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Superinfection Exclusion in Cells Infected with Hepatitis C Virus▽

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Superinfection exclusion is the ability of an established virus infection to interfere with infection by a second virus. In this study, we found that Huh-7.5 cells acutely infected with hepatitis C virus (HCV) genotype 2a (chimeric strain J6/JFH) and cells harboring HCV genotype 1a, 1b, or 2a full-length or subgenomic replicons were resistant to infection with cell culture-produced HCV (HCVcc). Replicon-containing cells became permissive for HCVcc infection after treatment with an HCV-specific protease inhibitor. With the exception of cells harboring a J6/JFH-FLneo replicon, infected or replicon-containing cells were permissive for HCV pseudoparticle (HCVpp) entry, demonstrating a postentry superinfection block downstream of primary translation. The surprising resistance of J6/JFH-FLneo replicon-containing cells to HCVpp infection suggested a defect in virus entry. This block was due to reduced expression of the HCV coreceptor CD81. Further analyses indicated that J6/JFH may be toxic for cells expressing high levels of CD81, thus selecting for a CD81^{low} population. CD81 down regulation was not observed in acutely infected cells, suggesting that this may not be a general mechanism of HCV superinfection exclusion. Thus, HCV establishes superinfection exclusion at a postentry step, and this effect is reversible by treatment of infected cells with antiviral compounds.

Hepatitis C virus (HCV) is an important human pathogen associated with the development of chronic liver disease. It is the type member of the *Hepacivirus* genus, which in addition to the *Pestivirus* and *Flavivirus* genera comprises the family *Flaviviridae*. Viruses within the family *Flaviviridae* are enveloped with a single-stranded RNA genome of positive polarity (reviewed in reference 33). Translation of the viral RNA genome is driven by an internal ribosome entry site (IRES), generating a polyprotein that is processed into structural and nonstructural proteins. The structural proteins, core (C), E1, and E2, form the physical virion. The nonstructural proteins, p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B, form the viral replication complex or assist in the assembly of virions.

Current understanding of HCV entry, translation, and replication events in cell culture has been learned through the use of HCV full-length and subgenomic replicons (reviewed in reference 2) and retroviral pseudoparticles bearing HCV E1 and E2 glycoproteins (HCVpp) (4, 19, 24). The recent development of an infectious system that permits the growth of HCV in cell culture (HCVcc) has enabled study of the complete viral life cycle (30, 44, 51). Both HCVpp and HCVcc entry is mediated by the E1 and E2 envelope glycoproteins, proceeding in a CD81- and pH-dependent manner (23, 30, 44, 50, 51).

Superinfection exclusion, or homologous interference, is the ability of an established virus infection to interfere with secondary virus infection. Multiple mechanisms that contribute to superinfection exclusion have been demonstrated for various viruses, including interference with receptor-mediated attachment (10, 11, 41, 42) and penetration into cells (39, 40, 47), as well as downstream replication events (1, 22, 26). We and others have previously described superinfection exclusion for the *Pestivirus* bovine viral diarrhea virus (BVDV) (29, 35) and established that cells acutely infected with BVDV exhibit blocks to superinfection at both the level of entry and viral RNA replication (29).

In this study, we observed that cells acutely infected with HCV genotype 2a chimeric strain J6/JFH and cells harboring HCV RNAs from a range of genotypes were resistant to HCVcc superinfection. Further analysis revealed that superinfection was blocked downstream of viral entry. Unlike cells acutely infected with J6/JFH and cells supporting Con1 and H77 full-length, and Con1, H77, and JFH subgenomic replicons, cells containing a persistent, full-length J6/JFH replicon were nonpermissive for HCVpp. Study of this HCVpp-resistant, stable J6/JFH replicon population suggested that J6/JFH replication/infection applies a strong negative selection for CD81-expressing cells. This observation could be mimicked at later time points after infection with J6/JFH HCVcc.

MATERIALS AND METHODS

Cells and HCV-specific inhibitors. Huh-7.5 cells (9) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1 mM nonessential amino acids. Media and reagents were purchased from GIBCO-BRL, Life Technologies Ltd. Replicon cells were maintained in 0.5 mg/ml G418. BILN 2061 (28) and 2′-C-methyladenosine (12) were resuspended at a concentration of 10 mM in dimethyl sulfoxide (DMSO).

VSV stocks and plaque assays. Vesicular stomatitis virus (VSV) San Juan strain stocks were generated by infection (multiplicity of infection [MOI] of 0.01) of BHK-J cells (31) and collection of cell supernatants at approximately 17 h

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postinfection (hpi). For plaque assays, cells were seeded in six-well plates and infected in duplicate with dilutions of virus. Cells were overlaid with 1× minimal essential medium, 0.6% LE agarose, and 2.5% FBS, and incubated for 16 h when they were fixed with 7% formaldehyde and stained with crystal violet. Plaques were enumerated, and titers were expressed as PFU per milliliter.

Plasmid construction. pFLneo-J6/JFH was created via three-piece ligation of a 9,903-bp Acc65I/SpeI fragment from pSGR/JFH1 (27), a 1,040-bp Acc65I/MfeI fragment from pCR2/EMC-J6, and a 2,849-bp MfeI/SpeI fragment from pFL-J6/JFH1 (30). pCR2/EMC-J6 was generated by TOPO-TA cloning into pCR2.1 (Invitrogen) of a PCR product containing the encephalomyocarditis virus (EMCV) IRES fused to the core through the NS2 gene region of HCV strain J6. This product was generated in two steps via overlap extension. One first-round PCR utilized oligonucleotides RU-O-3841 (5′-CTCTCCTCAAGCGTATTCAA C-3′) and RU-O-5720 (5′-GGTATTATCGTGTTTTTCAAAGGAAAACCA-3′) to amplify the EMCV IRES from a derivative of Con1-SGneo (I) (7). The other first-round PCR utilized oligonucleotides RU-O-5726 (5′-TGGTTTTCCTTTGA AAAACACGATAATACCATGAGCACAAATCCTAAACCTCAAAG-3′) and RU-O-5727 (5′-GGCATAAGCAGTGATGGGAGCGAGAAGACTCCACCC CTTG-3′) to amplify the core to NS2 region of HCV-J6 from pJ6CF (48). First-round PCR products were purified by using the QIAquick PCR purification kit (QIAGEN) and used as templates in a second-round PCR with oligonucleotides RU-O-3841 and RU-O-5727. pSGR-JFH1 has been described previously (27), and cells harboring this replicon (see below) will be referred to as JFH-SGneo cells. Con1-FLneo (I) and Con1-SGneo (I) are full-length and subgenomic genotype 1b (Con1 isolate) constructs, respectively, which contain an S2204I mutation in NS5A and have been described previously (7, 9) and will be referred to here as Con1-FLneo and Con1-SGneo. H-FLneo and H-SGneo are HCV genotype 1a (H77 isolate) full-length and subgenomic replicons, respectively, that contain the adaptive mutations P1496L, V1880A, A1940V, and C1968R, allowing for persistent replication of the H77 genome in Huh-7.5 cells. These adaptive mutations were identified after transfection of Huh-7.5 cells with H-SGneo (L) (8) and selection with G418 and subsequently reengineered into the H-SGneo (L) or H-FLneo (L) backbone (J. Fan and C. M. Rice, unpublished data).

RNA transcription and generation of HCV full-length and subgenomic replicon cell populations. FL-J6/JFH-5′C19Rluc2AUbi, pFLneo-J6/JFH, and pSGR-JFH1 RNAs were synthesized as described previously (43) by in vitro transcription of an XbaI-linearized template using the T7 MEGAscript kit (Ambion) and purification with the RNeasy mini kit (QIAGEN) with on-column DNase treatment. Con1 and H77 constructs were linearized with ScaI or HpaI, respectively, and utilized as templates for in vitro transcription as described above. J6/JFH-FLneo, JFH-SGneo, Con1-FLneo, Con1-SGneo, H-FLneo, and H-SGneo replicon cell populations were generated by electroporation of Huh-7.5 cells with 1 μg of the corresponding in vitro-transcribed RNA, as described previously (43). Replicon-containing cells were selected and maintained with 0.5 mg/ml G418.

Generation of FL-J6/JFH and HCVcc-Rluc virus stocks and HCVcc infections. Stocks of FL-J6/JFH and FL-J6/JFH-5′C19Rluc2AUbi (HCVcc-Rluc) were generated as described previously (30, 43) by electroporation of in vitro-transcribed RNA into Huh-7.5 cells. For HCVcc-Rluc infections, cells were incubated with undiluted virus (MOI of ~0.01) for 1 to 3 h at 37°C. Cells were then washed twice with media, incubated for 24 h, and harvested for luciferase assays. For infections including 2′-methyladenosine, drug (1.35 μM) was added to the media at the time of infection and maintained during the 24-h incubation. At each time point, cells (untreated or treated with inhibitor) were harvested from triplicate wells for luciferase assays, and cells from duplicate wells were analyzed for HCV RNA levels. All infections of neomycin-resistant cell populations were performed in the absence of G418.

Generation of HCVpp stocks and HCVpp infection. HCV pseudoparticles expressing firefly luciferase (HCVpp-Luc), VSV G protein pseudoparticles expressing firefly luciferase (VSVGpp-Luc,) and no-envelope control viruses were generated in 293T cells as described previously (24) by cotransfection of an envelope-deficient human immunodeficiency virus (HIV) genome (pNL4-3.Luc.R$^-$.E$^-$) and a plasmid expressing HCV H77 (21) or J6 (34) viral glycoproteins E1 and E2, VSV G, or an empty vector (no envelope). For infection, cells that had seeded in a 96-well plate were incubated with HCVpp, VSVGpp, or no-envelope pseudoparticles in the presence of Polybrene (4 μg/ml) for 6 to 12 h at 37°C. Luciferase activity was determined at 72 hpi. HCVpp relative luciferase units (RLU) values were normalized to VSVGpp values for each sample. Infections of neomycin-resistant cell populations were performed in the absence of G418. To generate HCVpp expressing a green fluorescent protein (GFP) reporter (HCVpp-GFP) and control VSVGpp-GFP, 293T cells were cotransfected as described above with the CSGW HIV provirus encoding only GFP (18), a plasmid expressing the HIV *gag-pol* genes, and either an HCV H77 E1 and E2 or the VSV G-protein expression plasmid. Infections were performed as described above, and cells were harvested for flow cytometry at 48 hpi.

Luciferase assays. For *Renilla* (HCVcc expressing *Renilla* luciferase [HCVcc-Rluc]) and firefly luciferase assays (HCVpp-Luc and VSVGpp-Luc), cells were lysed with *Renilla* or cell culture lysis buffer (Promega), respectively. Lysates were harvested and mixed with luciferase assay substrate (Promega), as suggested by the manufacturer. Luciferase activity was measured using a Centro LB960 luminometer (Berthold Technologies, Bad Wildbad, Germany). For HCVcc-Rluc infections, RLU values were normalized to the number of viable cells in each sample by quantitating total cellular ATP levels using the CellTiter-Glo luminescent cell viability assay (Promega) according to the manufacturer's instructions. The normalization step was performed to control for differences in cell size or viability that might exist among the various cell populations used in this study.

Immunostaining and flow cytometry. For live-cell surface staining, cells were detached by a brief treatment with trypsin and resuspended in 3% FBS-0.05% sodium azide in phosphate-buffered saline (PBS). Cells were incubated with anti-CD81 1.3.3.22 (Santa Cruz Biotechnology, Santa Cruz, CA) or purified mouse immunoglobulin G1 (IgG) (BD Pharmingen, Franklin Lakes, NJ) primary antibodies, washed with WB, then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Molecular Probes, Carlsbad, CA). Alternatively, cells were incubated with phycoerythrin-conjugated CD81 antibody (monoclonal antibody [MAb] JS-81; BD Pharmingen). Cells were washed, fixed in 0.5% paraformaldehyde, and analyzed by flow cytometry on a FACS-Calibur (Becton Dickinson), counting 10^4 cells. For total cell staining, cells were detached as described above and fixed in 0.5% paraformaldehyde. Fixed cells were permeabilized in 0.5% FBS-0.1% saponin in PBS and incubated with CD81 or isotype control primary antibody, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody. After the cells were washed, they were analyzed by flow cytometry as described above. For CD81/NS5A costaining, live cells were detached and stained with phycoerythrin-conjugated CD81 antibody (MAb JS-81; BD Pharmingen), fixed with paraformaldehyde, permeabilized, and stained for NS5A using anti-NS5A primary antibody (MAb 9E10) (30), followed by Alexa Fluor 647-conjugated goat anti-mouse IgG2a-specific secondary antibody (Invitrogen). Cells were then analyzed by flow cytometry as described above.

Immunoblotting. Cells were lysed in sodium dodecyl sulfate (SDS) loading buffer (2% SDS, 100 mM Tris-Cl, pH 6.8, 20% glycerol). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. CD81 was detected using MAb JS-81 (BD Pharmingen), followed by horseradish peroxidase-conjugated anti-mouse secondary antibody (Pierce). β-Actin was detected with MAb AC-15 (Sigma) and an anti-mouse secondary antibody as described above. Detection was performed with the SuperSignal West Pico chemiluminescent substrate (Pierce).

Transduction of J6/JFH-FLneo cells with TRIP-CD81 or TRIP-CD9. The lentiviral vector TRIP expressing wild-type human CD81 (TRIP-CD81) or CD9 (TRIP-CD9) protein has been described previously (50). For transduction, cells were incubated overnight with undiluted TRIP-CD81 or TRIP-CD9 lentivirus in the presence of Polybrene (4 μg/ml). Forty-eight hours posttransduction, CD81 surface expression was confirmed by immunostaining and flow cytometry, and cells were infected with HCVpp-Luc (H77) as described above.

Real-time quantitative reverse transcription-PCR (RT-PCR). To amplify CD81 RNA, total RNA was harvested using the RNeasy mini kit (QIAGEN). Fifty to 150 ng of total cellular RNA was mixed with primers and probe specific for CD81 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA. RNAs were amplified using the Platinum Quantitative reverse transcription-PCR ThermoScript one-step system (Invitrogen) and detected with the ABI Prism 7700 sequence detection system (PE Applied Biosystems, Foster City, CA). cDNAs were generated by reverse transcription at 50°C for 30 min, followed by inactivation of the reverse transcriptase at 95°C for 5 min. cDNAs were amplified with 40 cycles of 95°C for 15 s and 60°C for 1 min. The CD81-specific primers and 6-carboxyfluorescein-labeled probe were purchased from Applied Biosystems using a TaqMan inventoried gene expression assay for CD81 (assay identification code, Hs00174717_m1). GAPDH-specific primers and VIC-labeled probe were purchased from Applied Biosystems (catalog no. 4326317E). CD81 RNA levels were normalized to the levels of GAPDH RNA.

HCV RNA was amplified from 50 ng of total RNA using the 3′NTR Multi-Code real-time quantitative RT-PCR system (EraGen) and detected as described above with the ABI Prism 7700. cDNAs were generated by reverse transcription at 45 to 60°C for 15 min (1 min per degree) and amplified with 40 cycles of 95°C for 20 s, 60°C for 30 s, and 72°C for 30 s. The sequence of the forward primer, labeled with 5′ 6-carboxyfluorescein isoC (2′-deoxy-5-methylisocytidine) (Biosearch Technologies, Inc.), was 5′-GGCTCCATCTTAGCCCT

AGTC-3′, and the sequence of the reverse primer was 5′-AGTATCGGCACT CTCTCTGCAGT-3′.

Treatment of HCV replicon-containing cells with BILN 2061. HCV subgenomic replicon cell populations containing H-SGneo, Con1-SGneo, and JFH-SGneo replicons were treated with 40 nM (H-SGneo and Con1-SGneo) or 5 μM BILN 2061 (JFH-SGneo) in DMSO, 10× or 20× the 50% effective concentration (EC_{50}) determined in reference 30, respectively, in the absence of G418. The medium was changed every 2 or 3 days. HCV RNA levels were determined in BILN 2061- or DMSO-treated cells at 4 and 9 days posttreatment at which point the cells were challenged with HCVcc-Rluc. The RLU values from each cell population on day 4 or 9 were expressed as the percentage of the DMSO-treated Huh-7.5 cell RLU on day 4 or 9, respectively. J6/JFH-FLneo replicon-containing cells were treated with 5 μM BILN 2061 or DMSO for 9 days, after which the concentration of drug was raised to 20 μM. HCV RNA levels were determined at 4, 9, and 23 days posttreatment when they were analyzed for CD81 surface expression and HCV RNA levels, and challenged with HCVpp-Luc (H77).

RESULTS

Superinfection exclusion in HCVcc-infected and replicon-containing Huh-7.5 cells. To determine whether previous infection with HCV renders cells resistant to secondary HCV infection, we infected Huh-7.5 cells with the J6/JFH genotype 2a chimeric virus (MOI of 0.01). At 4 days postinfection (dpi), when approximately 77% of cells stained positive for NS5A antigen (data not shown), we challenged the cells that had been acutely infected with J6/JFH, in parallel with naïve Huh-7.5 cells, with the HCVcc *Renilla* luciferase reporter virus FL-J6/JFH-5′C19Rluc2AUbi (43) (HCVcc-Rluc). Luciferase activity resulting from HCVcc superinfection of J6/JFH-infected cells was <10% of the Huh-7.5 luciferase signal, suggesting that superinfection exclusion is established in HCV-infected cells (Fig. 1A).

To determine whether superinfection exclusion occurs across genotypes and in cells harboring long-term, persistent HCV replication, we generated HCV genotype 1a, 1b, and 2a full-length replicon-bearing cell populations. The bicistronic RNAs, H-FLneo (genotype 1a, H77), Con1-FLneo (genotype 1b, Con1), and J6/JFH-FLneo (genotype 2a, J6/JFH chimera), used to establish these replicon cells express the dominant selectable marker neomycin *N*-acetyltransferase (*neo*), which provides resistance to the drug G418 and thus allows stable maintenance of cells harboring replicating HCV RNA. The expression of *neo* is driven by the HCV IRES, while the EMCV IRES drives translation of the HCV polyprotein in the downstream cistron.

Following challenge of H-FLneo, Con1-FLneo, and J6/JFH-FLneo cells, as well as Huh-7.5 parental cells with HCVcc-Rluc (MOI of 0.01), all of the replicon-containing cells were able to efficiently exclude HCVcc, yielding <10% of the luciferase activity of infected Huh-7.5 cells (Fig. 1B). This result indicates that cells harboring HCV genotype 1a, 1b, or 2a, exhibit superinfection exclusion against genotype 2a HCVcc, similar to that observed in cells acutely infected with J6/JFH (Fig. 1A). To rule out the possibility that resistance to HCV infection was due to expression of the *neo* resistance gene by the HCV replicons, we challenged Huh-7.5 cells, which had been transiently transfected with a plasmid expressing *neo*, with HCVcc-Rluc. Luciferase activity from the transfected cells was >80% of naïve Huh-7.5 cells, despite similar levels of neomycin phosphotransferase II expression compared to the HCV replicon-containing cells (data not shown).

To establish if expression of the HCV structural-NS2 region was required for superinfection exclusion, we challenged Huh-7.5 cell populations selected for stable maintenance of H-SGneo, Con1-SGneo, or JFH-SGneo RNAs, the subgenomic counterparts of the full-length genomes described above. These genomes express *neo* and HCV NS3-NS5B via the HCV

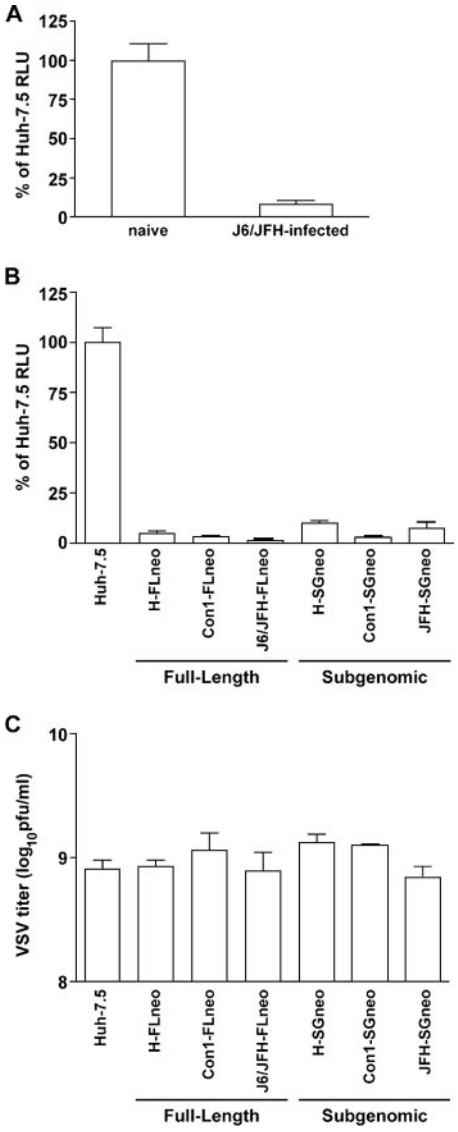

FIG. 1. Cells harboring HCV do not support HCVcc superinfection. (A) Huh-7.5 cells were infected with J6/JFH HCVcc and maintained in parallel with naïve cells. At 4 dpi, when approximately 77% of cells were NS5A positive, cells acutely infected with J6/JFH and naïve cells were superinfected with HCVcc-Rluc. Each bar, expressed as the percentage of the Huh-7.5 cell RLU, is the average value of triplicate wells; error bars show the standard deviations. RLU, relative light units. (B) Huh-7.5 cells and HCV full-length or subgenomic replicon-containing cells were infected with HCVcc-Rluc. Luciferase activity was measured at 24 hpi. Values, expressed as the percentage of Huh-7.5 RLU, are the combined data from two independent experiments done in triplicate; error bars represent the standard errors of the means. (C) Huh-7.5 cells and HCV replicon-containing cells were infected with VSV and overlaid with agarose. After 16 h of incubation, cells were fixed with formaldehyde and stained with crystal violet to visualize plaques. Each bar is the mean titer from duplicate wells; error bars represent the standard errors of the means.

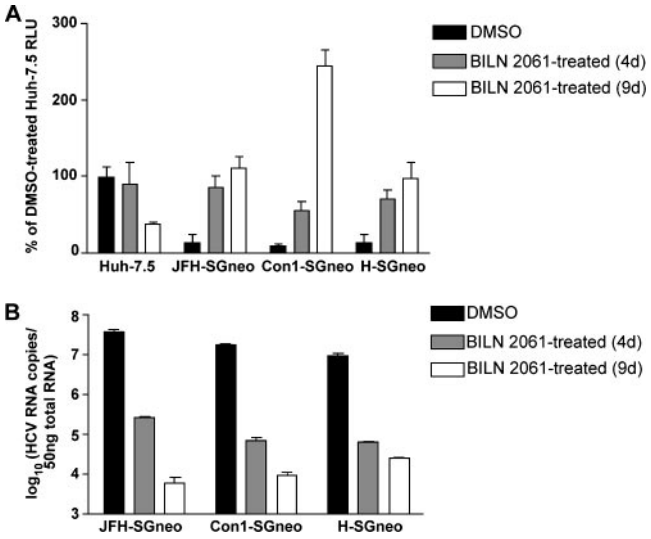

FIG. 2. HCV subgenomic replicon-containing cells treated with an HCV-specific protease inhibitor become permissive for HCVcc infection. (A) Huh-7.5, JFH-SGneo, Con1-SGneo, and H-SGneo cells were treated with BILN 2061 or DMSO in the absence of G418 for 4 days (4d) or 9 days (9d). BILN 2061- or DMSO-treated cells were infected with HCVcc-Rluc in the absence of inhibitor, and samples were harvested for luciferase assays at 24 hpi. Black bars (DMSO-treated cells) represent the average RLU value over days 4 and 9 for each DMSO-treated cell population compared to that in Huh-7.5 cells. RLU values from BILN 2061-treated cells (gray and white bars) on day 4 or 9 are expressed as the percentage of the DMSO-treated Huh-7.5 RLU on day 4 or 9, respectively. Each bar is the average value of triplicate wells; error bars show the standard deviations. The data shown are representative of at least two independent experiments. (B) HCV RNA levels in DMSO- or BILN 2061-treated JFH-SGneo, Con1-SGneo, and H-SGneo replicon-containing cells at 4 (gray bars) or 9 days (white bars) posttreatment were determined by real-time quantitative RT-PCR. Black bars (DMSO-treated cells) represent the average RNA level over days 4 and 9 for each DMSO-treated cell population. Error bars show the standard deviations.

IRES and EMCV IRES, respectively. Similar to the results observed with cells containing a full-length replicon, luciferase activity after infection of the cell populations containing HCV subgenomic replicons with HCVcc-Rluc was reduced to <15% compared to naïve Huh-7.5 cells (Fig. 1B).

Infection of replicon-containing cells with VSV yielded titers that were comparable to those observed on Huh-7.5 cells (Fig. 1C). This excludes the possibility that the observed HCVcc superinfection exclusion was due to the induction of a nonspecific antiviral state.

Treatment with BILN 2061 restores HCV permissiveness. We treated the H-SGneo, Con1-SGneo, and JFH-SGneo replicon-containing cells with the HCV-specific protease inhibitor BILN 2061 and subsequently challenged with HCVcc-Rluc. At 4 days posttreatment, HCVcc permissiveness was partially restored in H-SGneo, Con1-SGneo, and JFH-SGneo cells that had been treated with BILN 2061, as RLU values from these cells, and not from DMSO-treated cells, were at least 50% of the values for Huh-7.5 control cells (Fig. 2A). The increase in HCVcc permissiveness in BILN 2061-treated cells coincided with an approximately 100-fold decrease in HCV RNA levels compared to the DMSO-treated cells (Fig. 2B). By day 9 of BILN 2061 treatment, viral RNA levels had decreased further, and the cells became fully permissive for HCVcc infection. A slight but unexpected decrease in luciferase activity was observed in BILN 2061-treated Huh-7.5 cells at the later time points. This was observed in several repetitions of the experiment (data not shown), suggesting that prolonged BILN 2061 treatment may create a cellular environment less favorable for HCV replication or select a subpopulation of less permissive cells. In any case, the dramatic increase in HCVcc permissiveness observed in BILN 2061-treated replicon-containing cells indicates that superinfection exclusion requires the continuing presence of HCV RNA and/or proteins and is readily reversible by treatment with specific antivirals.

Superinfection exclusion occurs downstream of viral entry. Given that superinfection exclusion occurs at both entry and replication for BVDV (29), we used HCVpp to examine HCV entry in acutely infected or stable replicon-containing cell populations. HCV glycoprotein-mediated HCVpp entry was monitored via expression of luciferase from the encapsidated HIV RNA genome (HCVpp-Luc) (4, 24). The luciferase activity of cells acutely infected with J6/JFH (4 dpi, 77% HCV NS5A positive; Fig. 1A) was similar to that of naïve Huh-7.5 cells when infected with HCVpp-Luc bearing J6 glycoproteins (Fig. 3A). This suggested a block downstream of entry, given the resistance of this cell population to HCVcc.

Full-length and subgenomic HCV replicon-containing cells were also challenged with HCVpp-Luc bearing J6 or H77 glycoproteins. With the exception of cells harboring the stable J6/JFH-FLneo replicon, all of the replicon-containing cells were fully permissive for HCVpp (Fig. 3B), suggesting that the dominant mechanism(s) of HCVcc exclusion in these selected cell populations was also postentry.

Unexpectedly, HCVpp-expressed luciferase activity in the stable J6/JFH-FLneo replicon population was reduced 98% (J6 HCVpp) and 80% (H77 HCVpp) compared to that in control Huh-7.5 cells, in contrast to cells acutely infected with J6/JFH, which were fully permissive to HCVpp (Fig. 3A). These data suggest that in cells selected for long-term, persistent J6/JFH-FLneo replication, there is an additional block at the level of entry. We address this surprising observation in more detail below.

Examination of the postentry superinfection block. To further define the superinfection block, we determined whether translation of incoming HCV RNA was inhibited. To monitor translation in the absence of viral replication, JFH-SGneo replicon and parental Huh-7.5 cells were infected with HCVcc-Rluc in the presence of 2′-C-methyladenosine, a nucleoside inhibitor of the NS5B RNA-dependent RNA polymerase (12). 2′-C-methyladenosine was used at a 50-fold excess over the EC_{50} reported for HCV genotype 2a (30), and we confirmed that HCV RNA levels in HCVcc-infected Huh-7.5 and JFH-SGneo cells declined over the 24-h time course (data not shown).

In the absence of 2′-C-methyladenosine, luciferase activity in HCVcc-infected Huh-7.5 cells steadily increased, and by 24 hpi, it was more than 10-fold greater than the level found in JFH-SGneo replicon cells (Fig. 4A). When 2′-C-methyladenosine was present during HCVcc-Rluc infection, luciferase activity was maximal at 6 hpi and then decreased over 24 h in both Huh-7.5 and JFH-SGneo cells (Fig. 4B). During this time, luciferase activity was equivalent and even slightly greater in

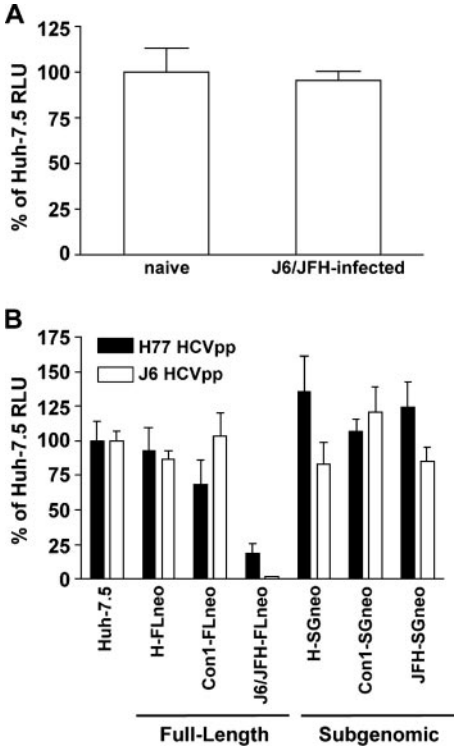

FIG. 3. Cells acutely infected with J6/JFH, but not cells harboring a stable J6/JFH-FLneo replicon, are permissive for HCVpp infection. (A) Huh-7.5 cells were infected with J6/JFH HCVcc and maintained in parallel with naïve cells. At 4 dpi, when approximately 77% of cells were NS5A positive, cells acutely infected with J6/JFH and naïve cells were superinfected with HCVpp-Luc-bearing strain J6 glycoproteins. Each bar, expressed as the percentage of the Huh-7.5 RLU, is the average value of triplicate wells; error bars show the standard deviations. (B) Huh-7.5 cells and HCV full-length or subgenomic replicon-containing cells were infected with HCVpp-Luc bearing HCV genotype 1a strain H77 (black bars) or genotype 2a strain J6 (white bars) glycoproteins or with VSVGpp-Luc. Luciferase activity was measured at 72 hpi. HCVpp-Luc RLU were normalized to VSVGpp-Luc RLU and expressed as a percentage of the Huh-7.5 cell RLU. Each bar shows the combined data from at least two independent experiments done in triplicate; error bars represent the standard errors of the means.

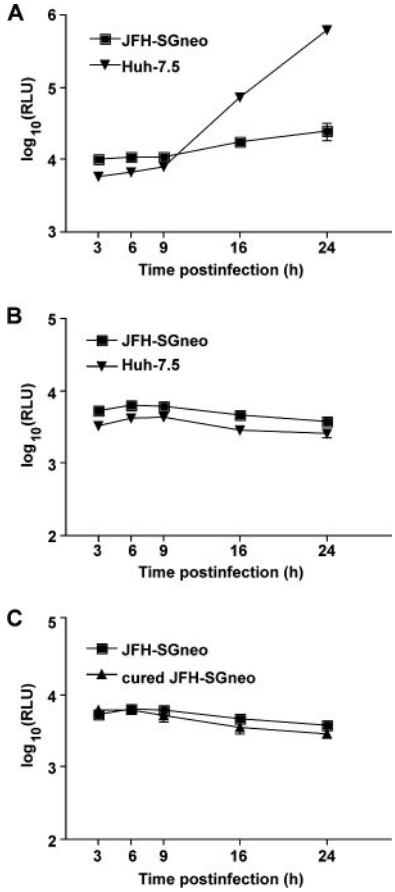

FIG. 4. JFH-SGneo cells exhibit a postentry superinfection block. (A) Huh-7.5 and JFH-SGneo cells were infected with HCVcc-Rluc. At each time point, luciferase activity was determined from triplicate wells and normalized to the average cellular ATP content for each cell type at that time point. Each bar is the average value of triplicate wells; error bars show standard deviations. (B and C) Huh-7.5 cells (B) or BILN 2061-cured JFH-SGneo (C) and JFH-SGneo cells were infected with HCVcc-Rluc in the presence of 2′-C-methyladenosine. At each time point, luciferase activity was determined from triplicate wells and normalized to the average cellular ATP content for each cell type at that time point. Each bar is the average value of triplicate wells; error bars show standard deviations.

JFH-SGneo cells compared to Huh-7.5 cells (Fig. 4B), suggesting that entry, uncoating, and primary translation were unaffected by the presence of the HCV replicon. Similar levels of luciferase activity were also observed in JFH-SGneo and Huh-7.5 cells after transfection with a polymerase-defective luciferase reporter HCV RNA (data not shown). In addition, we compared HCVcc-Rluc primary translation in BILN 2061-cured JFH-SGneo cells to untreated JFH-SGneo cells. Similar to what we had observed above in the presence of 2′-C-methyladenosine, the two cell types had similar HCV translation capacities (Fig. 4C). Moreover, we also found that the BILN 2061-cured and untreated JFH-SGneo cells supported similar levels of translation after infection with a temperature-sensitive Sindbis virus mutant, TOTO1101/Luc:ts110 (6), at the nonpermissive temperature, which allows translation, but is blocked for RNA replication (data not shown). Thus, these data indicate that the superinfection block in JFH-SGneo cells lies downstream from entry, uncoating, and primary translation.

An additional defect in J6/JFH-FLneo selected cells. We further investigated the surprising observation that HCVpp entry was blocked in the J6/JFH-FLneo cell population (Fig. 3B). A possible mechanism for restriction at the level of entry is down regulation of or interference with an essential viral receptor or coreceptor. The tetraspanin CD81, originally identified as an HCV E2 binding protein (37), is an essential HCV coreceptor required for entry of HCVpp and HCVcc (reviewed in references 3 and 16). We examined CD81 surface expression on J6/JFH-FLneo, JFH-SGneo, and Huh-7.5 parental cell lines by flow cytometry. While JFH-SGneo and Huh-7.5 cells expressed similar levels of CD81 at the cell surface (Fig. 5A), levels were greatly reduced on the J6/JFH-FLneo cells (Fig. 5B). In addition, staining of permeabilized cells, Western blot analysis of whole-cell lysates, and real-time

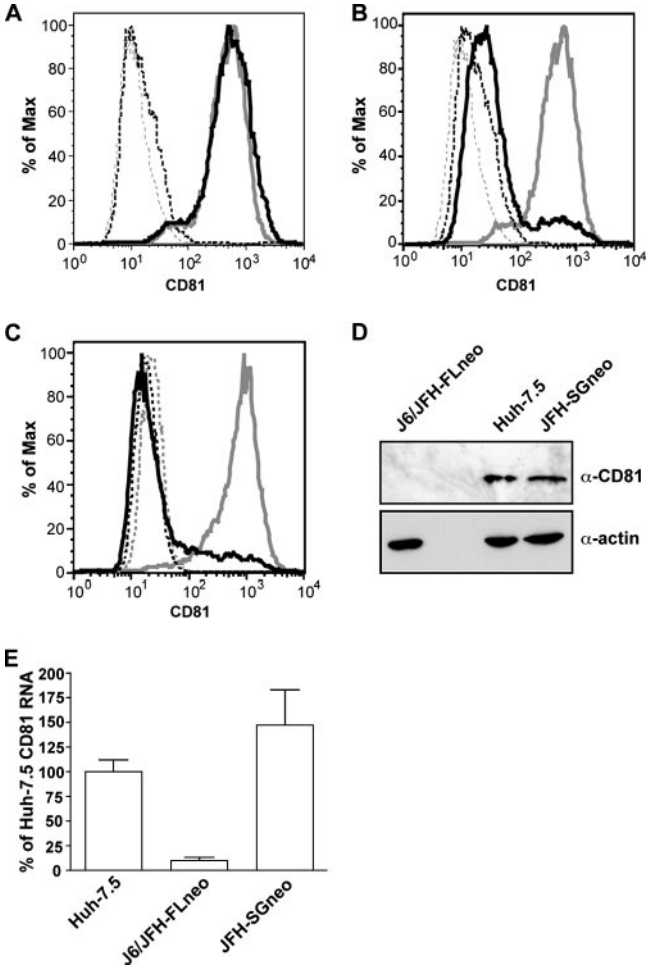

FIG. 5. Stable J6/JFH-FLneo replicon-containing cells have decreased CD81 protein and RNA levels. (A and B) Huh-7.5 (gray line), JFH-SGneo (black line in panel A), and J6/JFH-FLneo (black line in panel B) cells were surface stained for CD81 and analyzed by flow cytometry. Dashed gray and black lines indicate the IgG isotype controls for Huh-7.5 cells and the JFH-SGneo (A) or J6/JFH-FLneo cells (B), respectively. Max, maximum. (C) Permeabilized Huh-7.5 (gray line) and J6/JFH-FLneo (black line) cells were stained for total cell CD81 protein expression and analyzed by flow cytometry. Dashed gray and black lines indicate the IgG isotype controls for Huh-7.5 and J6/JFH-FLneo cells, respectively. (D) Western blot for CD81 (α-CD81) or actin (α-actin) protein expression in lysates from J6/JFH-FLneo, Huh-7.5, and JFH-SGneo cells. (E) CD81 RNA was amplified using real-time quantitative RT-PCR from total RNA derived from Huh-7.5, J6/JFH-FLneo, and JFH-SGneo cells. Values were normalized to GAPDH RNA levels for each sample and are expressed as relative levels compared to those in Huh-7.5 cells. Values are the combined data from three independent experiments done in triplicate; error bars show standard errors of the means.

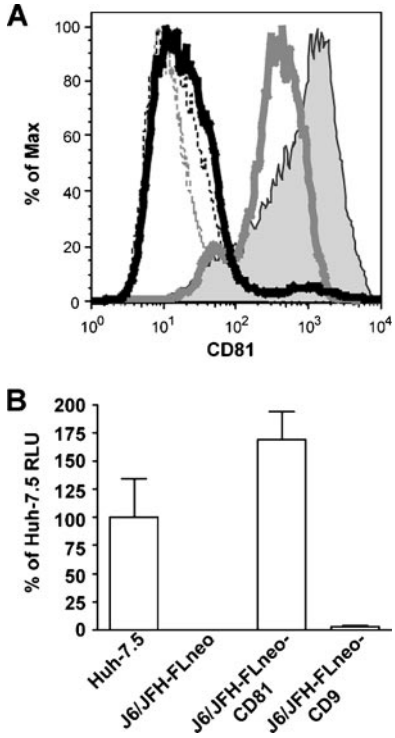

FIG. 6. Ectopic expression of CD81 renders stable J6/JFH-FLneo replicon cells permissive for HCVpp infection. (A) Transduction of J6/JFH-FLneo cells with TRIP-CD81 restores CD81 expression. J6/JFH-FLneo (black lines), J6/JFH-FLneo-CD81 (shaded area), and Huh-7.5 (gray lines) cells were surface stained for CD81 and analyzed by flow cytometry. Dashed lines indicate the IgG isotype controls. Max, maximum. (B) Huh-7.5, J6/JFH-FLneo, J6/JFH-FLneo-CD81, and J6/JFH-FLneo-CD9 cells transduced with TRIP-CD9 were infected with HCVpp-Luc (genotype 1a strain H77) or VSVGpp-Luc, and luciferase activity was measured at 72 hpi. HCVpp-Luc RLU were normalized to VSVGpp-Luc RLU. Each bar is the average value of triplicate wells; error bars show the standard deviations.

quantitative RT-PCR revealed that total cellular CD81 protein and RNA levels were also decreased in J6/JFH-FLneo cells compared to Huh-7.5 cells (Fig. 5C, D, and E). In contrast, CD81 expression on the surfaces of cell populations containing HCV genotype 1a and 1b full-length and subgenomic replicons and on cells acutely infected with J6/JFH was also examined and found to be similar to parental Huh-7.5 cells (data not shown), in keeping with the previous observation (Fig. 3) that these cells were permissive for HCVpp infection. Additionally, the levels of scavenger receptor BI, another proposed HCV entry factor (5, 38) were similar to those in Huh-7.5 parental cells in all of the replicon-containing cells tested, including cells harboring the stable J6/JFH-FLneo replicon and cells acutely infected with J6/JFH (data not shown).

We next determined whether reduced CD81 on J6/JFH-FLneo-containing cells was responsible for the entry defect by restoring CD81 expression. Transduction of the J6/JFH-FLneo cells with the lentiviral vector TRIP-CD81 (50) efficiently restored CD81 expression at the cell surface and permissiveness to HCVpp-Luc to 150% of control Huh-7.5 cells (Fig. 6A and B, J6/JFH-FLneo-CD81). In contrast, J6/JFH-FLneo cells transduced with a lentivirus expressing the tetraspanin CD9 (TRIP-CD9), which does not bind E2 or support HCV entry, was less than 3% of the level in Huh-7.5 cells (Fig. 6B). Thus, overexpression of CD81 rescues infection by HCVpp, indicating that reduced CD81 surface expression is responsible for the entry defect observed in J6/JFH-FLneo cells.

It was unclear whether RNA replication and HCV protein expression by the J6/JFH-FLneo replicon was somehow down regulating CD81 mRNA and/or protein levels or whether G418 selection of the replicon-containing population was fa-

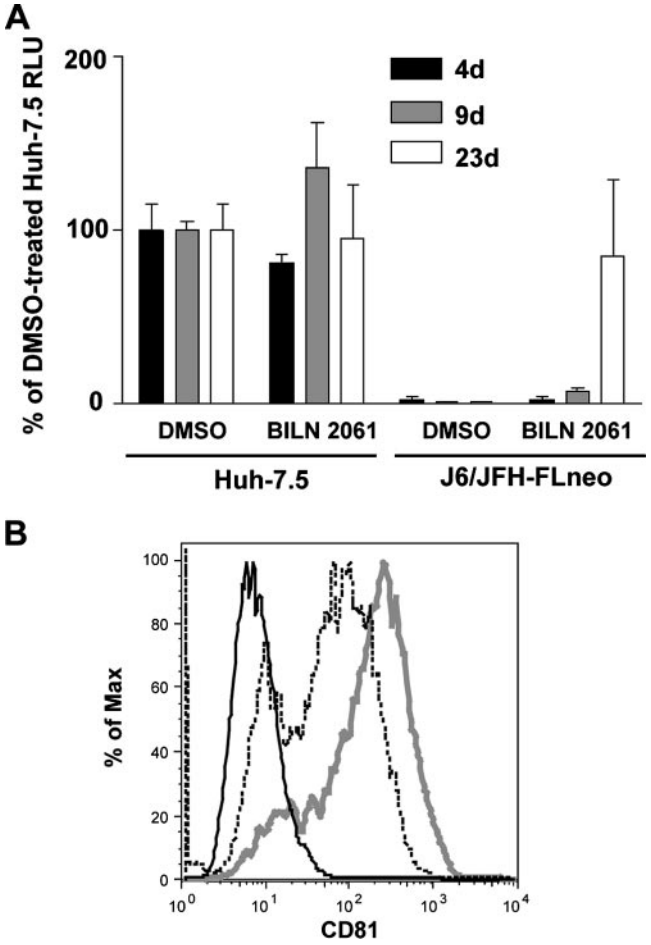

FIG. 7. BILN 2061-treated, J6/JFH-FLneo replicon-containing cells have increased CD81 surface expression and are permissive for HCVpp infection. (A) J6/JFH-FLneo and Huh-7.5 parental cells were treated with BILN 2061 or DMSO for a period of 23 days. At 4 days (4d), 9 days (9d), and 23 days (23d) posttreatment, DMSO- and BILN 2061-treated cells were challenged with HCVpp-Luc or VSVGpp-Luc. Luciferase activity was measured at 72 hpi, and HCVpp-Luc RLU were normalized to VSVGpp-Luc RLU. Each bar, expressed as the percentage of DMSO-treated Huh-7.5 RLU, is the average value of triplicate wells; error bars show the standard deviations. (B) At 23 days posttreatment, BILN 2061 (dashed, black line)- and DMSO (black line)-treated J6/JFH-FLneo and Huh-7.5 (gray line) cells were immunostained for CD81 surface expression and analyzed by flow cytometry. Max, maximum.

voring cells with low or absent CD81. We first examined whether J6/JFH-FLneo cells would regain CD81 expression and HCVpp permissiveness after treatment with BILN 2061. Cells were treated with BILN 2061 at 20× to 50× the reported EC_{50} (30) over a period of 23 days in the absence of G418. Cell surface expression of CD81 and HCVpp-Luc permissiveness were assessed after 4, 9, and 23 days of treatment. At 4 days, J6/JFH-FLneo cells remained resistant to HCVpp-Luc infection despite a 100-fold reduction in HCV RNA levels (Fig. 7A and data not shown). By 9 days, when HCV RNA levels had fallen 1,000-fold, a small increase in luciferase signal was observed. After 23 days of BILN 2061 treatment, HCV RNA levels had fallen to background levels (<0.005 RNA copies/cell), and the cells became permissive for HCVpp-Luc infection with a concomitant increase in CD81 surface expression (Fig. 7B). These results indicate that permissive cells expressing CD81 can emerge after curing the population of replicating J6/JFH-FLneo.

We also examined this phenomenon during long-term J6/JFH infection of Huh-7.5 cells. Cells were infected at an MOI of approximately 0.3 (infected population) and maintained in parallel with naïve cells (naïve population) for a period of 17 days. Both populations were analyzed for CD81 surface expression, NS5A expression, and permissiveness to HCVpp-GFP or to VSVGpp-GFP as a control. Pseudoparticle-challenged cells were harvested at 48 hpi, stained for NS5A, and analyzed by flow cytometry. The percentage of total GFP-positive, i.e., HCVpp-infected cells, both NS5A positive and NS5A negative, was normalized to the percentage of GFP-positive cells from the VSVGpp-GFP infection.

After 1 or 2 days, the infected population was fully permissive for HCVpp (Fig. 8A). Beginning at day 3 and continuing through day 8, there was a decrease in the percentage of GFP-positive cells compared to the naïve population, which correlated with increasing numbers of NS5A-positive cells in the J6/JFH-infected population (Fig. 8A). However, the vast majority of the cells that were HCVpp susceptible (>80%) were also NS5A positive (Fig. 8B). This demonstrated that cells productively infected with J6/JFH were fully permissive for HCV entry. Regarding surface CD81 expression, NS5A-positive and -negative cells had CD81 expression levels comparable to those of naïve cells early in infection (3 and 4 days) (Fig. 8C). After 6 days, surface CD81 on NS5A-positive cells remained comparable (70%) to naïve cells; however, CD81 expression on NS5A-negative cells was greatly reduced (10% of the naïve cell intensity; Fig. 8C). Interestingly, the appearance of this distinct $CD81^{low}$ NS5A-negative population was coincident with an observed cytopathic effect in the infected population (data not shown) and a 65% reduction in HCVpp-GFP permissiveness in the infected population (Fig. 8A). After 6 days and 8 days, NS5A-negative cells made up only a small proportion (approximately 10%) of the J6/JFH-infected cell population. However, this NS5A-negative fraction persisted and increased in frequency to >70% by the final time point, day 17. Notably, the modest decrease in CD81 expression in NS5A-positive cells at 6 days and 8 days was more apparent by 17 days (Fig. 8C). Unlike the J6/JFH-FLneo population, which had been selected with G418 and undergone multiple passages, we saw no decrease in CD81 RNA levels during the 17-day J6/JFH infection (data not shown). These data suggest that J6/JFH infection selects against cells expressing high levels of CD81, possibly due to a cytopathic effect or retarded growth kinetics, leaving $CD81^{low}$, uninfected cells to emerge. This scenario may also explain the low levels of CD81 and HCVpp resistance that we observed in G418-selected cell populations harboring the J6/JFH-FLneo replicon.

DISCUSSION

This report describes and investigates the mechanisms of superinfection exclusion in human hepatoma cells infected with HCV. Superinfection exclusion of HCVcc was observed in cells acutely infected with J6/JFH and in cells containing full-length and subgenomic replicons derived from genotype 1a, 1b,

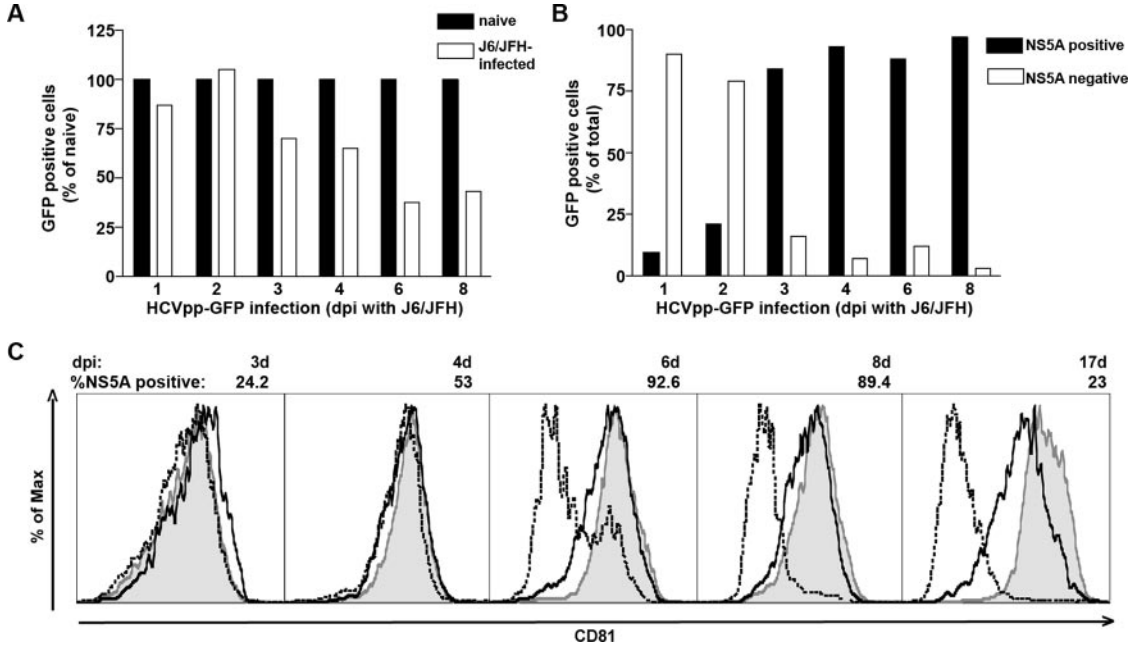

FIG. 8. J6/JFH infection of Huh-7.5 cells promotes the emergence of a CD81^{low} cell population. Huh-7.5 cells were infected with HCVcc (J6/JFH) at an MOI of 0.3 and maintained in parallel with naïve cells. (A) At 1, 2, 3, 4, 6, and 8 dpi with J6/JFH, the infected and naïve cell populations were infected with HCVpp-GFP or VSVGpp-GFP. Cells were harvested at 48 hpi. Cells were stained for NS5A and analyzed by flow cytometry for GFP and NS5A expression. The total number of GFP-positive cells resulting from HCVpp-GFP infection was normalized to the total number of GFP-positive cells in the VSVGpp-GFP-infected samples. Values are expressed as a percentage of GFP-positive cells in the naïve population. (B) The J6/JFH-infected cell population, which was infected with HCVpp-GFP and stained for NS5A in panel A, was grouped into NS5A-positive and NS5A-negative cells. GFP expression in NS5A-positive and NS5A-negative cells was determined and plotted as a percentage of the total GFP-positive cells. (C) The J6/JFH-infected and naïve cell populations were analyzed for CD81 surface expression and NS5A expression using immunostaining and flow cytometry at 3, 4, 6, 8, and 17 dpi with J6/JFH. Cells were gated on NS5A-positive (black line) or NS5A-negative (dashed, black line) cells, and CD81 expression was compared to the naïve population (shaded area). The day postinfection (dpi) and percentage of NS5A-positive cells at each time point are indicated above the plots. 3d, 3 days; Max, maximum.

or 2a genomes. Treatment with an HCV-specific antiviral agent restored permissiveness in these cells, indicating that the superinfection exclusion phenotype was fully reversible and that it required the presence of HCV RNA or proteins. We determined that the block to superinfection in cells harboring HCV replication was downstream from virus entry and translation of the incoming genome RNA, possibly at the level of RNA replication. An additional, unexpected defect at virus entry was observed in cells harboring a persistent full-length J6/JFH-FLneo replicon. Further analyses revealed that this block to HCV entry was due to the loss of CD81^{high} cells within selected or infected cell populations rather than to a classical superinfection exclusion mechanism.

During acute infection, the *Pestivirus* BVDV establishes superinfection blocks to both virus entry and RNA replication (29). Our data indicate that the principal mechanism of superinfection exclusion in cells harboring HCV replication lies downstream from virus entry, uncoating, and translation of the incoming genome RNA. Although it remains possible that a transient block at the level of primary translation may exist during acute infection, our data suggest that in JFH-SGneo replicon-containing cells, the superinfection block is due to a direct effect on a downstream event, most likely at one or more steps in RNA replication.

Interference at the level of RNA replication has been previously examined in the HCV replicon system. HCV replication efficiency was inversely correlated with the amount of replicon RNA transfected into Huh-7 cells (32). This effect was dependent on the presence of the HCV NS3-NS5B replicase proteins. In another study, replication capacity of a transfected replicon was reduced in cells that harbored an existing, replicating HCV genome (20). Similarly, in cells acutely infected with BVDV, the block to superinfecting virus at the level of replication was directly correlated with the level of the primary virus replication (29). These results suggest that viral replication can sequester factors that are limiting and saturable in the host cell, thereby interfering with superinfecting virus replication. There may also be competition for replication sites, which are in specialized membrane compartments similar to those observed during classical *Flavivirus* RNA replication (13–15, 45, 46).

In the course of our HCV superinfection exclusion study, we made the surprising observation that G418-selected cells harboring the J6/JFH-FLneo replicon have an additional defect in virus entry. J6/JFH-FLneo cells were resistant to HCVpp bearing glycoproteins from J6 or H77, whereas cells acutely infected with J6/JFH and cells harboring either genotype 1a or 1b replicons, and genotype 2a subgenomic replicon-bearing cells were fully permissive. Given that the defect was specific to selected J6/JFH-FLneo, but not subgenomic replicon-containing cells suggested that the phenotype was dependent on J6 C-NS2 expression (or an RNA element harbored in this re-

gion). Further analyses revealed that CD81 down regulation was the mechanism responsible for this phenotype. Total and cell surface CD81 protein and CD81 mRNA levels were dramatically reduced in J6/JFH-FLneo cells compared to parental cells. Furthermore, ectopic expression of CD81 rendered J6/JFH-FLneo cells fully permissive for HCVpp infection.

Two possibilities, among others, might explain the low levels of CD81 mRNA in J6/JFH-FLneo cells: (i) HCV-dependent down regulation of CD81 expression or (ii) a selective advantage for cells with low or absent CD81 during G418 selection. Consistent with the latter possibility, prolonged J6/JFH infection yielded a cell population that was increasingly resistant to HCVpp infection, although NS5A-positive cells were readily infected with HCVpp. When CD81 surface expression was examined, it became evident that a population of $CD81^{low}$ NS5A-negative cells had emerged within the J6/JFH-infected cell culture, coincident with and subsequent to a virus-induced cytopathic effect. The pathway by which J6/JFH infection leads to cell death is unknown, but our data indicate that expression of CD81 and the HCV J6 C-NS2 region are involved. Although we did find some $CD81^{low}$ NS5A-positive cells at later stages of J6/JFH infection, the absence of CD81 down regulation during acute infection suggests that this may be due to inefficient infection of cells with lower levels of CD81. Therefore, the main mechanism of this resistance could not be attributed to a classical superinfection exclusion block at the level of virus entry, where replicating HCV prevents a secondary HCV infection. Rather, selection occurs at the population level, and resistance is due to an emerging population of $CD81^{low}$ cells that are less permissive for HCV infection. Similar observations during persistent JFH-1 infection have been recently reported by Zhong et al. (52).

The J6/JFH-FLneo population cured with BILN 2061 in the absence of G418 regained CD81 expression and became permissive for HCVpp entry. Several possibilities either at the population or single-cell level could account for restored CD81 expression. At the population level, $CD81^{low}$ cells might lose their growth advantage over surviving $CD81^{high}$ cells in the absence of J6/JFH-FLneo replication or infection. Restoration of CD81 expression was slow and occurred after HCV RNA reached background levels. This might indicate that $CD81^{high}$ cells have only a subtle growth advantage over $CD81^{low}$ cells in the absence of virus. Alternatively, at the single-cell level, slow recovery of CD81 expression could be due to an intrinsically low rate of CD81 protein accumulation or slowly reversible epigenetic changes that down regulated CD81 expression and occurred due to persistent J6/JFH-FLneo replication and infection.

$CD81^{low}$ cells emerged after prolonged J6/JFH HCVcc infection or selection of G418-resistant populations harboring J6/JFH replicons. However, this was not the case for subgenomic replicons or for full-length Con1 or H77 replicons. One possible explanation for this genotype-specific difference is that high-level expression of the J6 C-NS2 region driven by the JFH replicase is required for CD81-dependent cytostatic or cytotoxic effects. Similar high levels of expression may not be achieved by the Con1- or H77-adapted replicons. Alternatively, whatever toxic determinants lie within the C-NS2 region may be specific for the genotype 2a isolates tested thus far (J6 and JFH [52]) and not present in the corresponding genotype 1a (H77) or 1b (Con1) proteins. However, another potentially important difference is the ability of J6/JFH constructs to produce infectious virus in cell culture. In future experiments, it will be interesting to explore these possibilities by examining the properties of H77/JFH chimeras that are defective for virus production versus those harboring adaptations that allow release of infectious virus (34a, 49; M. Evans et al., unpublished data).

The in vivo relevance of HCV-induced cytotoxicity and the role of the C-NS2 region and CD81 in this process requires further study. One might argue that the genotype-specific differences, the high level of replication driven by the thus far unique nonadapted JFH-1 replicase, and the use of Huh-7 or Huh-7.5 hepatoma cells make it unlikely that similar effects will be seen in the HCV-infected liver. Certainly, the generally accepted view that HCV-associated liver damage is immune mediated, rather than a direct consequence of hepatocyte infection, is consistent with this view. However, some instances of acute fulminant hepatitis C or rapidly progressive hepatitis in the posttransplant setting could be the result of HCV variants that are cytotoxic, either due to higher replication levels, the production of toxic viral components, or both. In addition, the level of hepatocyte CD81 expression is clearly one mechanism for controlling HCV infection that may also influence pathogenesis. This could be modulated by the virus, the host response to virus infection, or therapeutically. In this regard, it will be interesting to examine possible heterogeneity in the level of CD81 expression in HCV-infected and uninfected liver tissue and determine whether there is a relationship between CD81 levels, virus infection, and cell death.

The phenomenon of HCV superinfection exclusion, namely, that infected cells are less permissive for replication of a second infecting virus, has potentially important implications for understanding HCV biology in vivo. First, the efficient block for replication of the superinfecting genome would limit the potential for RNA recombination to cells simultaneously infected by two viruses or infected cells that were successfully superinfected before the replication block was established. This may, in part, explain the dearth of natural HCV recombinants that have been identified thus far despite numerous examples of patients harboring more than one HCV genotype (17, 25). Second, even less-fit HCV variants generated during error-prone HCV replication have a chance for at least transient survival, assuming that they could productively infect a target cell and efficiently exclude replication of the more-fit parental virus. This could provide HCV with a larger pool of variants to deal with immune or other selective pressures that emerge during the course of infection (or treatment). The observation that superinfection exclusion is reversible at the cellular level suggests that interferon (9) or specific antiviral (36; this study) treatment may "cure" cells, thereby expanding the number of hepatocytes that are permissive for replication of inhibitor-resistant variants. Finally, the phenomenon of superinfection exclusion and the fact that HCV RNA levels are regulated and plateau after transfection or infection suggest that there are one or more limiting cellular components required for HCV RNA replication. Future work will hopefully lead to the identification of these limiting factors and an understanding of how they regulate HCV RNA replication.

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