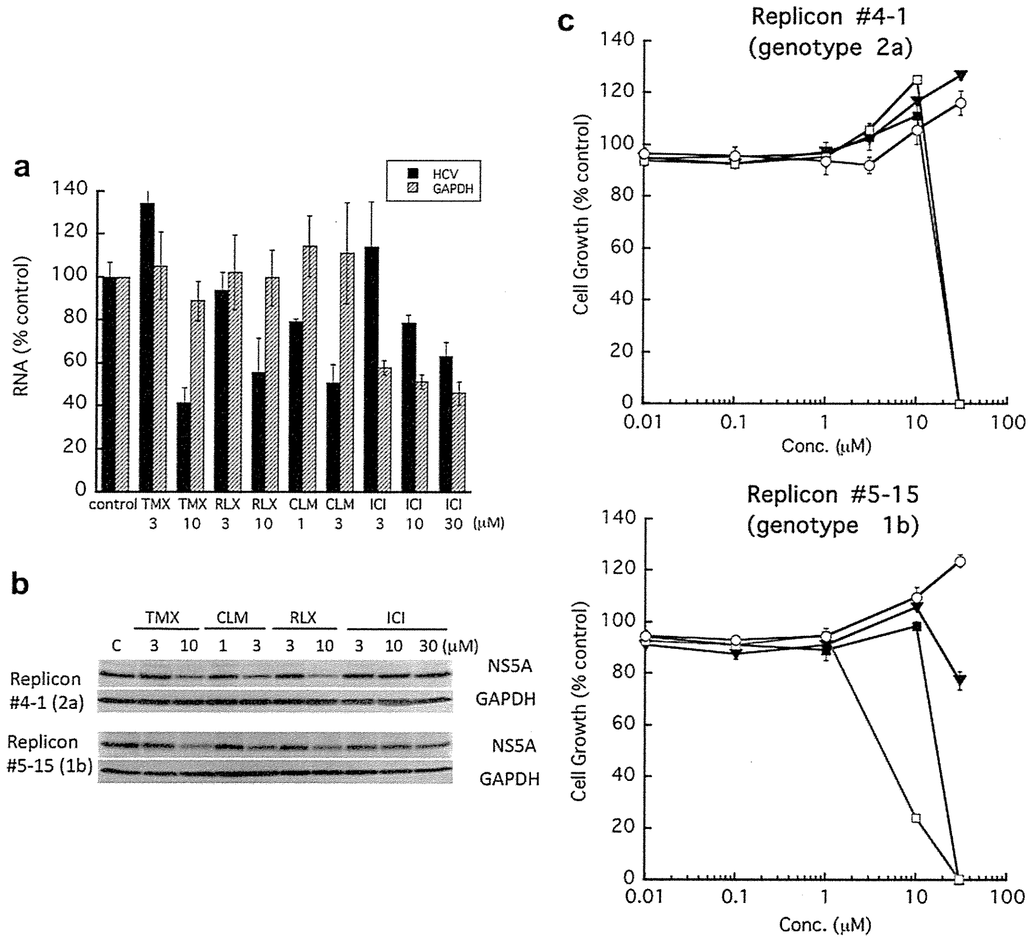

Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

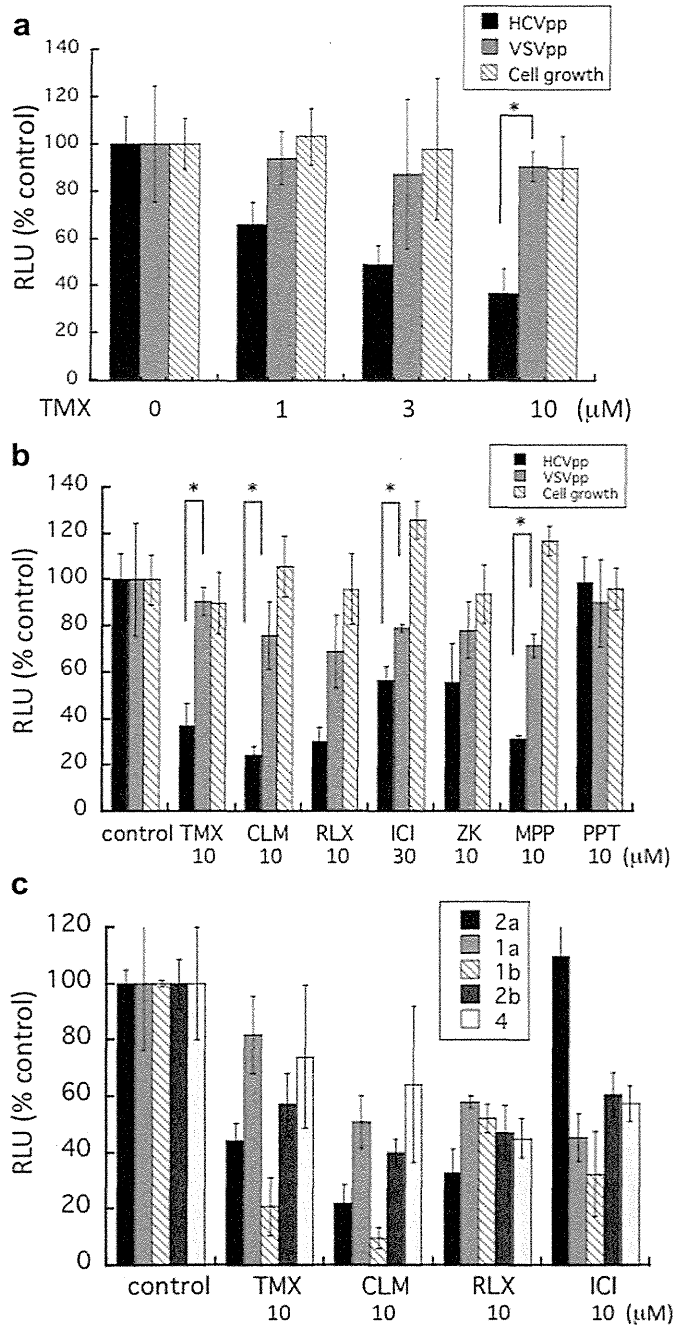

Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. *P-value < 0.05. One representative experiment of three independent experiments is shown.

3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.

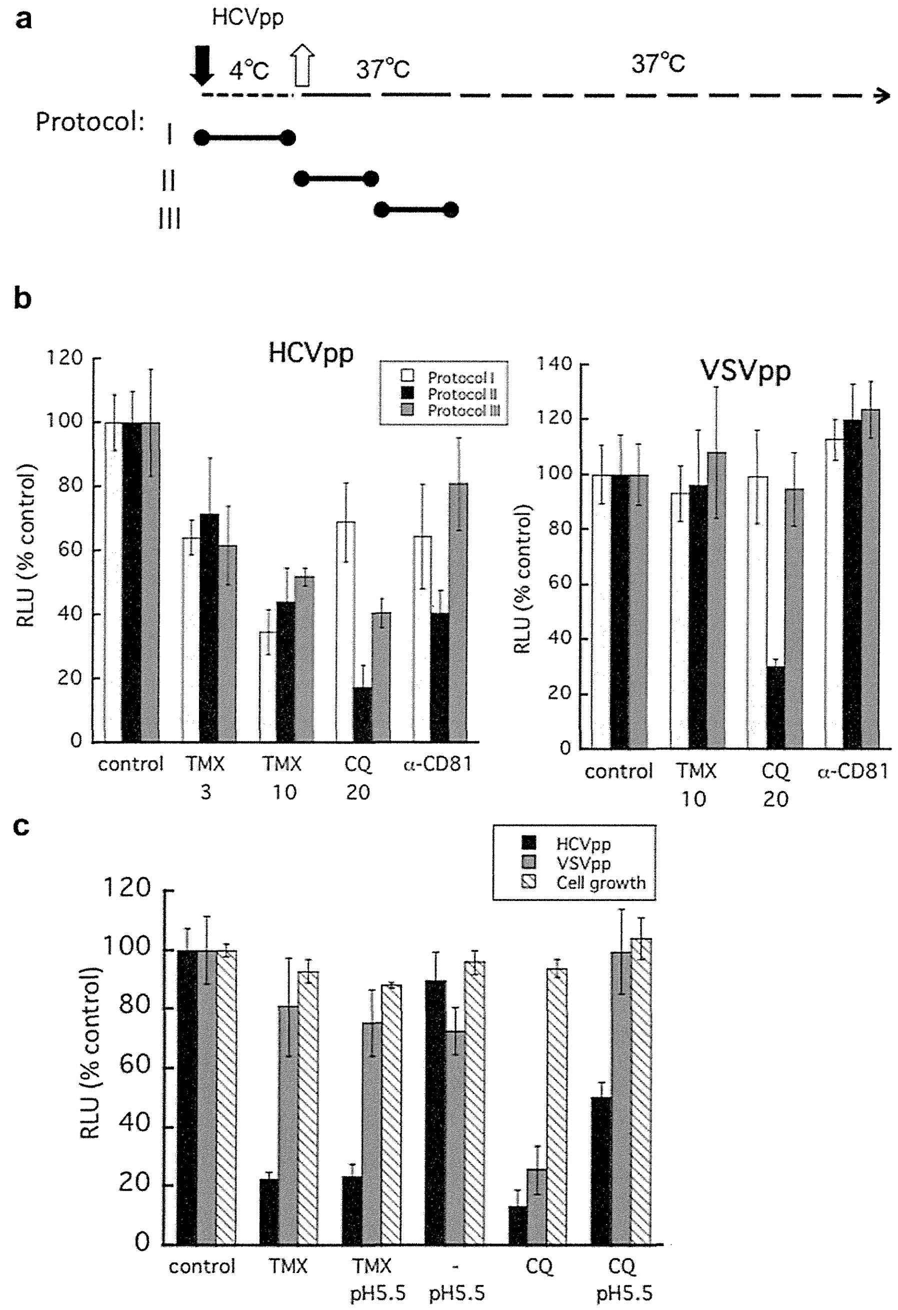

Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. The cells were treated with drug during the binding period at 4 °C (protocol I), during first hour after the shift to 37 °C (protocol II), or 1 h after the shift to 37 °C (protocol III). The drug-containing medium was removed for every treatment, and new medium was supplied to the cells. b) Effects of tamoxifen (TMX) (3 and 10 μM), chloroquine (CQ) (20 μM) and anti-CD81 antibody (20 μg/ml) on HCVpp attachment (protocol I) and post-binding events (protocol II and III) (left). Effects of tamoxifen, chloroquine and anti-CD81 on VSVpp (right). c) Effects of exposure to low pH on the inhibition of HCVpp entry by tamoxifen and chloroquine. The cells were incubated with HCVpp at 4 °C for 1.5 h in the absence of drug. After removing the inoculum, regular (pH 7.1) or acidic medium that was adjusted with HCl to pH 5.5, either with or without drug (tamoxifen, 10 μM, chloroquine, 20 μM), was added to the cells. The cells were subsequently incubated at 37 °C. The drug-containing medium was removed after 2 h of incubation, and the cells were incubated for an additional 3 days with fresh, regular medium. The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown.

4. Discussion

We screened for HCV inhibitors using the JFH-1-Huh 7.5.1 cell culture system and found that tamoxifen and ERα antagonists, but not ERα agonists, inhibited HCV JFH-1 infection. Although there are some reports about the HCV inhibitory effects of tamoxifen and other SERMs, we presented further information about the inhibitory effects of these substances. The time-of-addition experiments (Fig. 2a–c) suggested that these SERMs inhibit the entry and replication steps in the HCV life cycle. These SERMs, except ICI 182,780, reduced level of HCV genome (genotype 2a) and NS5A (genotypes 1b and 2a) in the subgenomic replicon cells (Fig. 3), which supports the hypothesis that the inhibitory effect of the SERMs occurred during the replication steps. Further we observed that SERMs preferentially reduced extracellular HCV RNA compare to intracellular HCV RNA in the newly (Fig. 2d) and persistently infected cells (Fig. 2e). It suggests that the SERMs also target post replication step(s) in the viral life cycle, such as assembly and release. A low concentration of tamoxifen (0.1 μM) accumulated intracellular HCV RNA (Fig. 2d), which suggests that SERMs target post replication step(s) more efficiently than replication steps. Additionally, these compounds inhibited HCVpp infection (Fig. 4), which supports an inhibitory effect during the entry step. The inhibition of entry was mediated through the inhibition of viral binding to cells and through the inhibition of a post-binding event (Fig. 5b). Taken together, SERMs seemed to target multiple steps of the HCV viral life cycle.

Among the SERMs, ICI 182,780 did not exhibit distinct inhibition of replication in the replicon cells (Fig. 3a and b), although the compound seemed to inhibit entry and replication steps according to the result of time-of-addition experiment (Fig. 2c). The replicon cells are derived from Huh 7 cell. Although viral sensitivity to the SERMs might be dependent on the cell that HCV infect, it remains unclear whether ICI 182,780 inhibits replication step or not. However, the compound affected post replication step in a similar manner to other SERMs (Fig. 2d and e). It is unlikely that ICI 182,780 is an inhibitor with different mechanisms.

The inhibitory effect of tamoxifen on HCV replication has been previously reported by Watashi et al. [23]. They also demonstrated that ICI 182,780 inhibited HCV replication. This effect was discovered using a cell line that harbored a subgenomic replicon (genotype 1b). Additionally, these researchers reported that RNA interference-mediated knockdown of ERα, not ERβ, reduced HCV replication, but the reduction was not related to ERE-mediated transactivation activity. They suggested that ERα interacts with the HCV viral polymerase NS5B and that ERα promotes the participation of NS5B with the HCV replication complex. Using the Huh 7.5.1-JFH-1 screening system, Gastaminza et al. and Chockalingam et al. found that toremifene and raloxifene, respectively, function as HCV inhibitors. Gastaminza et al. [24] reported that toremifene inhibited HCV infection by inhibiting both the entry and release steps of the viral life cycle. Chockalingam et al. [25] determined that raloxifene inhibited the entry and replication steps, as we also observed. Our results are in accordance with these previous reports and other information about the inhibitory effects of SERMs.

Pseudo-particle experiments confirmed that SERMs affected the entry step of HCV viral life cycle (Fig. 4b), although the inhibitory effects were lower compare to those against HCVcc (Fig. 2a–c). The reason for the difference in sensitivity may account for some difference in the two entry systems. Otherwise, in the treatment with the drugs for the first 2 h of HCVcc infection, some amount of the drugs might enter the cell and remain affecting the other steps. The SERMs affected not only genotype 2a but also other genotypes of HCVpp that were examined, suggests that these chemicals have effects on various genotypes of HCV. Although the SERMs appeared to inhibit multiple steps of the HCV life cycle, the primary target step in the viral life cycle might be the entry step. In the time-of-addition experiments, treatment with tamoxifen or raloxifene during the first 2 h was more effective than treatment during the subsequent 2–48 h (Fig. 2a). These SERMs are thought to primarily prevent viral entry and to inhibit post replication step and replication at higher concentrations.

As shown in Fig. 5, an experiment that could discriminate an effect on viral attachment from an effect at the post-binding processes indicated that tamoxifen inhibited both steps. The inhibition of endocytosis by tamoxifen was not rescued by exposure to a low pH. This suggests that the observed inhibition is the result of a mechanism that is independent of the compound's function as a base. HCV entry is a highly complicated process that involves numerous viral and cellular factors. Tamoxifen is thought to target multiple steps that are involved in the attachment and entry steps of the HCV life cycle, which results in high levels of inhibition.

At present, the mechanism of the entry inhibition by SERMs is not clear. It is possible that tamoxifen targets viral molecules, but we have no evidence to support this hypothesis. ERα might be a target molecule because all of the antagonists of ERα that were examined had an inhibitory effect. Watashi et al. indicated that ERα is involved in HCV replication [23]. ERα is thought to be present in the cytoplasm, which is where HCV replicates. However, it is doubtful that ERα is present on the cell surface where viral entry occurs. The addition of 17β-estradiol with tamoxifen did not prevent the inhibitory effect of tamoxifen in the HCVpp experiment (data not shown). This result suggests that tamoxifen does not compete with 17β-estradiol for the target molecules involved in HCV entry. Additionally, a pure ERα antagonist, ICI 182,780, was a less effective inhibitor of the entry step. Based on these results, it is thought that the molecule responsible for HCV entry that is targeted by SERMs is not ERα.

Tamoxifen has various targets other than ERα, such as P-glycoprotein (GPR30), calmodulin, and protein kinase C [26]. GPR30 (G protein-coupled receptor protein 30) is a membrane-associated estrogen receptor that is distinct from the classical ER [27]. Tamoxifen and ICI 182,780 are agonists of GPR30 [28]. We examined the effect of a specific GPR30 agonist, G-1, and a GPR30 antagonist, G-15, on HCVpp infection [29]. G-1 and G-15 did not inhibit HCVpp infection. Conversely, HCVpp infection was observed to increase upon addition of these compounds (data not shown). This result suggested that GPR30 is not involved in the inhibition of HCV entry.

We previously reported that a typical PKC inhibitor, bisindolylmaleimide I (BIM I), inhibited HCV replication [2]. BIM I (10 μM) inhibited both HCVpp and VSVpp infection in a similar manner by approximately 50% (data not shown). This suggests that BIM I has a different mechanism for the inhibition of entry compared to tamoxifen. PKC is not thought to be involved in the HCV-specific inhibition of entry by SERMs.

There were few reports of HCV entry inhibitors until the development of the cell-culture JFH-1 infection system. It has recently been reported that fluphenazine, trifluoperazine and related chemicals exhibit a strong, dose-dependent inhibition of HCV entry without significantly affecting the entry of VSVpp [24,25]. These compounds are structurally similar to chlorpromazine, which is an inhibitor of the clathrin-coated pit formation that is required for HCV entry [14]. Interestingly, these compounds and the SERMs have a common structural characteristic: planar, multiple aromatic rings with a tertiary amine side chain. Tamoxifen, raloxifene and ERα antagonists all have this structure, but the ERα agonists do not have these structures. Fluphenazine and related chemicals may inhibit HCV entry through a mechanism that is similar to tamoxifen.

In summary, we observed a significant HCV inhibitory effect of various SERMs using the Huh 7.5.1 cell-JFH-1 infection system. Additionally, we demonstrated that SERMs could be useful for the treatment of HCV. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen, toremifene, and raloxifene are all drugs that have been in use for an extended period of time. In our present *in vitro* study, the effective concentrations for the HCV inhibitory effects of the SERMs were approximately 0.1–10 μM. In the case of tamoxifen, 20 mg per day, administered for 8 weeks resulted in plasma concentrations of approximately 0.5 μM. These concentrations could be sufficient to exert an anti-HCV effect. SERMs should be investigated to determine their efficacy for treating HCV clinically. Further examination of the mechanism of the entry inhibition mediated by SERMs would produce significant new data relevant to the understanding of HCV entry.

Acknowledgments

We thank Drs. Kyoko Murakami, Kenichi Morikawa, Tomoko Date, and Koichi Watashi for helpful advice. We also thank Drs. François-Loïc Cosset (INSERM, France) and Yoshiharu Matsuura (Osaka University, Japan) for generously providing plasmids. This study was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan.

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HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases
AASLD

Hepatitis C Virus NS4B Protein Targets STING and Abrogates RIG-I–Mediated Type I Interferon-Dependent Innate Immunity

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Hepatitis C virus (HCV) infection blocks cellular interferon (IFN)-mediated antiviral signaling through cleavage of Cardif by HCV-NS3/4A serine protease. Like NS3/4A, NS4B protein strongly blocks IFN-β production signaling mediated by retinoic acid–inducible gene I (RIG-I); however, the underlying molecular mechanisms are not well understood. Recently, the stimulator of interferon genes (STING) was identified as an activator of RIG-I signaling. STING possesses a structural homology domain with flaviviral NS4B, which suggests a direct protein-protein interaction. In the present study, we investigated the molecular mechanisms by which NS4B targets RIG-I–induced and STING-mediated IFN-β production signaling. IFN-β promoter reporter assay showed that IFN-β promoter activation induced by RIG-I or Cardif was significantly suppressed by both NS4B and NS3/4A, whereas STING-induced IFN-β activation was suppressed by NS4B but not by NS3/4A, suggesting that NS4B had a distinct point of interaction. Immunostaining showed that STING colocalized with NS4B in the endoplasmic reticulum. Immunoprecipitation and bimolecular fluorescence complementation (BiFC) assays demonstrated that NS4B specifically bound STING. Intriguingly, NS4B expression blocked the protein interaction between STING and Cardif, which is required for robust IFN-β activation. NS4B truncation assays showed that its N terminus, containing the STING homology domain, was necessary for the suppression of IFN-β promoter activation. NS4B suppressed residual IFN-β activation by an NS3/4A-cleaved Cardif (Cardif1-508), suggesting that NS3/4A and NS4B may cooperate in the blockade of IFN-β production. *Conclusion:* NS4B suppresses RIG-I–mediated IFN-β production signaling through a direct protein interaction with STING. Disruption of that interaction may restore cellular antiviral responses and may constitute a novel therapeutic strategy for the eradication of HCV. (HEPATOLOGY 2013;57:46-58)

Type I interferon (IFN) plays a central role in eliminating hepatitis C virus (HCV) both under physiological conditions and when used as a therapeutic intervention.[1-3] In experimental acute-resolving HCV infection in chimpanzees, numerous IFN-related genes are expressed during clinical course of infection.[4] Viruses are recognized by cellular innate immune receptors, such as toll-like receptors, and a family of RIG-I–like receptors, such as retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5); host antiviral responses are then activated, resulting in the

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Received September 16, 2011; accepted July 24, 2012.

BiFC, bimolecular fluorescence complementation; CARD, caspase recruitment domain; DAPI, 4′,6-diamidino-2-phenylindole; dsRNA, double-stranded RNA; ER, endoplasmic reticulum; FACL4, fatty acid-CoA ligase, long chain 4; HCV, hepatitis C virus; IFN, interferon; IKKε, IκB kinase ε; IRF-3, interferon-regulatory factor 3; ISRE, interferon-stimulated response element; MAM, mitochondria-associated ER membrane; mKG, monomeric Kusabira-Green; PDI, protein disulphide-isomerase; pIRF-3, phosphorylated IRF3; poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; RIG-I, retinoic acid–inducible gene I; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; STAT1, signal transducer and activator of transcription protein-1; STING, stimulator of interferon genes; TBK1, TANK binding kinase 1.

**These authors contributed equally to this work.*

production of cytokines such as type I and type III IFNs.[5] RIG-I is activated through recognition of short double-strand RNA (dsRNA) or triphosphate at the 5′ end of dsRNA as pathogen-associated molecular patterns,[6,7] forming a homo-oligomer that binds with the caspase recruitment domain (CARD) of Cardif (also known as MAVS, VISA, or IPS-1).[8-11] Cardif subsequently recruits TANK binding kinase 1 (TBK1) and IκB kinase ϵ (IKKϵ) kinases, which catalyze phosphorylation and activation of IFN regulatory factor-3 (IRF-3).[12] Activation of TBK1 and IKKϵ results in the phosphorylation of IRF-3 or IRF-7, translocation to the nucleus, and induction of IFN-β mRNA transcription.

Several HCV proteins can block host cellular antiviral responses. HCV core protein blocks IFN signaling by interacting with signal transducer and activator of transcription protein-1 (STAT1).[13] The core protein also induces expression of suppressor of cytokine signaling-1 (SOCS1) and SOCS3, and blocks Janus kinase–STAT signaling.[14,15] A well-elucidated immune evasion strategy of HCV involves NS3/4A serine protease and its ability to inhibit host IFN signal pathways. Gale and colleagues[11,16,17] revealed that NS3/4A protease cleaves Cardif at Cys-508 resulting in dislocation of Cardif from mitochondria, and blocks downstream signaling of IFN-β production. On the other hand, Baril et al.[18] reported that Cardif was still able to form a homo-oligomer and to activate downstream IFN production signaling despite delocalization from the mitochondria. These reports suggest that homo-oligomerization of Cardif, and not mitochondrial anchorage, is essential for the activation of downstream IFN signaling and that other virus-derived molecules may cooperate with NS3/4A to abrogate the signaling of IFN production.

We reported previously that HCV-NS4B, as well as NS3/4A, inhibited RIG-I and Cardif-mediated interferon-stimulated response element (ISRE) activation, while TBK1- and IKKϵ-mediated ISRE activation were not suppressed.[19] These results indicate that NS4B suppresses IFN production signaling by targeting Cardif or other unknown signaling molecules between the level of Cardif and TBK1/IKKϵ.

Recently, a stimulator of interferon genes (STING, also known as MITA/ERIS/MPYS/TMEM173) was identified as a positive regulator of RIG-I–mediated IFN-β signaling.[20-23] STING is a 42-kDa protein localized predominantly in the endoplasmic reticulum (ER) that binds RIG-I, Cardif, TBK1, and IKKϵ. STING is thought to act as a scaffold for Cardif/TBK1/IRF-3 complex upon viral infection.[22] It has been reported that NS4B of yellow fever virus, which is a member of the flaviviridae family of viruses, inhibits STING activation probably through a direct molecular interaction.[24] These reports have led us postulate that HCV-NS4B may also inhibit RIG-I dependent IFN signaling through association with STING.

In the present study, we further investigated the molecular mechanisms by which HCV-NS4B protein inhibits RIG-I–mediated IFN expression signaling. We demonstrated that HCV-NS4B specifically binds STING, blocks the molecular interaction between STING and Cardif, and suppresses the RIG-I–like receptor–induced activation of IFN-β production signaling.

Materials and Methods

Plasmids. The ΔRIG-I and RIG-IKA plasmids express constitutively active and inactive RIG-I, respectively.[5] Full-length Cardif (Cardif) and CARD-truncated Cardif (ΔCARD) plasmids were provided by J. Tschopp.[11] Plasmids expressing STING were provided by G. N. Barber.[20] Plasmids expressing HCV NS3/4A, NS4B, and truncated NS4B have been described.[25] Plasmid pIFNβ-Fluc was provided by R. Lin.[26]

Cell Culture. HEK293T and Huh7 cells were maintained in Dulbecco's modified minimal essential medium (Sigma) supplemented with 2 mM L-glutamine and 10% fetal calf serum at 37°C with 5% CO_2.

HCV Replicon Constructs and HCV-JFH1 Cell Culture. An HCV subgenomic replicon plasmid, pRep-Feo, expressed fusion protein of firefly luciferase and neomycin phosphotransferase.[27,28] Huh7 cells were transfected by Rep-Feo RNA, cultured in the presence of 500 μg/mL of G418, and a cell line that stably expressed Feo replicon was established. For HCV cell culture, the HCV-JFH1 strain was used.[29,30]

Antibodies. Antibodies used were anti–IRF-3 (FL-425, Santa Cruz Biotechnology), anti-HA (Invitrogen), anti-myc (Invitrogen), mouse anti-PDI (Abcam),

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DOI 10.1002/hep.26017

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

rabbit anti-PDI (Enzo Life Science), anti-Flag (Sigma Aldrich), anti-Cardif (Enzo Life Science), anti-phospho–IRF-3 (Ser396, Millipore), anti-monomeric Kusabira-Green C- or N-terminal fragment (MBL), and anti-FACL4 (Abgent).

Luciferase Reporter Assay. IFN-β reporter assays were performed as described.[19,31] The plasmids pIFN-β-Fluc and pRL-CMV were cotransfected with NS3/4A or NS4B, and ΔRIG-I, Cardif, STING or poly(deoxyadenylic-deoxythymidylic) acid [poly(dA:dT)] (Invivogen). RIG-IKA, ΔCARD, and pcDNA3.1, respectively, were used as controls. Luciferase assays were performed 24 hours after transfection by using a 1420 Multilabel Counter (ARVO MX PerkinElmer) and Dual Luciferase Assay System (Promega). Assays were performed in triplicate, and the results are expressed as the mean ± SD.

Immunoblotting. Preparation of total cell lysates was performed as described.[19,28] Protein was separated using NuPAGE 4%-12% Bis/Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane. The membrane was immunoblotted with primary followed by secondary antibody, and protein was detected by chemiluminescence.

Immunoprecipitation Assay. HEK-293T or Huh7 cells were transfected with plasmids as indicated. Twenty-four hours after transfection, cellular proteins were harvested and immunoprecipitation assays were performed using an Immunoprecipitation Kit according to the manufacturer's protocol (Roche Applied Science). The immunoprecipitated proteins were analyzed by immunoblotting.

Indirect Immunofluorescence Assay. Cells seeded onto tissue culture chamber slides were transfected with plasmids as indicated. Twenty-four hours after transfection, the cells were fixed with cold acetone and incubated with primary antibody and subsequently with Alexa488- or Alexa568-labeled secondary antibodies. Mitochondria were stained by MitoTracker (Invitrogen). Cells were visualized using a confocal laser microscope (Fluoview FV10, Olympus).

BiFC Assay. Expression plasmids of NS4B, Cardif, or STING that was fused with N- or C-terminally truncated monomeric Kusabira-Green (mKG) were constructed by inserting polymerase chain reaction–amplified fragments encoding NS4B, Cardif, or STING, respectively, inserted into fragmented mKG vector (Coral Hue Fluo-Chase Kit; MBL). HEK293T cells were transfected with a complementary pair of mKG fusion plasmids. Twenty-four hours after transfection, fluorescence-positive cells were detected and counted by flow cytometry, or observed by confocal laser microscopy.

Small Interfering RNA Assay. Nucleotide sequences of STING-targeted small interfering RNAs (siRNAs) were as follows: (1) 5′-gcaacagcatctatgagcttctggagaac-3′, (2) 5′- gtgcagtgagccagcggctgtatattctc;-3′, (3) 5′-gctggcat ggtcatattacatcggatatc-3′.[22] Stealth RNAi Negative Control Duplex (Medium GC Duplex, Invitrogen) was used. Forty-eight hours after siRNA transfection, expression levels of STING were detected by immunoblotting.

Statistical Analyses. Statistical analyses were performed using unpaired, two-tailed Student t test. $P < 0.05$ were considered to be statistically significant.

Results

NS4B Suppressed RIG-I, Cardif, and STING-Mediated Activation of IFN-β Expression Signaling. First, we performed a reporter assay using a luciferase reporter plasmid regulated by native IFN-β promoter. Consistent with our previous study,[19] overexpression of NS4B, as well as NS3/4A, inhibited the IFN-β promoter activation that was induced by ΔRIG-I and Cardif, respectively (Fig. 1A). We next studied whether NS4B targets STING and inhibits RIG-I pathway–mediated activation of IFN-β production. Expression of NS4B protein significantly suppressed STING-mediated activation of the IFN-β promoter reporter, whereas expression of NS3/4A showed no effect on STING-induced IFN-β promoter activity (Fig. 1A). To study whether NS4B blocks the STING-mediated DNA-sensing pathway, we performed a reporter assay using a luciferase reporter plasmid cotransfection with poly(dA:dT), which is a synthetic analog of B-DNA and has been reported to induce STING-mediated IFN-β production and NS4B. NS4B significantly blocked poly(dA:dT)-induced IFN-β promoter activation, suggesting that NS4B may block STING signaling in the DNA-sensing pathway (Fig. 1A).

Activation of RIG-I signaling induces phosphorylation of IRF-3, which is a hallmark of IRF-3 activation.[32] Thus, we examined the effects of NS3/4A and NS4B expression on phosphorylation of IRF-3 by immunoblotting analysis. As shown in Fig. 1B, overexpression of ΔRIG-I, Cardif, or STING in HEK293T cells increased levels of phosphorylated IRF-3 (pIRF-3). Expression of NS4B impaired the IRF-3 phosphorylation that was induced by ΔRIG-I, Cardif, or STING. NS3/4A also blocked production of pIRF-3 induced by ΔRIG-I or Cardif. Intriguingly, NS3/4A did not block STING-induced pIRF-3 production. These results demonstrate that both NS3/4A and

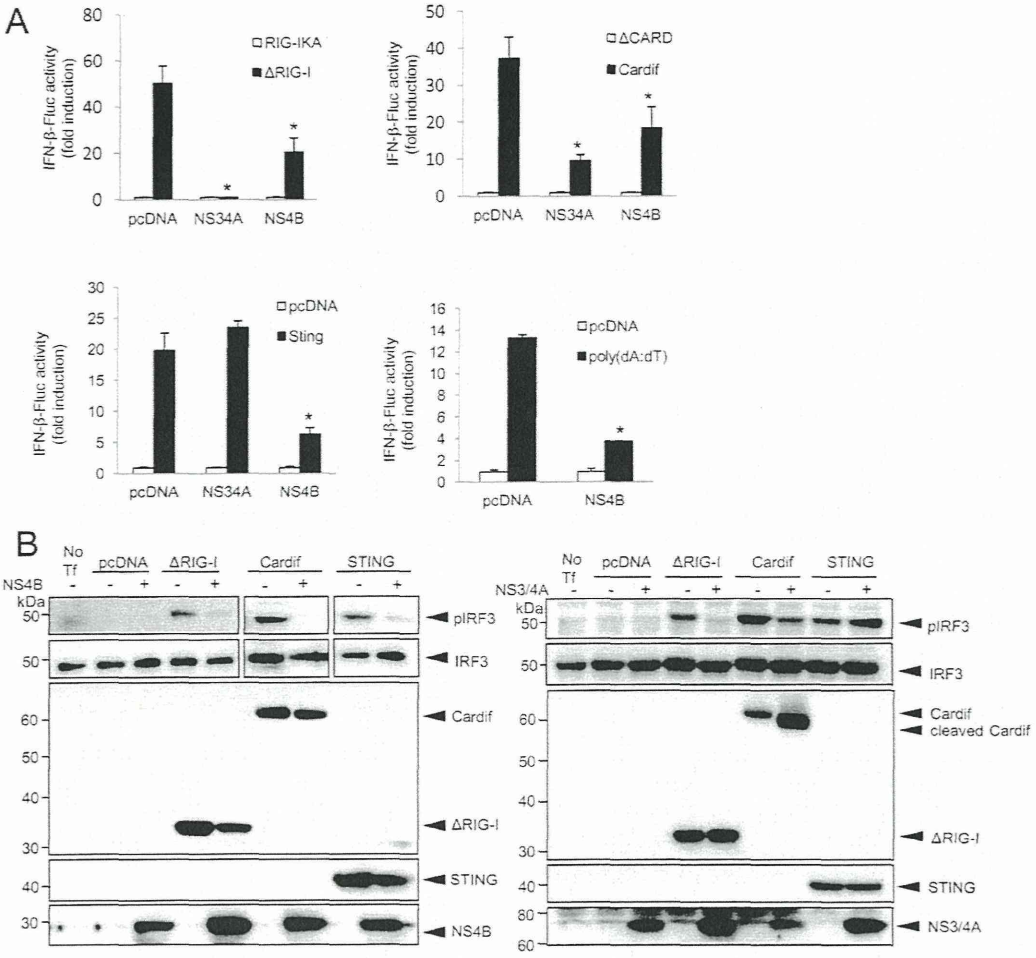

Fig. 1. NS4B suppressed IFN-β signaling mediated by RIG-I, Cardif, or STING. (A) Plasmids expressing ΔRIG-I, Cardif, or STING or poly(dA:dT) as well as NS3/4A or NS4B were cotransfected with pIFN-β-Fluc and pRL-CMV into HEK293T cells. After 24 hours, dual luciferase assays were performed. Plasmids expressing RIG-IKA, ΔCARD, or an empty plasmid (pcDNA) were used as a corresponding negative control. The experiments were performed more than three times and yielded consistent results. The *y* axis indicates relative IFN-β-Fluc activity. Assays were performed in triplicate and error bars indicate mean ± SD. $^*P < 0.05$. (B) HEK293T cells were cotransfected with indicated plasmids. On the day after transfection, the cells were lysed and immunoblot analyses were performed. No Tf, transfection-negative controls. pIRF-3 and IRF-3, phosphorylated and total IRF-3, respectively.

NS4B suppress RIG-I–mediated IFN-β production, but they do so by targeting different molecules in the signaling pathway.

Subcellular Localization of NS4B, Cardif, and STING. We next studied the subcellular localization of NS4B following its overexpression and measured the colocalization of NS4B with Cardif and STING in both HEK293T cells and Huh7 cells by indirect immunofluorescence microscopy. NS4B was localized predominantly in the ER, which is consistent with previous reports[33] (Fig. 2A). Cardif was localized in mitochondria but did not colocalize with the ER-resident host protein disulphide-isomerase (PDI). Interestingly, Cardif and NS4B colocalized partly at the boundary of the two proteins, although their original localization was different (Fig. 2A,C). STING was localized predominantly in the ER[20,21] (Fig. 2B,D). STING colocalized partly with Cardif, which is consistent with a previous report by Ishikawa and Barber[20] (Fig. 2B,D). In cells cotransfected with NS4B and STING expression plasmids, NS4B colocalized precisely with STING (Fig. 2B,D). To examine the region of NS4B-STING interaction, we next observed the two proteins by performing staining for them along with mitochondria-associated ER membrane (MAM), which is a physical association with mitochondria[34] and has been reported the site of Cardif-STING association.[24] Both NS4B and STING were adjacent to and partially colocalized

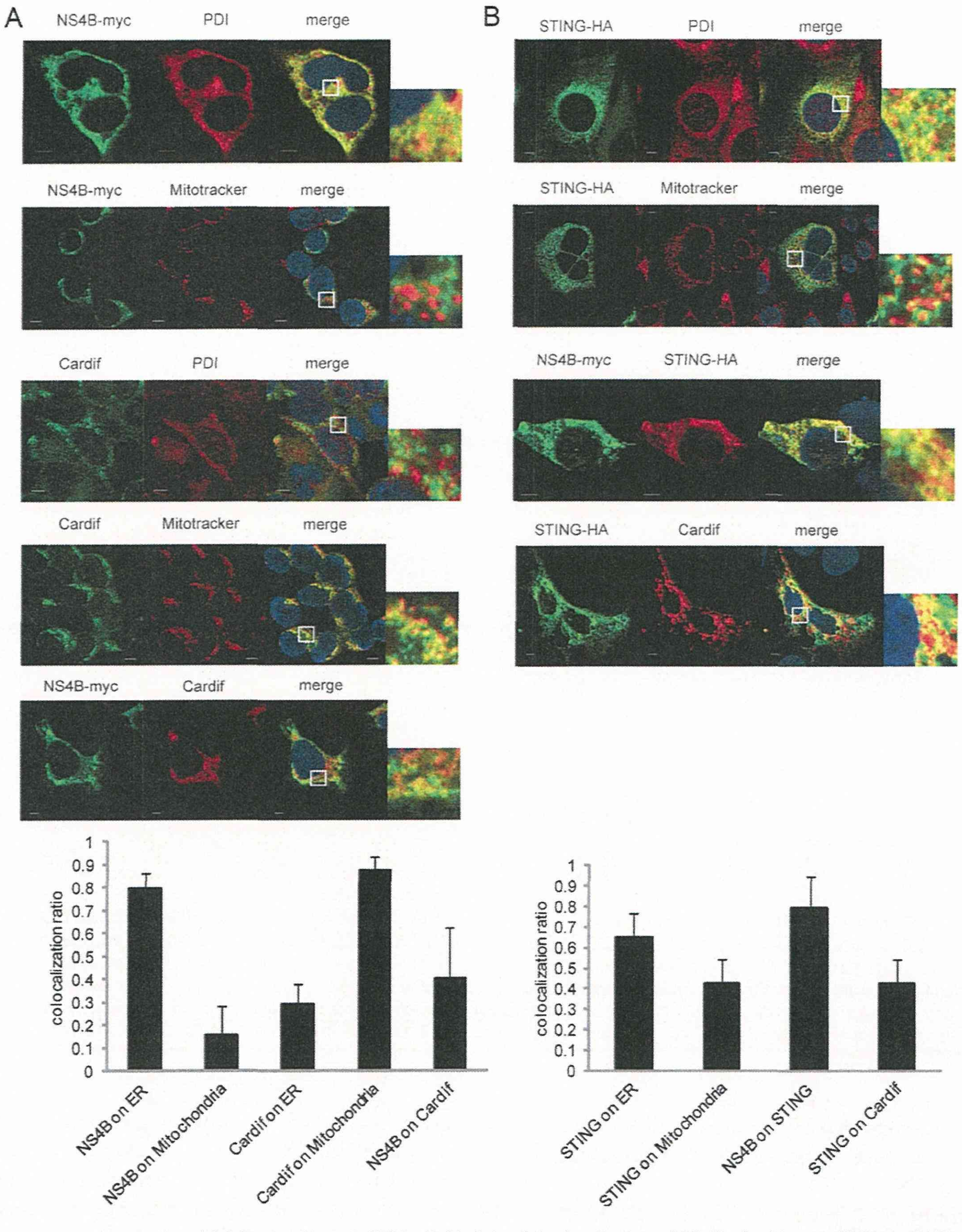

Fig. 2. Subcellular localization of NS4B, Cardif, and STING. (A-D) Subcellular localization of NS4B, Cardif, and STING in 293T (A,C) and Huh7 (B,D) cells. (A,C) NS4B-myc (first, second, and fifth panels of A and third panel of C) was transfected, and 24 hours later the cells were fixed and immunostained with anti-myc. In the third, fourth, and fifth panels of A, and the first and second panels of C, endogenous Cardif was detected with anti-Cardif antibody. ER was immunostained with anti-PDI antibody (first and third panels of A and first panel of C). Mitochondria were stained using Mitotracker (second and fourth panels of A and second panel of C). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). (B,D) STING-HA (all panels) and NS4B-myc (third panels) were transfected, and after 24 hours the cells were fixed and immunostained with anti-HA or anti-myc, respectively. In the fourth panels, endogenous Cardif was detected with anti-Cardif antibody. ER was immunostained with anti-PDI antibody (first panels). Mitochondria were stained using Mitotracker (second panels). Nuclei were stained with DAPI. (E) NS4B-myc and STING-HA were transfected into Huh7 cells and after 24 hours the cells were fixed and immunostained with anti-HA, anti-myc, and anti-FACL4 (MAM) antibody. Cells were visualized by confocal microscopy. Scale bars indicate 5 μm. In each microscopic image, the grade of protein colocalization in a single cell was quantified and is shown in the graphs at the bottom of each panel. Values are shown as the average colocalization ratio in 8 cells. Error bars indicate the mean + SD.

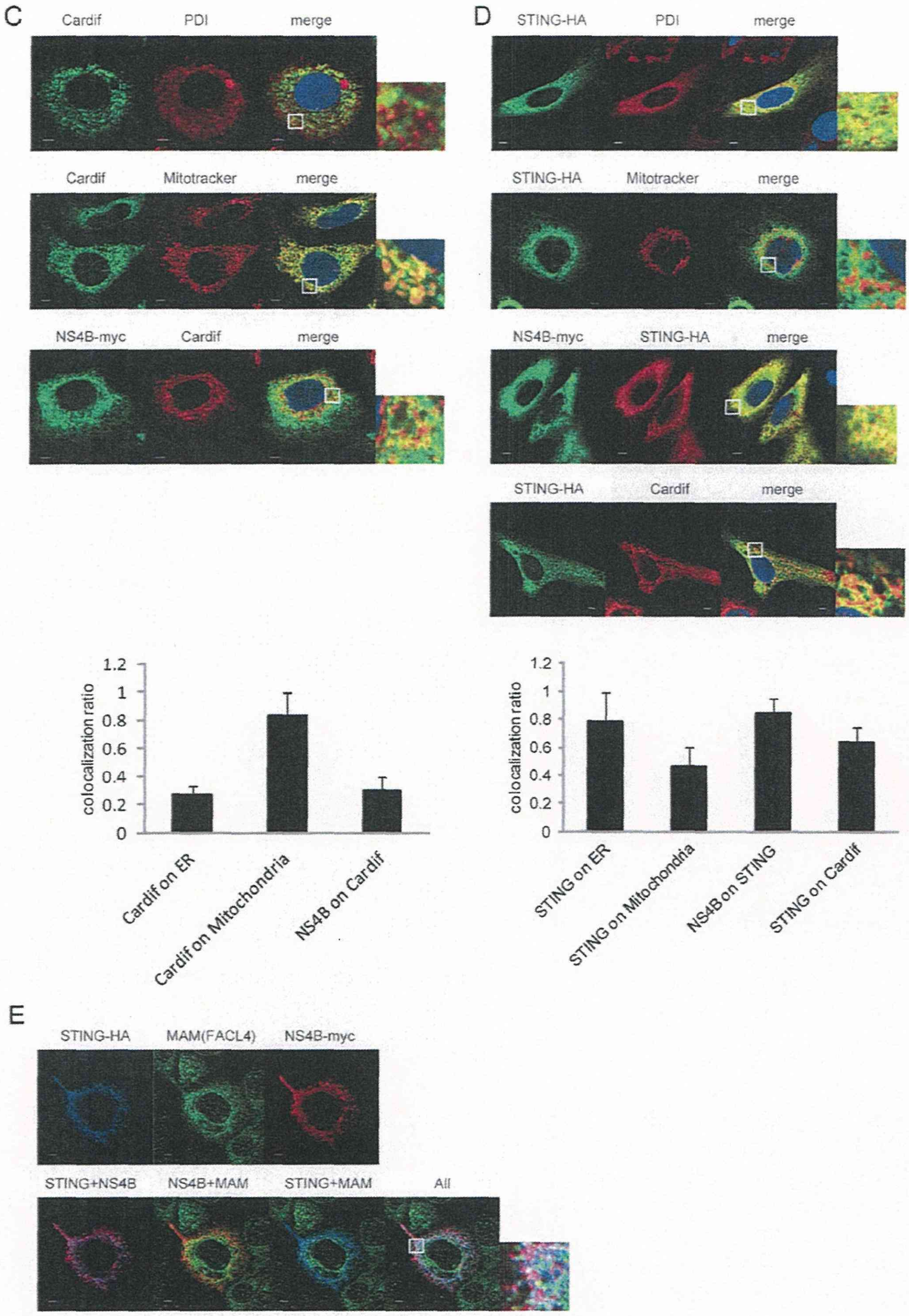

Fig. 2. Continued

with fatty acid-CoA ligase long chain 4 (FACL4), which is a MAM marker protein[35,36] (Fig. 2E). These findings suggest that NS4B might interact with STING on MAM more strongly than with Cardif.

Protein-Protein Interaction Between NS4B, Cardif, and STING. Knowing that NS4B was colocalized strongly with STING and only partly with Cardif, we next analyzed direct protein-protein interactions

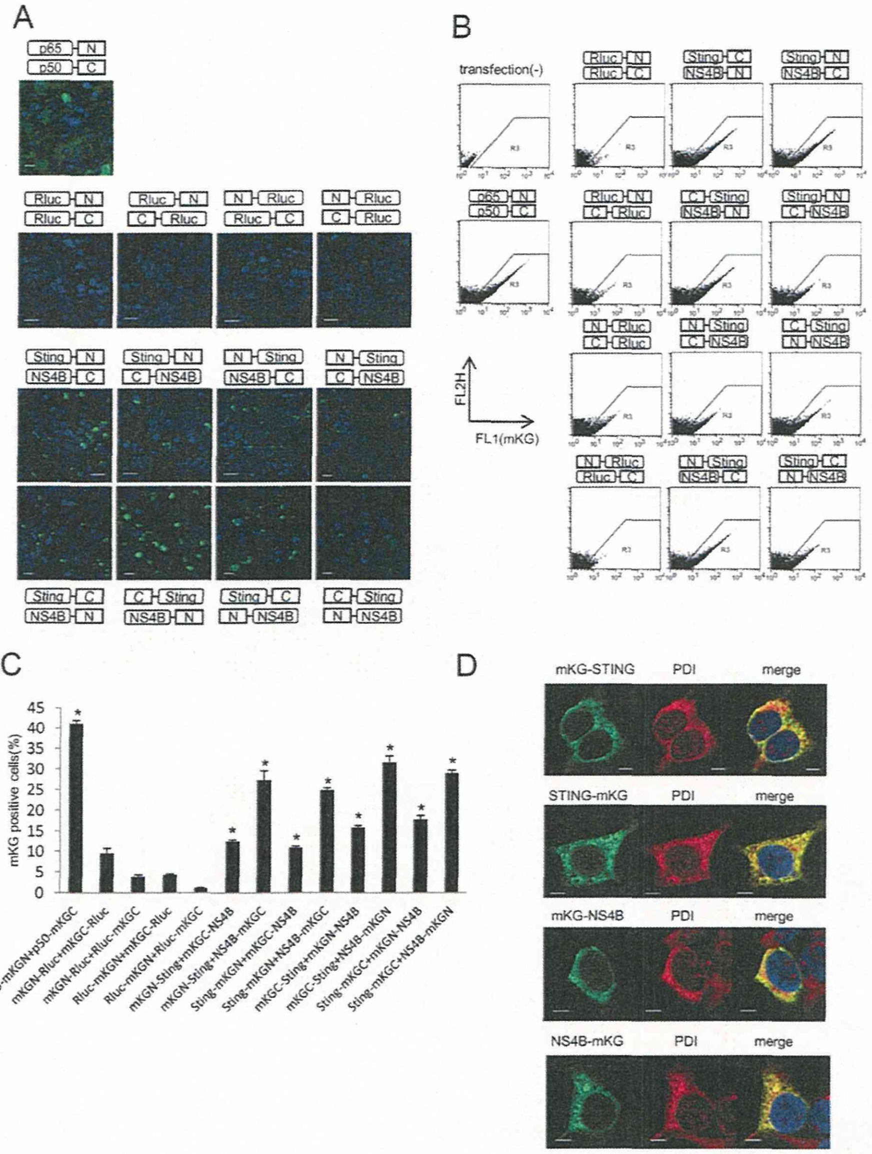

Fig. 3. BiFC assays of STING and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and STING expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B,C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC-positive control and plasmids expressing N- or C-terminally mKG fused Rluc were used as a negative control. The letters N and C denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were performed in triplicate and error bars indicate the mean ± SD. Scale bars indicate 10 μm (A). *$P < 0.05$ compared with corresponding negative controls. (D) Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG and anti-PDI (ER) antibody. Nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars = 5 μm.

between NS4B, Cardif, and STING. To detect those interactions in living cells, we performed BiFC assays.[37,38] We constructed NS4B, Cardif, and STING expression plasmids that were N- or C-terminally fused with truncated mKG proteins, respectively. First, we cotransfected several different pairs of NS4B and STING expression plasmids that were fused with complementary pairs of N- or C-terminally truncated mKG. Strong fluorescence by mKG complexes (BiFC signal) was detected in all pairs of cotransfections, suggesting significant molecular interaction (Fig. 3A). In flow cytometry, all pairs of NS4B- and STING-mKG fusion proteins were positive for strong BiFC signal (Fig. 3B). The percentages of cells positive for BiFC signal were significantly higher in STING-mKG and NS4B-mKG fusion complexes than in corresponding controls (Fig. 3C). These results demonstrate that HCV-NS4B and STING proteins interact with each other strongly and specifically in cells. Fluorescence microscopy indicated that N- and C-terminal fusion of mKG onto NS4B and STING did not affect subcellular localization (Fig. 3D).

We next studied the molecular interaction between NS4B and Cardif by BiFC assay using NS4B and Cardif fusion plasmids that were tagged with complementary pairs of truncated mKG. Weak fluorescence was detected in cells transfected with the pairs N-Cardif and NS4B-C, N-Cardif and C-NS4B, C-Cardif and

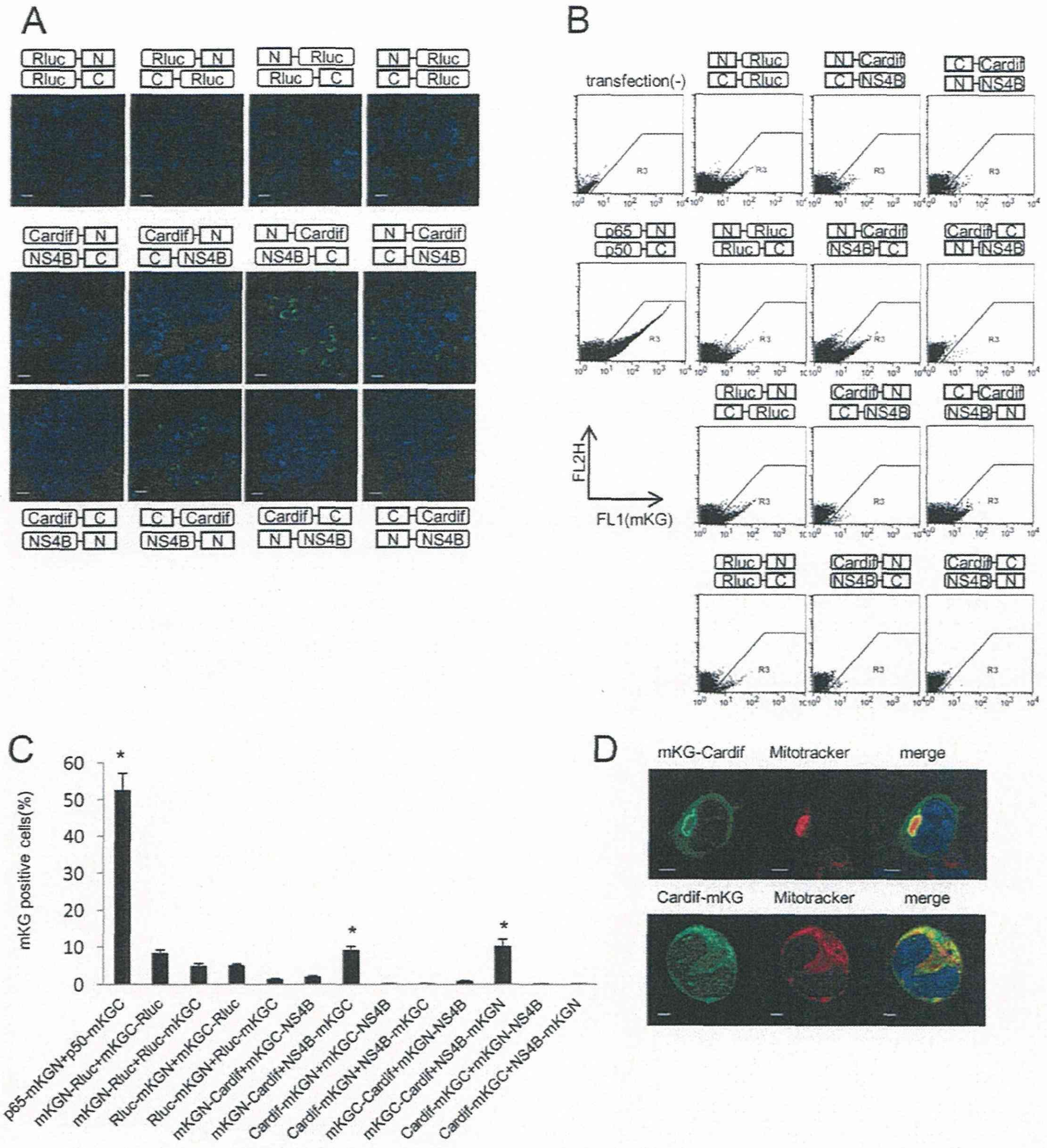

Fig. 4. BiFC assays of Cardif and NS4B. The complementary pairs of N- or C-terminally mKG-fused NS4B and Cardif expression plasmids were cotransfected in HEK293T cells. After 24 hours, the cells were fixed and observed by confocal microscopy (A) or subjected to flow cytometry to measure mKG-emitted fluorescence (BiFC signal) and to count BiFC signal-positive cells (B,C). Plasmids expressing p65-mKGN and p50-mKGC individually were used as a BiFC-positive control and plasmids expressing N- or C-terminally mKG-fused Rluc were used as a negative control. The letters N and C denote complimentary N- and C-terminal fragments of mKG, respectively. Assays were performed in triplicate, and error bars indicate the mean ± SD. Scale bars indicate 10 μm (A). *$P < 0.05$ compared with corresponding negative controls. (D) Plasmids expressing mKG fragment-fused STING or NS4B were transfected in HEK293T cells. After 24 hours, the cells were fixed and immunostained with anti-mKG antibody. Mitochondria were stained using Mitotracker, and nuclei were stained with DAPI. Cells were observed by confocal microscopy. Scale bars = 5 μm.

NS4B-N, and C-Cardif and N-NS4B (Fig. 4A,B). The percentage of cells positive for BiFC signal increased with the combination of N-Cardif and NS4B-C, and C-Cardif and NS4B-N (Fig. 4C). Fluorescence microscopy indicated that mKG-Cardif, but not Cardif-mKG, was partially colocalized with mitochondria, possibly due to disruption of mitochondria anchor domain by C-terminal fusion with mKG (Fig. 4D). These results indicate the lack of significant molecular interactions between NS4B and Cardif.

Binding of NS4B to STING Blocks Molecular Interaction Between Cardif and STING. It has been reported that STING binds Cardif directly.[20,22] Thus, we hypothesized that NS4B, through a competitive

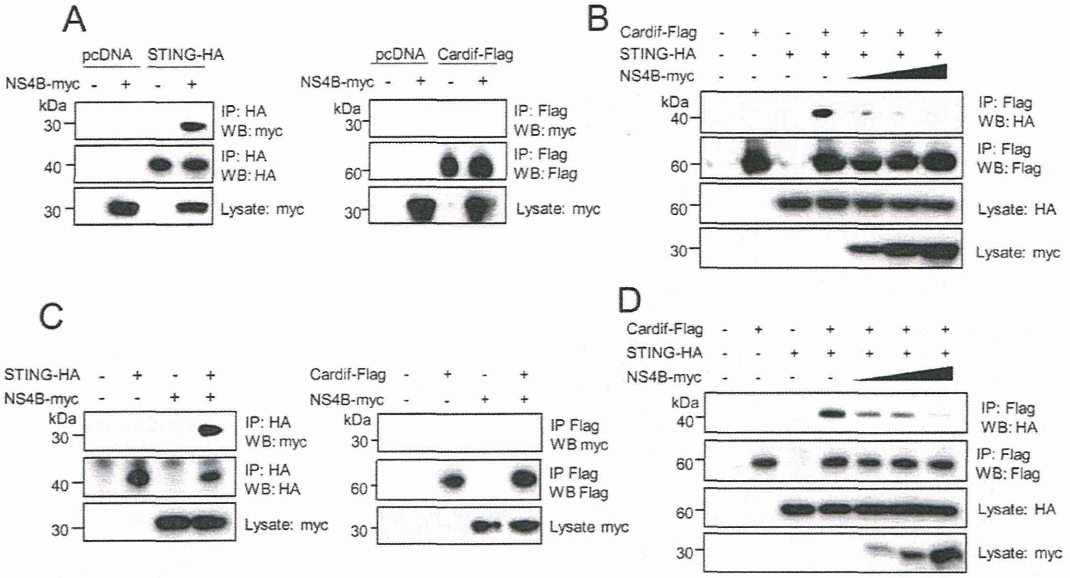

Fig. 5. Binding of NS4B to STING blocks molecular the interaction between Cardif and STING. (A,C) NS4B expression plasmid was cotransfected with STING or Cardif expression plasmid into HEK293T cells (A) or Huh7 cells (C). After 24 hours, cell lysates were subjected to immunoprecipitation using anti-HA or anti-Flag and were immunoblotted with anti-myc. (B,D) Cardif and STING expression plasmids were cotransfected with various amounts of NS4B plasmid in HEK293T cells (B) or Huh7 cells (D). After 24 hours, cells lysates were subjected to immunoprecipitation using anti-Flag and were immunoblotted with anti-HA.

interaction with STING, may hinder the direct molecular interaction between Cardif and STING. To verify this hypothesis, we performed immunoprecipitation assays. First, we transfected plasmids that expressed NS4B and Cardif, or NS4B and STING, in HEK293T cells or Huh7 cells, and performed immunoprecipitation. NS4B strongly bound to STING in both HEK293T cells and Huh7 cells, suggesting specific molecular interactions, whereas NS4B and Cardif did not show any obvious interaction (Fig. 5A,C). Consistent with previous reports, STING and Cardif showed significant interaction (Fig. 5B,D). Interestingly, those interactions were decreased by coexpression of NS4B, depending on its input amount, and finally blocked completely in both HEK293T and Huh7 cells (Fig. 5B,D). Collectively, the results above demonstrate that NS4B disrupts the interaction between Cardif and STING possibly through competitive binding to STING.

Effects on HCV Infection and Replication Levels by STING Knockdown and NS4B Overexpression. We next studied the impact of STING-mediated IFN production and its regulation by NS4B on HCV infection and cellular replication. First, we transfected three STING-targeted siRNAs into Huh7/Feo cells (Fig. 6A). As shown in Fig. 6B, STING knockdown cells conferred significantly higher permissibility to HCV replication. We next transfected HCV-JFH1 RNA into Huh7 cells that were transiently transfected with NS4B. As shown in Fig. 6C, HCV core protein expression was significantly higher in NS4B-overexpressed cells. Furthermore, HCV replication was increased significantly in Huh7/Feo cells overexpressing NS4B (Fig. 6D). Taken together, the results above demonstrate that STING and NS4B may negatively or positively regulate cellular permissiveness to HCV replication.

The N-terminal Domain of NS4B Is Essential for Suppressing IFN-β Promoter Activity Mediated by RIG-I, Cardif, and STING. It has been reported that the N-terminal domain of several forms of flaviviral NS4B shows structural homology with STING.[24] We therefore investigated whether the STING homology domain in NS4B is responsible for suppression of IFN-β production. We constructed two truncated NS4B expression plasmids, which covered the N terminus (NS4Bt1-84, amino acids 1 through 84) containing the STING homology domain and the C terminus (NS4Bt85-261, amino acids 85 through 261), respectively (Fig. 7A). Immunoblotting showed that NS4Bt1-84 and NS4Bt85-261 yielded protein bands of ~9 kDa and ~20 kDa, respectively. Aberrant bands in the truncated NS4B may be due to alternative post-translational processing. HEK293T cells were transfected with ΔRIG-I, Cardif, or STING, and NS3/4A or the truncated NS4B, along with IFN-β-Fluc plasmid, and a reporter assay was performed. NS4Bt1-84 significantly suppressed RIG-I, Cardif, and STING-