JOURNAL OF VIROLOGY, Oct. 2011, p. 10451–10463 Vol. 85, No. 20
0022-538X/11/$12.00 doi:10.1128/JVI.05259-11

Mapping a Region of Hepatitis C Virus E2 That Is Responsible for Escape from Neutralizing Antibodies and a Core CD81-Binding Region That Does Not Tolerate Neutralization Escape Mutations▽†

Zhen-Yong Keck,[1] Anasuya Saha,[1] Jinming Xia,[1] Yong Wang,[1] Patrick Lau,[1] Thomas Krey,[2] Felix A. Rey,[2] and Steven K. H. Foung[1]*

Department of Pathology, Stanford University School of Medicine, Stanford, California 94305,[1] and Institut Pasteur, CNRS URA3015, Unite de Virologie Structurale, Paris, France[2]

Received 30 May 2011/Accepted 22 July 2011

Understanding the interaction between broadly neutralizing antibodies and their epitopes provides a basis for the rational design of a preventive hepatitis C virus (HCV) vaccine. CBH-2, HC-11, and HC-1 are representatives of antibodies to overlapping epitopes on E2 that mediate neutralization by blocking virus binding to CD81. To obtain insights into escape mechanisms, infectious cell culture virus, 2a HCVcc, was propagated under increasing concentrations of a neutralizing antibody to isolate escape mutants. Three escape patterns were observed with these antibodies. First, CBH-2 escape mutants that contained mutations at D431G or A439E, which did not compromise viral fitness, were isolated. Second, under the selective pressure of HC-11, escape mutations progressed from a single L438F substitution at a low antibody concentration to double substitutions, L438F and N434D or L438F and T435A, at higher antibody concentrations. Escape from HC-11 was associated with a loss of viral fitness. An HCV pseudoparticle (HCVpp) containing the L438F mutation bound to CD81 half as efficiently as did wild-type (wt) HCVpp. Third, for HC-1, the antibody at a critical concentration completely suppressed viral replication and generated no escape mutants. Epitope mapping revealed contact residues for CBH-2 and HC-11 in two regions of the E2 glycoprotein, amino acids (aa) 425 to 443 and aa 529 to 535. Interestingly, contact residues for HC-1 were identified only in the region encompassing aa 529 to 535 and not in aa 425 to 443. Taken together, these findings point to a region of variability, aa 425 to 443, that is responsible primarily for viral escape from neutralization, with or without compromising viral fitness. Moreover, the region aa 529 to 535 is a core CD81 binding region that does not tolerate neutralization escape mutations.

Up to 170 million people worldwide are chronically infected with hepatitis C virus (HCV), with many at significant risk for liver failure and hepatocellular carcinoma (http://www.who.int/vaccine_research/diseases/viral_cancers/en/index2.html). The virus is transmitted primarily by parenteral routes and injection drug use in developed countries, whereas contaminated injection equipment appears to be the major risk factor for HCV infection in developing countries. From unsafe needle injections alone, the World Health Organization estimates an annual increase in the global burden by 2 million new infections (35). Current therapy with combined pegylated interferon and ribavirin has led to clinical improvement for some patients, but treatment is associated with adverse side effects and a high relapse rate off therapy. Clearly, additional approaches are needed for treatment and prevention of infection. However, an effective HCV vaccine has yet to be achieved, despite considerable effort. A major impediment is the genetic diversity of the virus. The phylogenetic tree of HCV contains seven major genotypes with more than 30% divergence between genotypes, and each genotype contains a large number of related subtypes that differ between 20 and 25% at the nucleotide level (13, 37). Furthermore, the virus replicates at a high rate (10^{12} copies per day) using an error-prone viral RNA-dependent polymerase with an estimated mutation rate of 2.0×10^{-3} base substitutions per genome per year and exists in an infected individual as a swarm of quasispecies (7, 28, 38). This high rate of quasispecies formation contributes to the emergence of viral variants that escape immune surveillance.

A required step in the design of a vaccine for HCV is the identification of relevant mechanisms of immune protection. The induction of neutralizing antibodies following vaccination provides a first line of adaptive immune defense against a number of viral pathogens. For HCV, emerging evidence indicates a protective role of virus-neutralizing antibodies and the ability of B cell responses to modify the course of infection (3, 26, 32). A significant challenge is defining conserved epitopes in this highly diverse virus that are capable of eliciting protective antibodies. The envelope glycoproteins of HCV display some of the highest levels of genetic diversity found in HCV, with E2 being more variable than E1. A hypervariable region (HVR1) found at the N terminus of E2 is highly immunogenic and is a major determinant of isolate-specific neutralizing-antibody responses (11, 36). The limited role of the B

* Corresponding author. Mailing address: Stanford Medical School Blood Center, 3373 Hillview Avenue, Palo Alto, CA 94304. Phone: (650) 723-6481. Fax: (650) 725-6610. E-mail: sfoung@stanford.edu.

▽ Published ahead of print on 3 August 2011.

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cell response to this region in recovery from infection was demonstrated in a study of sequential HCV sequences isolated from one patient over a 26-year period. While they were capable of neutralizing earlier quasispecies obtained from this patient, autologous serum antibodies failed to neutralize the concurrent dominant HCV E1E2 species present in the blood (40). Escape was associated with mutations within HVR1 leading to decreased binding and neutralization by monoclonal antibodies directed to the earliest E2 HVR1 sequence obtained from this patient.

Broadly neutralizing antibodies are usually directed against conformational epitopes within E2 (2, 6, 14, 15, 34). We previously described a panel of neutralizing and nonneutralizing human monoclonal antibodies (HMAbs) to conformational epitopes on HCV E2 that were derived from peripheral B cells of individuals infected with either genotype 1a or 1b HCV. Cross-competition analyses delineated at least three immunogenic clusters of overlapping epitopes with distinct functions and properties (18, 20, 21). Neutralizing HMAbs segregated into two clusters, designated domains B and C, that inhibit E2 binding to the essential viral coreceptor CD81 (18, 21). Domain B HMAbs display various degrees of virus neutralization activity in assays with HCV pseudoparticles (HCVpp) containing glycoproteins of HCV genotypes 1 to 6. Some showed neutralizing activity against all genotypes (21, 22, 29). Alanine scanning mutagenesis revealed that two conserved E2 residues at G530 and D535 are required for binding by all domain B HMAbs, while G523 or W529 is required for some, but not all, of these antibodies (21, 22, 29). Importantly, W529, G530, and D535 have been shown to participate in the interaction of E2 with CD81 (30, 33). The data thus suggest that domain B HMAbs exert potent and potentially broad neutralizing effects on HCV by competing with CD81 for binding to conserved residues on E2 that are important for viral entry. Consistent with this, broadly neutralizing HMAbs derived from combinatorial libraries isolated from individuals with chronic hepatitis C also recognize epitopes containing G523, W529, G530, and D535 (17, 27, 31). The conserved nature of this cluster of overlapping epitopes makes them of interest for vaccine development. A critical concern is the likelihood that immune selection could lead to escape from neutralization by domain B HMAbs. For example, CBH-2 is an HMAb recognizing a broadly conserved epitope in domain B that contains residues G530 and D535 (22). However, a single amino acid substitution at residue 431 in a naturally occurring variant, which is located at a considerable distance within the linear sequence of E2 from CBH-2 contact residues 530 and 535, results in complete escape from CBH-2-mediated neutralization in a genotype 1a virus (22). In addition, a pattern of virus escape from CBH-2 is observed with sequential HCVpp isolates derived from a patient with chronic HCV infection over several decades (19). For other domain B antibodies, neutralizing activities are maintained against these sequential isolates. To obtain new insights into mechanisms of escape from neutralization, infectious cell culture virus, 2a HCVcc, was propagated under increasing concentrations of a neutralizing antibody. We describe in this report three patterns of virus escape from three neutralizing antibodies to overlapping epitopes. One antibody led to escape mutant viruses without affecting viral fitness, a second led to escape but with compromised viral fitness, and a third led to complete virus elimination without escape mutants. Epitope mapping of these three antibodies identified a region of variability on the E2 glycoprotein that is responsible for escape from neutralization with or without compromising viral fitness.

MATERIALS AND METHODS

Cell culture, antibodies, virus, and reagents. HEK-293T cells were obtained from the ATCC. Huh7.5 cells (generously provided by C. Rice, Rockefeller University) were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich Co., St. Louis, MO) and 2 mM glutamine. HMAbs CBH-2, HC-1, HC-11, and H-111 against HCV E1 and E2 were generated as described previously (15, 18, 24). A MAb against HCV NS3 protein was generously provided by G. Luo (University of Kentucky). A MAb against human CD81 (clone JS-81) was purchased from BD Bioscience (San Jose, CA). A molecular clone encoding the CD81 large extracellular loop fused to glutathione *S*-transferase was generously provided by S. Levy (Stanford University) and affinity purified over a GSTrap FF affinity column according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). JFH-1 2a HCVcc was generously provided by T. Wakita (National Institute of Infectious Diseases, Japan).

Selection of antibody-resistant neutralization escape mutants. Infectious genotype 2a JFH-1 virus was produced essentially as described previously (41), and employed for the escape mutation studies. Briefly, a linearized plasmid carrying HCVcc genomic cDNA was used as a template to generate viral genomic RNA by *in vitro* transcription (MEGAscript; Ambion, Austin, TX) and electroporation into Huh7.5 cells. Ten micrograms of *in vitro*-transcribed RNA was mixed with 0.4×10^6 Huh7.5 cells in a 4-mm cuvette in calcium-free phosphate-buffered saline (PBS) containing 10 μg calf liver tRNA and pulsed at 0.27 kV and 960 μF using the Gene Pulser Xcell (Bio-Rad) electroporator. Electroporated cells were seeded into 10-cm cell culture dishes with 10 ml complete DMEM containing 10% FCS after resting from electroporation for 10 min. The transfected cells were passaged at 4- to 5-day intervals with a 1:4 to 1:5 split into fresh culture flasks. Expression of HCV E2 was confirmed by indirect immunofluorescence assay (IFA) at each passage of the cells. The infectious-virus titer of pooled cell culture supernatant fluids was measured in focus-forming units (FFU), as described below, and virus harvests were placed in small aliquots and kept at −80°C.

Huh7.5 cells (3.2×10^4/ml) seeded 24 h previously in a 24-well plate were infected with 2a HCVcc (1×10^4 FFU). The initial concentration of the neutralizing antibody employed to isolate escape HCVcc mutants was adjusted to the 50% inhibitory concentration (IC_{50}) of the antibody against 2a HCVcc. Infectious virus was first incubated with the selection antibody for 1 h at 37°C prior to inoculation onto naïve Huh7.5 cells. This was followed by a second incubation for 3 h at 37°C before the medium was replaced with fresh medium containing the same antibody concentration. The cultures were maintained for 3 days in the presence of HMAb CBH-2, HC-1, HC-11, or R04 (as mock human IgG selection), and the extracellular virus was harvested for the next round of selection. The entire process constituted one passage of infectious virus under antibody selection. At each antibody concentration, the virus was repeatedly passaged until the virus titer reached 1×10^4 FFU prior to subjecting the virus to the next higher antibody concentration. The number of rounds of amplification required for this purpose varied from antibody to antibody. Starting at the IC_{50}, the antibody concentration was progressively increased (0.05, 0.1, 0.5, 1, 5, 10, and 100 μg/ml). Viral growth and the emergence of escape variants were monitored weekly by two-color confocal immunofluorescence microscopy staining with the respective neutralizing antibody and an anti-NS3 antibody. The viral supernatant and cells were collected weekly and stored at −80°C for sequence analysis. Viral supernatants were used for neutralization studies against escape mutants and as a source of virus stock.

Virus titration. Virus titers were determined by an FFU assay. Huh7.5 cells were seeded at 3.2×10^4 cells/well in 8-well chamber slides (Nalge Nunc, Rochester, NY) 1 day before inoculation with 100 μl of a dilution of the wild-type (wt) virus or neutralization escape variant sample. After 3 h of incubation at 37°C in the presence of 5% CO_2, the inoculum was replaced with 400 μl of fresh complete medium and incubated for an additional 72 h. After the supernatant fluids were removed, the cells were washed and fixed in methanol-acetone (1:1) at room temperature for 9 min and then stained with 100 μl per well of anti-HCV NS3 antibody at 10 μg/ml. The cells were extensively washed before the addition of a secondary antibody labeled with Alexa-Fluor 594-conjugated goat anti-mouse IgG antibody (Invitrogen, Carlsbad, CA) at 1:100 dilution, and

the nuclei were counterstained with Hoechst 33342 at 1 μM. The slides were mounted and examined under a Leica DM 2500 UV fluorescence microscope for foci of antigen-positive cells. Each cluster of ≥3 infected cells, identified by staining for NS3 antigen expression, was considered to constitute a single infectious focus. The entire well was visualized in approximately 16 nonoverlapping fields, and the virus titers were calculated accordingly in FFU/ml.

Confocal immunofluorescence microscopy. Cells from mock- or virus-infected cultures were harvested by trypsinization, counted, resuspended in DMEM, and plated onto a 24-spot Teflon-coated slide. The cells were air dried and fixed with 100% acetone. The slides were incubated with a mixture of mouse anti-NS3 (10 μg/ml) and either CBH-2, HC-1, or HC-11 (10 μg/ml) as primary antibodies for 30 min, followed by fluorescein isothiocyanate (FITC)-labeled anti-human IgG (Jackson Immune Research) or goat anti-mouse IgG conjugated to Alexa 594 (Invitrogen, Carlsbad, CA) as a secondary antibody for 30 min at 37°C. The slides were washed, counterstained with Hoechst 33342, mounted in mounting medium (Vector Laboratories, Burlingame, CA), sealed, and then examined with a Leica DM 2500 laser scanning confocal microscope. The captured images were superimposed using LaserSharp software (Bio-Rad Laboratories).

Neutralization assay by focus-forming unit reduction. The neutralization activities of HMAbs against 2a HCVcc and neutralization escape HCVcc mutants were evaluated as previously described (19). Briefly, a virus inoculum (containing 50 FFU) was incubated with serial dilutions of antibodies for 1 h at 37°C before inoculation onto Huh7.5 cells (3.2×10^4 cells/well) seeded 24 h previously into 8-well chamber slides (Nalge Nunc, Rochester, NY). After 3 h of incubation at 37°C in the presence of 5% CO_2, the inoculum was replaced with 400 μl of fresh complete medium, followed by incubation for an additional 72 h. The infected cells were fixed and examined for NS3 protein expression by immunofluorescence detection of foci, as described in "Virus titration" above. The entire well was visualized in approximately 16 nonoverlapping fields to obtain the number of foci. Each experiment was performed in triplicate. The antibody concentrations (μg/ml) causing 50% reductions in FFU were determined by linear regression analysis. The percent neutralization was calculated as the percent reduction in FFU compared with virus incubated with an irrelevant control antibody.

HCV-pseudotyped retroviral particle production, infection, and neutralization assay. HCVpp were produced as described previously (5, 20) by cotransfection of 293T cells with the pNL4-3.Luc.R^-E^- plasmid containing the *env*-defective HIV proviral genome and an expression plasmid encoding the HCV glycoproteins. The virus-containing extracellular medium was collected 48 h after transfection and separated from cell debris by passing through a 0.45-μm filter. For the neutralization assay, the virus-containing medium was incubated with each HMAb at various concentrations, or with phosphate-buffered saline instead of the antibodies as an infectivity control, plus 4 μg/ml Polybrene at 37°C for 60 min (4, 39).The HCVpp-antibody mixture was transferred to Huh7.5 cells (8×10^3 cells/well) preseeded in 96-well plates, and infected cultures were centrifuged at $730 \times g$ for 2 h at room temperature. After incubation at 37°C in the presence of 5% CO_2 for 15 h, the unbound virus was replaced with fresh complete medium, followed by additional incubation for a total of 72 h. For detection of HCVpp entry, 100 μl of reconstituted Bright-Glo (Promega, Madison, WI) was added to each well, followed by 2 min of mixing and light detection in a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA). The neutralizing activity of an antibody was calculated as the percent reduction of luciferase activity compared with an inoculum containing PBS. For HCVpp infectivity studies, the virus-containing extracellular medium was normalized for HIV p24 expression by using a QuickTiter lentivirus titer kit (Cell Biolabs, San Diego, CA).

Quantitative ELISA. Enzyme-linked immunoassays (ELISA) were performed to measure antibody binding to the wt or mutant E2 glycoproteins and to measure E2 binding to CD81, as described previously (19). Briefly, microtiter plates were prepared by coating each well with 500 ng of *Galanthus nivalis* agglutinin (GNA) and blocking with 2.5% nonfat dry milk and 2.5% normal goat serum. Lysates of cells expressing wt HCV, mutant E1E2, or pelleted virus were captured by GNA on the plate and later bound by a range of 0.01 to 100 μg/ml of HMAb. The bound HMAb was detected by incubation with alkaline phosphatase-conjugated goat anti-human IgG (Promega; Madison, WI), followed by incubation with *p*-nitrophenyl phosphate for color development. Absorbance was measured at 405 nm and 570 nm.

For CD81 binding analysis, 20% sucrose cushion-pelleted HCVpp were resuspended in NTE buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and captured on GNA-coated plates. Bound antigen was first incubated with purified CD81 at concentrations from 0.5 to 500 μg/ml. The amount of HCVpp captured in each well was determined by probing the cells with H-111, an HMAb directed against a linear epitope within E1 (24). The bound CD81 was visualized with alkaline phosphatase (AP)-conjugated mouse anti-human CD81 (Santa Cruz Biotechnologies, Santa Cruz, CA). Color development and absorbance measurement were performed as described above.

Virus yield assay. Virus-containing supernatants were inoculated onto Huh7.5 cells at a multiplicity of infection (MOI) of 0.01. The cells were seeded 24 h previously in a 24-well plate. After 3 h of incubation at 37°C and 5% CO_2, the inoculum was replaced with fresh complete medium and incubated for an additional 72 h. The supernatant fluids were then collected, and the titer of infectious virus was assessed by the FFU assay.

Inhibition of virus infection by anti-CD81. Huh7.5 cells seeded at 8,000 cells/well in a 96-well plate on the previous day were incubated with dialyzed anti-CD81 (clone JS81; BD Bioscience) at 37°C and 5% CO_2 for 1 h before infection with wt HCVcc or escape HCVcc mutants. After 3 h of incubation, the inoculum was replaced with fresh complete medium, followed by an additional incubation for 72 h. Infected cells were fixed and examined for NS3 protein expression by immunofluorescence detection of foci, as described in the virus titration assay. The percent inhibition was calculated as the percent reduction in FFU compared to virus incubated with an irrelevant control antibody.

Site-directed mutagenesis. All mutagenesis was conducted using a QuikChange II site-directed mutagenesis kit (Agilent, La Jolla, CA) in accordance with the manufacturer's instructions. A plasmid containing the 2a JFH-1 E1E2-encoding sequence was used as the template for introducing single and double mutations. Alanine substitution mutants of full-length E2 (residues at 834 to 746) or more finite E2 regions were constructed in plasmids carrying, respectively, the H77c 1a E1E2 sequence or a genotype 1b E1E2 coding sequence (GenBank accession no. AF009606 and AF348705) to epitope map CBH-2, HC-1, and HC-11. All the mutations were confirmed by DNA sequence analysis (Sequetech, Mountain View, CA) for the desired mutation and for exclusion of unexpected residue changes in the full-length E1E2-encoding sequence. The resulting plasmids were transfected into HEK293T cells for transient protein expression using the calcium-phosphate method. The mutated constructs were designated X#Y, where # is the residue location in H77c, X denotes the single-letter code for the H77c amino acid, and Y denotes the altered amino acid.

Sequence analysis. Total RNA or viral RNA from virus-infected cells or virus-containing culture supernatant was extracted using commercial kits (Qiagen, Valencia, CA). cDNA of the E2 glycoprotein was synthesized with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) by using primer p7rev (CCCGACCCCTGATGTGCCAAGC) in a 20-μl reaction mixture of the manufacturer's recommended buffer. Subsequent amplification was performed in a 50-μl reaction mixture using the Expend High Fidelity PCR system (Roche Applied Sciences, Indianapolis, IN) and primers E1fwd (GGTCATCATAGAC ATCGTTAGC) and p7rev (CCCGACCCCTGATGTGCCAAGC). The PCR consisted of 30 cycles at 94°C for 60 s, 45°C for 60 s, and 72°C for 90 s. A total of 2 μl of the resulting PCR product was used as a template for a nested amplification, using primer pair E2F (GGCACCACCACCGTTGGAGGC) and E2R (TGCTTCGGCCTGGCCCAACAAGAT). This second round of PCR comprised 25 cycles at 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s. In some cases, when the viral titer was low and failed to amplify the *E2* gene, the number of PCR cycles in the nested round was increased. The PCR products were purified with the QIAquick gel extraction kit (Qiagen, Valencia, CA) and ligated into the Topo cloning vector (Invitrogen, Carlsbad, CA), and individual clones containing an insert of the expected size were sequenced in both sense and antisense strands (Elim Biopharm, Hayward, CA).

RESULTS

We previously reported on multiple neutralizing HCV HMAbs to conserved overlapping conformational epitopes in HCV E2, designated domain B (21, 23). Each of these epitopes contains contact residues that are also involved in the binding of E2 to CD81, which explains their broad reactivity to different HCV genotypes and subtypes. However, these antibodies exhibited different neutralization patterns against a panel of sequential HCV variants isolated from a patient over several decades of chronic hepatitis (19). Some antibodies (such as HC-1 and HC-11) consistently neutralized the sequential isolates, while others (such as CBH-2) neutralized with waxing and waning patterns suggestive of virus escape from neutralization. We postulated that the epitopes of these antibodies contain contact residues beyond those involved in E2 binding

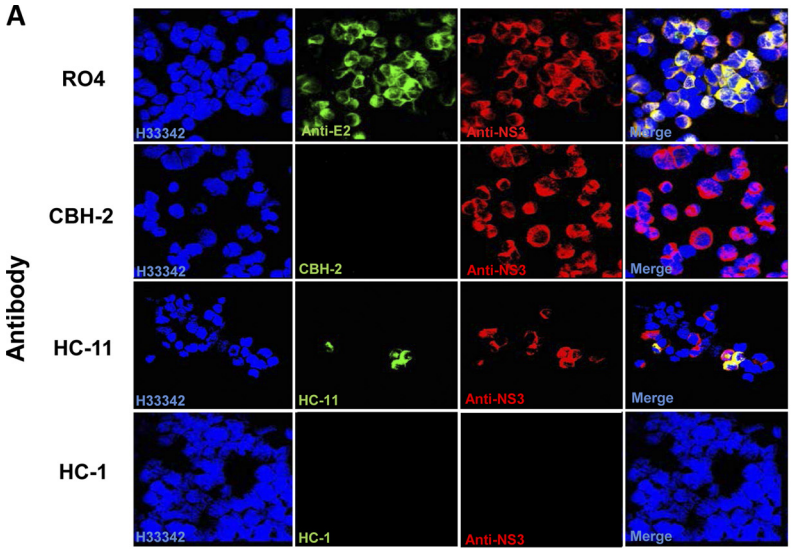
to CD81 that influenced their different neutralization escape profiles. Our current studies focused on characterizing selected antibodies having different neutralization patterns, first by *in vitro* escape selection and then by epitope mapping.

Identifying contact residues in an epitope responsible for virus escape from neutralization. The analysis of escape mutants should provide new insights into the compositions of selected domain B epitopes and the amino acid substitutions responsible for the emergence of escape mutant viruses. HC-1, HC-11, and CBH-2 were chosen on the basis of their neutralization profiles against naturally occurring sequential HCV variants (19). Initial attempts to elicit escape mutants by subjecting 2a JFH-1 HCVcc to repetitive rounds of neutralization at 100 μg/ml of CBH-2, HC-1, or HC-11, followed by amplification of the surviving virus by passage of infected cells in the absence of antibody, led to complete elimination of the virus. This approach was successful in an earlier effort with a monoclonal antibody to a linear epitope (12). An alternative approach was implemented as described in Materials and Methods. The *in vitro* escape selection was designed to maximize the likelihood of escape variants by subjecting wt HCVcc to increasing concentrations of the selection antibody from 0.05 μg/ml to 100 μg/ml. At each antibody concentration, the extracellular virus was passaged repeatedly to reach a titer of 1×10^4 FFU/ml before subjecting the virus to the next higher antibody concentration. Each passage cycle was approximately 3 days in duration, after which the secreted extracellular virus was overlaid onto uninfected cells for the next passage. Repeated passaging allowed minority variants to be amplified prior to the next round of selective pressure at a higher antibody concentration. As a control virus population, wt HCVcc was subjected to serial passages in increasing concentrations of R04, an isotype-matched HMAb to cytomegalovirus (CMV), to provide reference viral variants. This permitted specific discrimination between mutations introduced during long-term *in vitro* propagation of wt HCVcc and those mutations induced under the selective pressure of CBH-2, HC-1, or HC-11. At the designated antibody concentrations, escape mutants were noted by a loss in specific antibody binding by IFA (Fig. 1A). RNA from escape mutants was extracted from either cells or culture supernatants, reverse transcribed, and subcloned. Genomic residues 1491 to 2579 spanning the entire E2 coding region were sequenced from selected individual clones. The number of clones sequenced and analyzed per sample ranged from 20 to 60.

Figure 1A and B shows neutralization escape profiles for the three tested antibodies and the control antibody, R04. The concentration of R04 was increased rapidly, since the antibody has no effect on HCV, and only several passages at each antibody concentration were needed to reach 10^4 FFU/ml HCVcc. Identical spontaneous mutations were detected after repeated passages of wt HCVcc (without R04) and with R04. Two variants appeared, one containing amino acid substitutions at residues V402A and N417S and the other containing a mutation at N415D (Fig. 1B). The V402A plus N417S and N415D variants remained throughout the course of the study, as wt HCVcc was passaged and as R04 was increased to 100 μg/ml. There were no additional variants in R04-passaged virus. In agreement with a previous study, the N417S and N415D mutations are felt to be cell culture-adaptive mutations that spontaneously occurred with long-term passage of 2a HCVcc (8). As expected, Huh7.5 cells infected with R04-passaged HCVcc (between 10 and 100 μg/ml) were stained equally by a positive-control antibody to HCV E2 (CBH-5) and an anti-NS3 antibody (R04) (Fig. 1A). Huh7.5 cells infected with R04-passaged and wt HCVcc were also stained equally by CBH-2, HC-1, or HC-11 and the control anti-E2 and anti-NS3 antibodies (data not shown).

Under the selective pressure of CBH-2, whose concentration was increased progressively from 0.1 to 1 μg/ml, the virus required 3 to 4 rounds of amplification before reaching a titer of 10^4 FFU/ml. This is consistent with the antibody neutralizing a substantial portion of wt HCVcc. When the concentration increased from 1 to 10 μg/ml, a single mutation at D431G was observed in 80% of the infected cells not stained by CBH-2 but stained by anti-NS3 (Fig. 1A and B). To precisely determine the antibody concentration that led to escape, CBH-2 was increased in smaller increments from 1 to 10 μg/ml. The D431G mutation first appeared in the extracellular virus at a CBH-2 concentration of 2 μg/ml and remained stable at 4, 6, 8, and 10 μg/ml. When intracellular virus was isolated from infected cells, we detected mostly D431G mutants (24 of 25 sequenced clones) and one double-substitution mutant at A439E plus N578D. Interestingly, the D431G mutation is the same mutation that was identified in a naturally occurring variant shown to be resistant to neutralization by CBH-2 (22). This provided validation of our method to isolate escape mutants. In a separate experiment, after the third passage at 10 μg/ml CBH-2 and with 100% of infected cells not stained by CBH-2, two other mutants were identified among the extracellular variants: a double-substitution A439E plus N578D mutant and a triple-substitution N415D plus A439E plus N578D mutant were observed (Fig. 1B). From passages 4 to 8 at 10 μg/ml CBH-2 and with 100% of infected cells not stained by CBH-2 (Fig. 1A), the triple-substitution mutant became the only virus variant detected. These results linked two independent sets of escape mutants as a consequence of CBH-2 selective pressure. Moreover, to assess whether and how rapidly escape mutants can revert to wt HCVcc, each CBH-2-resistant mutant (either D431G or N415D plus A439E plus N578D) was passaged over 5 times in the absence of CBH-2. No sequence changes were detected at any passage, and the virus showed no apparent change in the replication rate, indicating that these substitutions were relatively stable. Collectively, CBH-2 neutralization-resistant mutants represent an escape profile that allows the virus to escape from some neutralizing antibodies to conserved conformational epitopes without affecting viral replication. The mutations leading to escape from CBH-2 are D431G, A439E, and N578D. The N415D mutation has been observed as a spontaneously occurring mutation independent of antibody selection, as noted in passaging wt HCVcc with or without R04.

Under the selective pressure of HC-11, at least 3 or 4 passages at each antibody concentration from 0.1 to 1 μg/ml were required to reach 10^4 FFU/ml. Similarly to CBH-2, this is consistent with a significant degree of HCVcc neutralization. At 1 μg/ml, a mutant with substitutions at residues V402A and N417S was observed, along with wt HCVcc. As noted above, this mutant was also detected in R04-treated controls. At 10 μg/ml HC-11, the remaining virus was wt HCVcc during the



B

Antibody	Mutant 1	Mutant 2	Mutant 3	Mutant 4
RO4	V402A+N417S	N415D		
CBH-2	D431G	N415D+A439E+N578D	A439E+N578D	
HC-11	V402A+N417S	L438F	L438F+N434D*	L438F+T435A**
HC-1	No mutation			

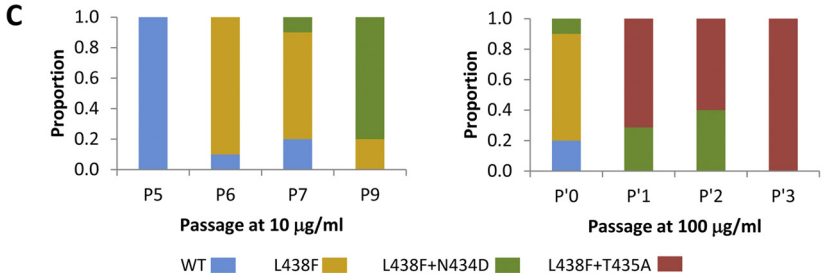


FIG. 1. Identification of neutralization escape mutants and associated amino acid changes in the HCV E2 glycoprotein. (A) Dual-antibody immunofluorescence staining of Huh7.5 cells infected with JFH1 2a virus after multiple rounds of neutralization by the respective antibody. R04, a human monoclonal antibody against CMV, was used as a mock selection. HCV E2 glycoprotein was stained with the antibody under which viral escape mutants were selected (green). Total virus-infected cells were stained with anti-NS3 antibody labeled with Alexa-594 (red). The cells were counterstained with Hoechst nuclear stain H33342 (blue). Escape mutants were assessed for CBH-2 at passage 4 in 10 μg/ml, for HC-11 at passage 6 in 10 μg/ml, and for HC-1 at passage 5 in 10 μg/ml. (B) Observed amino acid substitutions in neutralization escape mutants. *, amino acid changes in the escape mutants isolated from HC-11 at 10 μg/ml; **, amino acid changes in the escape mutants isolated from HC-11 at 100 μg/ml. (C) Appearance of escape mutants at different HC-11 concentrations and passages of extracellular virus. (Left) Proportions of wt HCVcc and HC-11 escape mutants bearing single and double mutations observed in extracellular virus during passages 5 to 9 in 10 μg/ml HC-11. (Right) Proportions of escape mutants bearing single and double mutations during three passages of extracellular virus at 100 μg/ml HC-11, designated P′1 to P′3. P′0 is extracellular virus from P7 in 10 μg/ml HC-11.

first 5 passages, but at passage 6, a mutant with a substitution at L438F appeared in 90% of the variants and mixed with 10% wt HCVcc (Fig. 1A to C). Increasing HC-11 to 10 μg/ml led to a greater virus titer reduction: only after 13 passages at this concentration did the virus titer increase to 10^4 FFU/ml. At 10 μg/ml HC-11, passage 7, a double-substitution L438F plus N434D mutant emerged in ~10% of the variants, along with the single-substitution L438F mutant and wt HCVcc (Fig. 1B and C). During the next 6 consecutive passages at 10 μg/ml, wt HCVcc disappeared, and the single L438F and double L438F plus N434D mutants remained in a ratio from 1:4 to 3:2. With the disappearance of wt HCVcc, infected Huh7.5 cells were detected only by anti-NS3 and not by HC-11.

In an attempt to reach 100% escape from HC-11, a split of the extracellular virus from passage 7 at 10 μg/ml HC-11 was placed in 100 μg/ml HC-11 (Fig. 1C) and designated P′0. The single-substitution L438F mutant disappeared after a single passage, P′1, while a new double-substitution L438F plus T435A mutant emerged in the mixture, in combination with the existing double-substitution L438F plus N434D mutant. In passage 3 (P′3) and later passages, the L438F plus N434D mutant disappeared while the L438F plus T435A mutant re-

mained as the surviving virus. The pattern of evolving escape mutations for HC-11 is different than for CBH-2 selection, in which the escape mutants remained unchanged. For HC-11 selection at 10 or 100 μg/ml, intracellular variants were also analyzed and found to have virus compositions similar to those observed from culture supernatants (data not shown). The implication is that substitutions at L438F, N434D, or T435A did not prevent virus assembly and release from infected cells. Interestingly, the composition of HCV variants at 10 μg/ml from passages 9 to 13, L438F and L438F plus N434D, remained stable, even when HC-11 was withdrawn from later passages (data not shown). However, a mixture of two double-substitution variants, L438F plus T435A and L438F plus N434D, appeared when the antibody concentration was 100 μg/ml. The L438F plus T435A mutant was the eventual dominant virus, suggesting that the T435A substitution contributes to escape at higher antibody selective pressure.

Under the selective pressure of HC-1, no escape variants were isolated in four independent experiments (Fig. 1B). At low antibody concentrations of ≤0.5 μg/ml, virus replicated less efficiently than wt HCVcc, indicating some degree of virus neutralization. When HC-1 was increased to 1 μg/ml, 5 passages were required to reach 10^4 FFU/ml. At 10 μg/ml, each passage led to less and less viral replication, and after the fourth passage, no extracellular virus and no infected cells were observed (Fig. 1A). During each of these passages, the decreasing percentages of infected cells stained by HC-1 were consistent with those detected using anti-NS3 antibody. We attempted to rescue the virus by passaging cultured supernatant from the fourth round onto naïve Huh7.5 cells in the absence of HC-1 for four additional passages, and no detectable virus emerged from the passaged supernatants. To confirm that HC-1 is able to prevent viral escape, a similar study was performed with an affinity-matured HC-1 HMAb with over 90-fold increase in antibody binding affinity (unpublished data). The results showed the virus disappearing under selection at 0.1 μg/ml without detectable escape mutants (data not shown). The 100-fold decrease in antibody concentration needed by the affinity-matured HC-1 compared to wt HC-1 was inversely proportional to the improvement in antibody binding efficiency. The failure to generate HC-1-induced HCV escape mutants could be due to the viral strain employed in these studies. However, it is also possible that the HC-1 epitope is the most conserved of the antibodies tested, so that each contact residue for HC-1 is essential and the induction of escape within this epitope leads to a lethal change in virus function or structure.

Characterization of mutations. To confirm that the mutations elicited under selection pressure contributed to the escape phenotypes, we first studied the cross-neutralization patterns of the escape mutants with HMAbs CBH-2, HC-1, and HC-11 (Fig. 2A). The control virus, HCVcc bearing two mutants with substitutions at N415D and V402A plus N417S, respectively, which were obtained during passages with increasing concentrations of R04, remained completely sensitive to the three neutralizing antibodies. This indicates an inability of the virus to escape spontaneously from either CBH-2, HC-1, or HC-11 under these experimental conditions. CBH-2 failed to neutralize the escape mutant HCVcc bearing N415D plus A439E plus N578D, as expected, but the mutant remained sensitive to HC-1 and HC-11 (Fig. 2A, CBH-2 mutant). The other escape mutant HCVcc from CBH-2, bearing a mutation at residue 431, was fully characterized in a previous study (22). In that study, a mutation at residue 431 led to a loss of CBH-2 neutralizing activity but retained sensitivity to other domain B or C neutralizing antibodies. Neutralization escape mutants from HC-11 selective pressure contained single (L438F) or double (L438F plus N434D or L438F plus T435A) substitutions. All three mutants resisted HC-11 neutralization while remaining sensitive to CBH-2 and HC-1 (Fig. 2A, P9 and P′3 mutants).

We further assessed the relative contribution of each mutation in the neutralization-resistant mutants of CBH-2 and HC-11 by introducing their respective single or combined substitutions into the JFH-1 full-length E1E2. Each mutant 2a E1E2 was then incorporated into HCVpp (Fig. 2B and C). For CBH-2, 2a HCVpp containing E2 mutations at A439E or N578D were studied initially by binding (Fig. 2B). The studies revealed the central role of A439E in viral escape from CBH-2. The A439E HCVpp was not bound by CBH-2 but was bound by a positive-control domain B antibody, CBH-5 (Fig. 2D). For N578D, CBH-2 retained dose-dependent binding to the mutant (Fig. 2B). Binding data are not shown for N415D, since that mutation also occurred in the R04 control. Neutralization studies showed that CBH-2 did not neutralize A439E HCVpp, which was neutralized by HC-11 (Fig. 2E). Taken together, these findings indicate that the A439E substitution accounts for escape from CBH-2 neutralization. Thus, a single amino acid substitution at residue 431 (22) or 439 leads to escape from neutralization by CBH-2.

HC-11 escape variants containing single (L438F) or double (L438F plus N434D or L438F plus T435A) mutations appeared to occur at different selection antibody concentrations. Of the three substitutions at residues 434, 435, and 438, the HCVpp mutants exhibiting substitutions at L438F, either single (L438F) or double (L438F plus N434D or L438F plus T435A) mutations, displayed complete loss of binding by HC-11 (Fig. 2C) but retained binding by CBH-5 (Fig. 2D). Compared to binding to wt HCVpp, HCVpp containing a single-substitution mutation at N434D or T435A showed no significant changes in binding by HC-11 (Fig. 2C). The change in the pattern of neutralization by HC-11 against these mutants correlated with antibody binding studies (Fig. 2E). Compared to wt HCVpp, HC-11 lost neutralization against the three L438F-related HCVpp mutants that remained sensitive to CBH-2. For both CBH-2 and HC-11, the residue responsible for loss of binding is the residue responsible for loss of neutralization. Although CBH-2 and HC-11 bind to overlapping epitopes, they each contain distinct residues, 431 and 439 for CBH-2 and 438 for HC-11, whose mutation leads to escape from neutralization by the respective antibody.

Fitness of escape mutants and their binding efficiencies to CD81. There are several differences in the progression of escape from CBH-2 and HC-11. The two substitutions, N434D and T435A, appeared to occur, respectively, in response to either prolonged HC-11 selection pressure at 10 μg/ml or an increase in the antibody concentration to 100 μg/ml. More importantly, when the HC-11 antibody reached a critical concentration of 10 μg/ml, virus replication significantly slowed, as shown by the necessity for 13 passages to reach a titer of 10^4

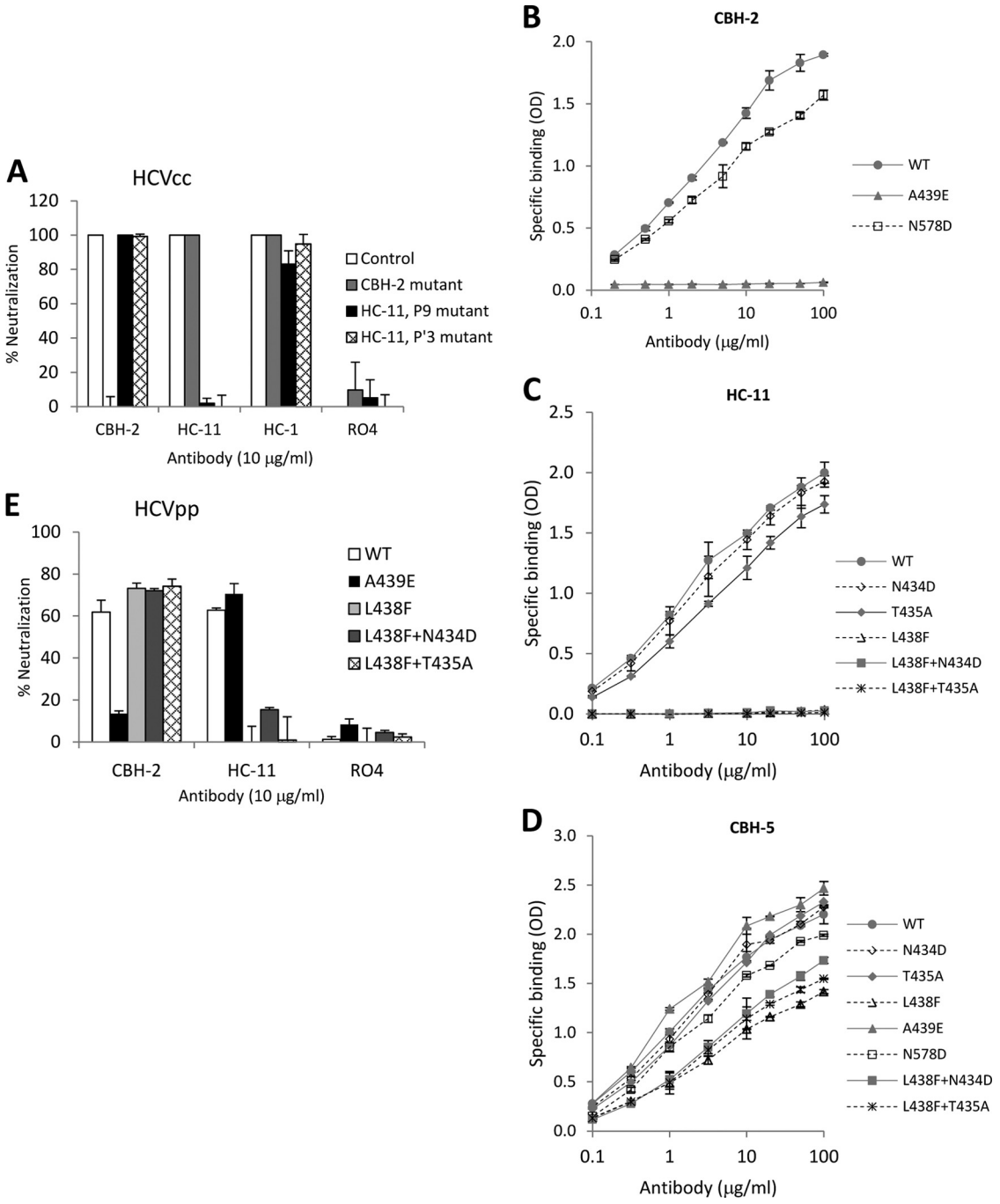
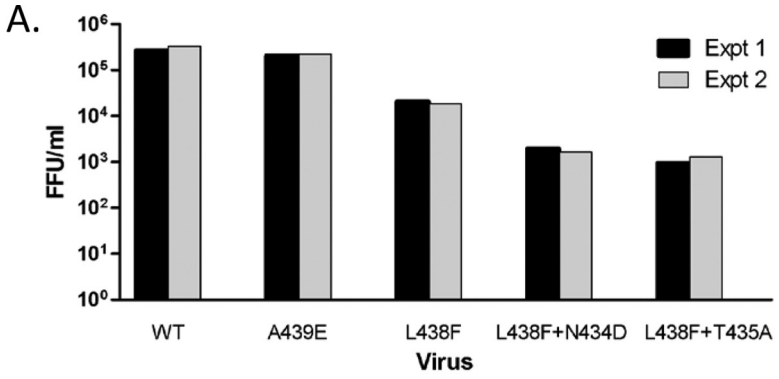


FIG. 2. Characterization of neutralization escape mutant viruses. (A) HMAbs HC-1, HC-11, and CBH-2 were tested for the ability to neutralize wt HCVcc (control) and HCVcc escape mutants isolated under CBH-2 (CBH-2 mutant) and HC-11 selection pressure. HC-11, P9, represents escape mutants from passage 9 in 10 μg/ml HC-11. HC-11, P′3, represents escape mutants from passage 3 in 100 μg/ml HC-11. Each virus preparation was tested for neutralization by CBH-2, HC-11, and HC-1 at 10 μg/ml by FFU reduction assay. R04 was used as an isotype-matched negative-control antibody. Each assay was performed in triplicate, and the data are shown as percent neutralization (the means from three experiments ± standard deviations [SD]). (B to D) Antibody binding to HCVpp containing mutations in E2 derived from HMAb CBH-2 and HC-11 selection pressure. (B) CBH-2 dose-dependent binding to wt HCVpp or HCVpp mutants with single mutations associated with escape from CBH-2 at A439E and N578D by ELISA. (C) HC-11 dose-dependent binding to wt HCVpp or HCVpp mutants with single and double mutations associated with escape from HC-11 at N434D, T435A, L438F, L438F plus N434D, and L438F plus T435A analyzed by ELISA. (D) CBH-5 is another domain B antibody employed as a positive control. For the experiments shown in panels B to D, the *y* axis shows the mean optical density values normalized to binding values obtained with H-111, an HMAb to a linear epitope in HCV E1. The data in panels B to D are derived from triplicate wells and are shown as the means from two experiments ± SD. (E) Neutralization of HCVpp containing wt E2 or specific mutations in E2 that conferred a loss in their respective binding by CBH-2 or HC-11. CBH-2 (A439E)- and HC-11 (L438F, L438F plus N434D, or L438F plus T435A)-related HCVpp mutants and wt HCVpp were tested for neutralization by CBH-2, HC-11, or a control antibody, R04, at 10 μg/ml. Neutralization is expressed as percent reduction in luciferase activity. The data are shown as the means from three experiments ± SD, with each experiment performed in triplicate.



B. HCVpp mutant binding to CD81*

HCVpp	CD81 (μg/ml) 5	10	20	50	100	200	500
L438F	40	38	45	47	52	79	86
N434D	35	37	38	47	53	79	90
T435A	33	37	37	47	55	76	86
N434D+L438F	20	20	23	30	38	69	80
T435A+L438F	35	37	41	45	49	71	78

*Percent binding of designated mutant to CD81 as compared to wt (set at 100 percent).

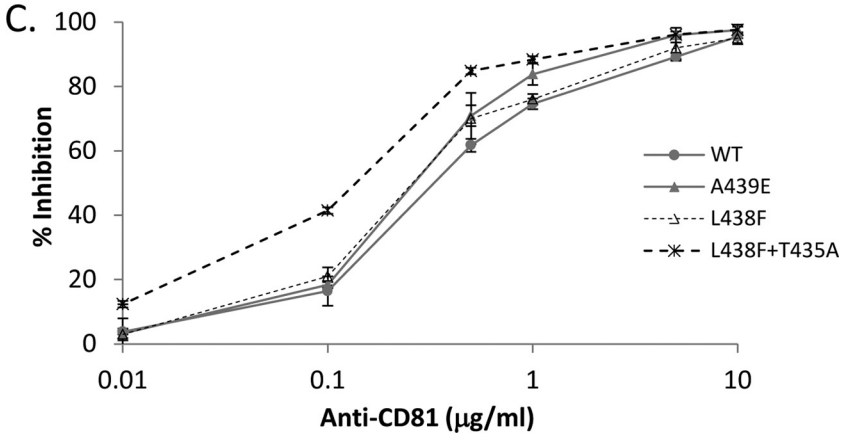


FIG. 3. HC-11 mutations confer loss of viral fitness associated with decreased affinity for CD81. (A) Wt, CBH-2 (A439E), and HC-11 (L438F and L438F plus T435A) virus stocks were inoculated onto Huh7.5 cells at an MOI of 0.01 in a virus yield assay. Supernatant fluids were collected at 72 h, and the titer of infectious virus was assessed by FFU assay. The results from two experiments (Expt) are plotted. (B) Binding of soluble CD81 to purified wt or mutant HCVpp captured on a solid-phase support. Each value is calculated as the mean optical density value of CD81 binding at the specified concentration to each mutant from triplicate wells of two experiments over the mean value of CD81 binding to the wt times 100. (C) Inhibition of virus infection by anti-CD81. Huh7.5 cells were preincubated with anti-CD81 at the indicated concentrations for 1 h at 37°C prior to infection using wt HCVcc CBH-2- or HC-11-resistant virus stocks. Inhibition of infection is expressed as a percentage of FFU reduction compared to a control antibody, R04. The data are derived from triplicate wells and are shown as the means from two experiments ± SD.

FFU/ml. Compared to the production of escape mutants after 3 or 4 passages in increasing concentrations of CBH-2, reaching 10^4 FFU/ml after 1 or 2 passages, the consequence of escape from HC-11 appears to have a negative effect on the structure and fitness of the virus. We then assessed the fitness of HC-11 escape mutants along with that of CBH-2 escape mutants (Fig. 3A). Huh7.5 cells were infected with either HC-11 or CBH-2 escape HCVcc variants or wt HCVcc at a low MOI of 0.01. The infectious-virus yield by FFU assay at 72 h postinfection revealed that the CBH-2-resistant mutant, A439E, grew as efficiently in Huh7.5 cells as did wt HCVcc. For HC-11 escape mutants, the single-substitution mutant observed at passage 6, L438F, grew 8-fold less than wt HCVcc, although 10% of the virus at this passage was composed of wt HCVcc (Fig. 1C, P6). The L438F plus N434D double-substitution mutant grew 164-fold less than wt HCVcc, with 20% of the virus at this passage composed of wt HCVcc (Fig. 1C, P9). The L438F plus T435A double-substitution mutant observed at passage 3 (Fig. 1C, P′3) in 100 μg/ml of HC-11 showed a 265-fold reduction in virus yield (Fig. 3A). Although the N434D and T435 residues do not appear to be part of the HC-11 epitope, the mutations at these sites, in combination with L438F, clearly imposed a significantly greater decrease in viral fitness than the single L438F mutation.

CBH-2 and HC-11 belong to a cluster of antibodies within domain B that share a mechanism of neutralization by blocking E2 binding to CD81. The residues responsible for escape from CBH-2 or HC-11 are less likely to participate as direct contact residues in E2 binding to CD81, although some of these residues are in a region previously associated with this function (9). If these residues are required in virus binding to CD81, we would not expect escape mutations at these sites. We next tested whether the loss of viral fitness resulted from a reduction in the efficiency of binding of the escape variants to CD81. The binding of recombinant soluble CD81 to immobilized HCVpp bearing the single E2 mutations L438F, N434D, and T435A began differentiating from the wt when the concentration of CD81 was ≥5 μg/ml (Fig. 3B). Binding by these mutants was reduced to 33 to 55% of binding by wt HCVpp to CD81 at 5 to 100 μg/ml. The L438F plus T435A double-substitution mutant showed similar reductions in binding to CD81, and the L438F plus N434D mutant was further reduced to 20 to 38% of wt HCVpp (Fig. 3B). Although the L438F plus T435A double-substitution mutant had a more profound effect on viral fitness than the L438F single-substitution mutant, their binding efficiencies to CD81 were similar. However, it remains possible that the combined effect of these two mutations, independently affecting interaction with CD81, accounts for the markedly reduced viral fitness observed with the combined mutations. This is more apparent for the L438F plus N434D mutant, where the incremental decrease in binding to CD81 could be a contributing factor to the greater effect on viral fitness than the L438F HCVcc mutant.

We next reasoned that the reduced affinity of the HCVpp bearing L438F plus T435A mutations for CD81 would be reflected in a reduced ability of the infectious L438F (Fig. 1C, P6) or L438F plus T435A (Fig. 1C, P′3) HCVcc mutants, compared to wt HCVcc, to bind to a decreasing number of CD81 molecules that are expressed on the surfaces of Huh7.5 cells. This was evaluated by blocking CD81 with anti-CD81 at increasing antibody concentrations. The readout was to determine the relative efficiency of anti-CD81 in blocking viral entry (Fig. 3C). Huh7.5 cells were preincubated with anti-CD81 prior to infection with either the L438F or L438F plus T435A HCVcc mutant. Anti-CD81 IC_{50}s for L438F plus T435A and L438F mutants and wt HCVcc were, respectively, 0.17 μg/ml, 0.33 μg/ml, and 0.39 μg/ml. Infection of Huh7.5 cells by the CBH-2 escape mutant A439E HCVcc and infection by wt HCVcc were blocked by anti-CD81 with similar efficiencies, an IC_{50} of 0.35 μg/ml (Fig. 3C). In contrast to the direct CD81 binding study, the greater ability of anti-CD81 to block entry by the L438F plus T435A than the L438F mutant is consistent with the double mutant interacting with CD81 less efficiently than the single L438F mutant. This can account for the further reduction in viral fitness observed with the L438F plus T435A mutant compared to the L438F mutant (Fig. 3A). Collectively, three neutralization escape patterns are observed with domain B antibodies. First, escape from CBH-2 is not associated with a cost to viral fitness, as mutations at residue 431 or 439 are beneficial for virus survival. Second, the mutations leading to escape from HC-11 significantly lower virus fitness and are associated with reduced virus binding to a required coreceptor, CD81. Third, the virus was unable to escape from HC-1. At a critical antibody concentration, complete virus deterioration occurred, indicating that each contact residue in the HC-1 epitope is necessary for virus function or structure.

Epitope mapping and E2 structural analysis. CBH-2, HC-1, and HC-11 bind to overlapping epitopes. However, the three escape profiles emphasize the important role of distinct contact residues in their respective epitopes, whose mutation can lead to viral escape with or without a cost in virus fitness or virus elimination. We consequently proceeded to identify the contact residues within each of the epitopes of CBH-2, HC-1, and HC-11. In addition, epitope mapping could provide additional information on regions of E2 that participate in E2 binding to CD81, since these antibodies mediate neutralization by blocking this interaction. We previously showed that the domain B antibodies cross-competed with each other by 70 to 90% and shared contact residues within the amino acid (aa) 520 to 540 region of E2 (21, 22). In the escape selection studies, however, no mutations occurred in aa 520 to 540. Mutations contributing to escape from CBH-2 and HC-11 were localized in aa 425 to 443, a sequence that includes another region of E2 that has been proposed to be involved in binding to CD81, aa 436 to 443 (9). This led us to postulate that the shared amino acids in aa 520 to 540 are the primary and invariant contact residues for CD81 binding. To fully determine the contact residues within the epitopes of HC-1 and HC-11, we performed sequential alanine substitutions for residues in the H77c 1a HCVpp covering the full-length E2 glycoprotein (aa 384 to 746) (Fig. 4A).

Binding by HC-1 and HC-11 to these proteins was examined by ELISA using lysates of transiently transfected HEK293 cells. The results were normalized according to the E2 abundance in each lysate, as determined by the binding of a nonneutralizing HMAb, CBH-17, to a linear E2 epitope (15). Because CBH-17 does not cross-compete with domain B antibodies, the contact residues of this antibody should not be involved in the domain B epitopes. To confirm that the E2 conformational structure was not altered with each alanine substitution, CBH-4G and CBH-7 binding were also measured. CBH-4G is a nonneutralizing antibody to an E2 conformational epitope, and CBH-7 is a neutralizing antibody to an E2 conformational epitope that has minimal to no cross-reactivity to domain B antibodies (23). Thus, a substitution that results in a reduction in binding to testing antibodies, as well as to either or both CBH-4G and CBH-7, was interpreted as having a global effect on E2 structure instead of being specific for the HC-1 or HC-11 epitope. Specific contact residues were defined as substitutions leading to ≥80% binding reduction. As shown in Fig. 4A, alanine scanning of the entire E2 revealed two regions, encompassing aa 424 to 444 and aa 528 to 535, that were HC-11 specific, with 9 contact residues at positions 425, 428, 436, 437, 438, 442, 443, 530, and 535. For HC-1, 3 amino acids were identified at residues 529, 530, and 535 (Fig. 4A). Since CBH-2 does not bind to the H77c 1a strain, we constructed alanine substitution mutants in the context of a 1b strain. Three separate segments of E2, 424 to 431, 436 to 443, and 529 to 535, were selected for this study based on earlier findings that CBH-2 binds to residues within these segments (22). Seven amino acids were identified as CBH-2 specific at residues 425, 426, 431, 437, 439, 530, and 535 (Fig. 4A).

The complete epitopes of the three HMAbs are summarized in Fig. 4C. On the basis of contact residue location,

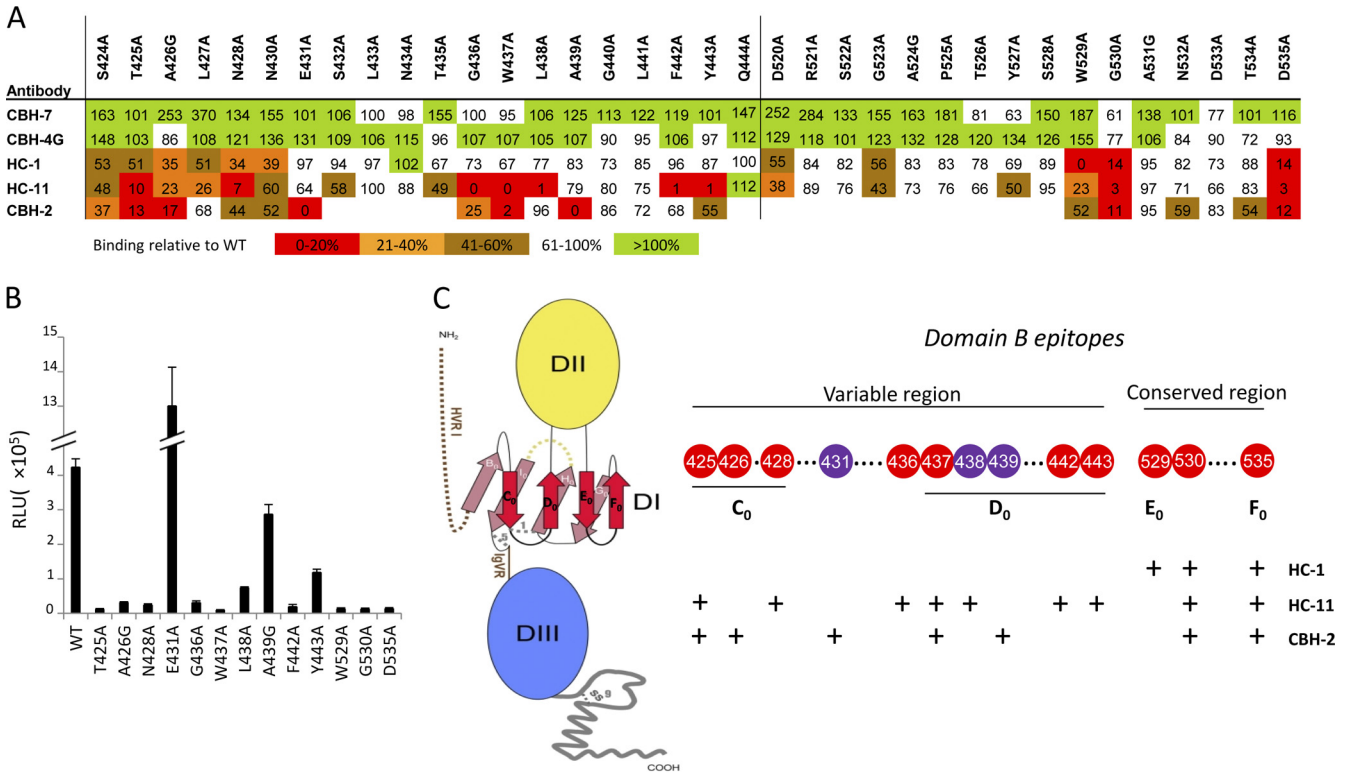


FIG. 4. Epitope mapping and effects of the identified residues on virus infectivity. (A) Two regions of the entire alanine substitution H77c E2 library encompassing S424 to Q444 and D520 to D535 contain contact residues for HC-1 and HC-11. For CBH-2, three regions of the alanine substitution library for E2 genotype 1b, S424 to E431, G436 to Y443, and W529 to D535, contain contact residues. E2 mutant proteins were expressed in HEK293T cells, and the cell lysates were analyzed by ELISA. Individual protein expression was normalized by binding of CBH-17, an HCV E2 HMAb, to a linear epitope (15). Red indicates 0 to 20%, orange 21 to 40%, white 61 to 100%, and green >100% binding when the residue was replaced by alanine relative to binding to the wt. CBH-7 and CBH-4G are two non-domain B HMAbs to conformational epitopes on E2. Retention of binding by these antibodies is necessary to ensure native E2 structure. The data are shown as mean values of two experiments performed in triplicate. (B) Effects of alanine substitutions within CBH-2 and HC-11 epitopes on virus infectivity. HCVpp bearing wt, CBH-2-, or HC-11-related mutations were produced as described in Materials and Methods and used to infect Huh7.5 cells. Virus infectivity was assessed by measuring luciferase activity on day 3 postinfection. The results are shown as luciferase activity signals in infected cells relative to signals from cells infected with vectors alone. The data are shown as the means from three experiments plus SD, with each performed in triplicate. (C) Comparative analysis of three domain B epitopes and their locations on a structure model of the E2 glycoprotein. Contact residues (red and blue circles) for CBH-2, HC-11, and HC-1 are located in two discontinuous regions on E2, aa 425 to 443 and aa 529 to 535. The mutations associated with neutralization escape from HMAb CBH-2 (D431G and 439E) and HC-11 (L438F) are all located in aa 425 to 443 (blue circles), labeled as a variable region. The aa 529 to 535 region is labeled as conserved. The specific contact residues for the three domain B antibodies (+) are located on four β-strands, C_0, D_0, E_0, and F_0, in the central domain I of a structural model of E2.

two regions spanning aa 425 to 443 and aa 529 to 535 are involved in binding to domain B antibodies. In addition, two regions can be identified on the basis of function. The first, at aa 424 to 444, contains contact residues that are unique for particular domain B antibodies, 431 and 439 for CBH-2 and 438 for HC-11, and are responsible for their neutralization escape profiles. In this region, some amino acids, such as 431 and 439, can mutate without compromising viral fitness; some amino acids, such as 438, are less flexible to mutation, and replacement of these residues is accompanied by partial loss in viral fitness through reduced binding affinity to CD81. This was further confirmed by assessing the relative infectivities of mutant HCVpp bearing specific alanine substitutions at each of these sites (Fig. 4B). Replacement of H77c HCVpp mutants associated with the CBH-2 epitope at positions 431 and 439 did not compromise virus entry, although the luciferase signal at 439 was reduced by 30%. Substitution of HCVpp mutants associated with unique contact residues in the HC-11 epitope, 425, 428, 436, 437, 438, 442, and 443, showed greater reductions in infectivity. For the substitution at 438, over 80% reduction was observed. It is possible that modifying the side chain at residue 438 has a structural impact on the CD81 binding region at aa 527 to 535. The second region, at aa 528 to 536, contains 2 residues, 530 and 535, common to all domain B antibodies and 1 residue, 529, shared by a subset of domain B antibodies. Collectively, these results show that residues responsible for escape from CBH-2 and HC-11 are all located in a region designated C_0-D_0 in domain I of a structural model of HCV E2 (25). Although CBH-2, HC-1, and HC-11 bind to overlapping epitopes, there are distinct residues within the respective epitopes located in C_0-D_0 that have major roles in the evolution of neutralization-resistant phenotypes. In addition, the specific contact residues located in this region are likely responsible for the different neutralization profiles exhibited by these antibodies.

DISCUSSION

Our laboratory and others have isolated broadly neutralizing HMAbs to overlapping epitopes of the E2 protein, which we designated domain B (21, 22, 29). The breadth of their protective potential is exemplified by one antibody in particular that completely neutralized the diversity of HCV quasispecies in an infectious inoculum in a human liver-mouse chimeric model (27). The successful isolation of these antibodies suggests that domain B is a highly immunogenic region on HCV E2 that contains a cluster of overlapping epitopes that are able to induce potent neutralizing antibodies in patients infected with HCV. The immunogenicity of this region is supported by a study showing that an HCVpp mutant with an alanine substitution at N532A exhibited greater sensitivity to neutralizing sera obtained from individuals infected with HCV genotype 1a, 1b, 2b, 3, 4, or 5 virus (10). The findings revealed that these sera contain domain B-like neutralizing antibodies directed at residues near N532, in close proximity to residues contributing to domain B epitopes. The elimination of this N-glycosylation site allowed more efficient binding by these domain B-like antibodies to their epitopes surrounding N532 and more efficient virus neutralization (16). Taken together, these results indicate that the epitopes comprising domain B are frequent targets of the humoral immune response in HCV-infected patients. Two questions are of concern from a vaccine perspective: which of the domain B epitopes are prone to accumulating mutations under immune pressure, leading to virus escape from neutralization, as observed with the antibody response to HVR1, and which domain B epitopes remain relatively invariant to accommodate the interactions of E2 with CD81 required for viral viability? We implemented in this study an approach that allowed escape variants to be amplified for detection by propagating infectious 2a HCVcc under increasing concentrations of a neutralizing antibody and by repeated passaging of the surviving virus to achieve a virus titer of 10^4 FFU/ml at each antibody concentration. This led to the isolation of escape mutants bearing mutations in a functional region on HCV E2 that has been associated with binding to CD81.

The studies reported here revealed three patterns contributing to the ability of HCV to escape from virus neutralization by domain B antibodies. For CBH-2, two sets of escape mutants containing mutations at D431G and A439E, respectively, were isolated. The induction of the escape mutant at D431G under the selective pressure of CBH-2 mimics the previously observed naturally occurring variant at this site, D431E, which was not neutralized by this antibody (22). Thus, the design of the *in vitro* escape selection method appears to recapitulate the evolution of viral antigenic determinants under immune pressure in humans. Furthermore, the similarity of the replication rate of infectious CBH-2 escape HCVcc mutants to that of wt HCVcc suggests that escape from CBH-2-like antibodies is similar to the observed rapid escape from the antibody response to HVR1 (40) and does not compromise viral fitness. This pattern of virus escape partially explains the persistent viremia in the context of broadly neutralizing antibodies in the sera of patients with chronic HCV infection.

In the escape studies with HMAb HC-11, wt HCVcc was completely eliminated, and no escape mutants were generated under a starting antibody concentration of 100 μg/ml. By modifying the approach to growing the virus at increasing antibody concentrations starting at a low IC_{50} level of 0.05 μg/ml, an escape mutant with a single substitution at L438F was observed when the antibody concentration reached 10 μg/ml. When the virus was maintained at a steady concentration of 10 μg/ml, wt HCVcc disappeared, and a second mutant bearing a double substitution, L438F plus N434D, appeared. The L438F plus N434D mutant probably evolved from the L438F single-substitution mutant rather than from wt HCVcc (Fig. 1C). When the mixture of variants composed of 20% wt HCVcc, 70% L438F, and 10% L438F plus N434D was exposed to 100 μg/ml HC-11, wt HCVcc disappeared in a single passage and a second double-substitution mutant, L438F plus T435A, appeared. The pattern of rapid elimination of wt HCVcc with the appearance of the L438F plus T435A mutant again suggested that this variant evolved from the L438F mutant. Surprisingly, the appearance of a second mutation at 434 or 435 was not a compensatory change leading to a virus with greater fitness than the single L438F mutation. In fact, the opposite occurred, and the viral fitness of the L438F plus N434D and L438F plus T435A mutants was substantially less than that of the L438F mutant. Although the fine epitope-mapping studies by alanine substitution indicated that neither residue 434 or 435 is part of the HC-11 epitope (as defined by >80% decrease in binding associated with alanine substitution), a possible explanation is that these 2 residues, in proximity to 438, provide some component (albeit minor) to the HC-11 epitope. This is perhaps more likely with residue T435, as HC-11 bound less than 50% to an alanine substitution mutant at T435A compared to wt HCVpp (Fig. 4A). If that is the case, a more "complete" escape is achieved with the L435F plus T435A double mutation under the increased immune pressure associated with a higher concentration of HC-11 at 100 μg/ml. If T435A does not contribute to the HC-11 epitope, the expected outcome is for the L438F single-substitution mutant to persist at 100 μg/ml of HC-11. The contribution by N434 to the binding pocket for HC-11 is less clear. Alanine substitution at this site, N434A, is associated with only a 12% decrease in binding by HC-11. It is also possible that the addition of the N434D mutation to the L438F mutant at 10 μg/ml of HC-11, which leads to the double-substitution mutant, is a random event. Supporting this possibility is the continued presence of both single (L438F) and double (L438F plus N434D) escape mutants with prolonged exposure to 10 μg/ml of HC-11. If N434 contributes to the HC-11 epitope, the L438F plus N434D double-substitution mutant should be a surviving escape mutant. One can speculate that a stepwise pattern occurs in which the virus first accumulates the L438F mutant and then the T435A mutation becomes a mechanism for the virus to escape from prolonged exposure to a broadly neutralizing antibody at increasing concentrations. The implication for vaccine design is a requirement for the immunogen, containing an HC-11-like epitope, to be able to elicit a high-titer antibody response to avoid virus escape. If the antibody response is of low titer, virus escape can occur. Nonetheless, the escape mutations result in a progressive decrease in viral fitness alongside the virus's survival under the selective pressure of HC-11. The L438F single-substitution mutant yielded at least eight times less virus and the L438F plus T435A double-substitution mutant yielded 256 times less virus than wt HCVcc.

Epitope mapping by alanine scanning localized the binding regions of CBH-2 and HC-11 to two regions of HCV E2 encompassing aa 425 to 443 and aa 529 to 535. Both regions have been reported to contain contact residues that form the site of E2 binding to CD81 (9, 30). In a proposed model of the tertiary organization of HCV E2, based on the 3-domain organization of the class II viral membrane fusion proteins (25), the residues in the CD81 binding site on HCV E2 lie in domain I and, more precisely, on its exposed $C_0D_0E_0F_0$ β-sheet (see reference 32a for domain and strand nomenclature). The proposed HCV E2 domain I is organized so that β-strands C_0D_0, as well as E_0F_0, are consecutive in sequence from aa 418 to 444 and aa 526 to 542, respectively. In contrast, there is a long insertion between D_0 and E_0, which is part of domain II. The locations of contact residues for the CBH-2 and HC-11 epitopes on C_0D_0 and E_0F_0 provide support for this model, in which these antibodies bind to the same tertiary structure that interacts with CD81.

The studies of the interaction between the HC-11 L438F escape mutant and CD81 suggested that the loss of viral fitness associated with this mutation is caused in part by a decreased ability of the escape mutant to interact with CD81. This implies that both discontinuous binding regions of HC-11, aa 425 to 443 and aa 529 to 535, contribute to the structure required for virus binding to CD81. This is consistent with a recent report that two HCV E2 regions encompassing aa 384 to 444 and aa 522 to 541 are required for proper protein folding to form the CD81 binding region (1), which is also consistent with the proposed E2 model described above. However, the relative contributions to the structure required for CD81 binding appear different for aa 425 to 443 and aa 529 to 535. The CD81 contact residues within aa 529 to 535 (e.g., 529, 530, and 535) remain absolutely invariant to permit the interactions of E2 with CD81 required for virus viability (Fig. 4) (30). However, the residues within aa 425 to 443, depending on location and substitution, may or may not adversely affect viral fitness or distort the E2 tertiary structure required for this interaction with CD81. The HC-11 L438F-related escape mutants indicate that mutations in this region are more variable and that substitution at L438F is more tolerable from the perspective of virus survival, though it significantly reduces efficiency in binding to CD81. Thus, there appear to be two groups of residues forming the CD81 binding structure of E2. The first group, as exemplified by aa 529, 530, and 535, includes primary contact residues, where any amino acid substitution leads to complete abrogation of CD81 binding. The second group of residues, located in aa 425 to 443, has different degrees of modulating effects on virus binding to CD81. Depending on the location of the residue, such as aa 431, 439, or 438, amino acid substitution may have no effect or variable effects on CD81 binding. It is possibly that the L438F substitution leads to some degree of steric hindrance affecting the binding of the primary contact residues located in aa 529 to 535 to CD81. HC-1 differs from the other two antibodies in that the HC-1 epitope does not contain contact residues in the aa 425 to 443 region. It is probable that the failure of HCV to escape from HC-1 is due to a lack of contact residues in aa 425 to 443, a region having a greater ability to mutate without causing a lethal change to the virus. Collectively, these findings reveal a region on E2 that is responsible for virus escape from broadly neutralizing antibodies, highlight the substantial challenges inherent in developing HCV vaccines, and show that an effective vaccine will need to induce antibodies to intrinsically conserved epitopes in order to lessen the probability of virus escape.

ACKNOWLEDGMENTS

This work was supported in part by PHS grant AI081903 and research support from Crucell NV to S.K.H.F. F.A.R. acknowledges support from ANRS, CNRS, and Institut Pasteur.

The CBH-2, HC-1, and HC-11 HMAbs have been licensed to Crucell for therapeutic development. S.K.H.F. serves as a scientific advisor to Crucell.

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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