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Mutations in Hepatitis C Virus E2 Located outside the CD81 Binding Sites Lead to Escape from Broadly Neutralizing Antibodies but Compromise Virus Infectivity

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Broadly neutralizing antibodies are commonly present in the sera of patients with chronic hepatitis C virus (HCV) infection. To elucidate possible mechanisms of virus escape from these antibodies, retrovirus particles pseudotyped with HCV glycoproteins (HCVpp) isolated from sequential samples collected over a 26-year period from a chronically infected patient, H, were used to characterize the neutralization potential and binding affinity of a panel of anti-HCV E2 human monoclonal antibodies (HMAs). Moreover, AP33, a neutralizing murine monoclonal antibody (MAb) to a linear epitope in E2, was also tested against selected variants. The HMAs used were previously shown to broadly neutralize HCV and to recognize a cluster of highly immunogenic overlapping epitopes, designated domain B, containing residues that are also critical for binding of viral E2 glycoprotein to CD81, a receptor essential for virus entry. Escape variants were observed at different time points with some of the HMAs. Other HMAs neutralized all variants except for the isolate 02.E10, obtained in 2002, which was also resistant to MAb AP33. The 02.E10 HCVpp that have reduced binding affinities for all antibodies and for CD81 also showed reduced infectivity. Comparison of the 02.E10 nucleotide sequence with that of the strain H-derived consensus variant, H77c, revealed the former to have two mutations in E2, S501N and V506A, located outside the known CD81 binding sites. Substitution A506V in 02.E10 HCVpp restored binding to CD81, but its antibody neutralization sensitivity was only partially restored. Double substitutions comprising N501S and A506V synergistically restored 02.E10 HCVpp infectivity. Other mutations that are not part of the antibody binding epitope in the context of N501S and A506V were able to completely restore neutralization sensitivity. These findings showed that some nonlinear overlapping epitopes are more essential than others for viral fitness and consequently are more invariant during earlier years of chronic infection. Further, the ability of the 02.E10 consensus variant to escape neutralization by the tested antibodies could be a new mechanism of virus escape from immune containment. Mutations that are outside receptor binding sites resulted in structural changes leading to complete escape from domain B neutralizing antibodies, while simultaneously compromising viral fitness by reducing binding to CD81.

Over 170 million people worldwide are infected with hepatitis C virus (HCV). While acute infection is usually silent, the majority of infected individuals develop persistent infections. Approximately 30% of acute infections are spontaneously resolved. Cellular immunity is clearly necessary, as robust and sustained CD4+ and CD8+ T-cell responses are temporally associated with virus clearance leading to disease resolution (7). Persistent infection is associated with an inability to sustain a vigorous CD4+ response. The role of antibodies in disease resolution is increasingly recognized but less understood. Clinical trials with gamma globulin administration prior to the discovery of HCV achieved prophylactic effects on transfusion-associated non-A, non-B hepatitis cases, most of which were subsequently shown to be HCV related (28, 46). Animal studies showed that gamma globulin therapy delayed the onset of acute HCV infection (29). Preincubation of the infectious inoculum with pooled gamma globulin from HCV-positive donors prevented infection in challenged chimpanzees (55). The protection afforded by gamma globulin preparations correlated with antibody titers blocking infection of target cells with retroviral pseudotype particles expressing HCV E1E2 glycoproteins (HCVpp) (4). In addition, chimpanzees vaccinated with recombinant HCV E2 glycoproteins were protected against infection in a manner that correlated with serum antibody titers inhibiting binding of E2 to CD81 (19, 40, 41), a receptor required for entry by both HCVpp and cell culture infectious HCV (HCVcc) (5, 17, 33, 53, 56). Two recent studies observed that patients with strong and progressive neutralizing antibody responses demonstrated decreasing viremia and control of viral replication (31, 39). A third study, however, reported the lack of neutralizing antibodies to heterologous HCVpp isolates in the sera of patients who eventually controlled their viremia during acute HCV infection (21). Furthermore, 10⁴ to 10⁶ virions per milliliter of serum are usually detected during chronic infection in the presence of high titers of serum neutralizing antibodies. A driver of persistent viremia is a high degree of viral vari-
ants, or “quasispecies.” Owing to a high viral replication rate (10^{12} copies per day) and an error-prone viral RNA-dependent polymerase, the estimated mutation rate is 2.0 × 10^{-3} base substitutions per genome per year (9, 34). This high rate of quasispecies formation contributes to the emergence of escape viral variants from immune surveillance. Mutations within major histocompatibility complex class I-restricted HCV epitopes lead to escape from cytotoxic T-cell responses (7). Mutations leading to escape from humoral immunity, particularly in E2 hypervariable region 1 (HVR1), known to be the target of host neutralizing antibodies, are also documented (10, 22, 30, 45). Protection in chimpanzees is achieved following challenge with an inoculum that had been preincubated with antibodies to autologous HVR1 (10). Yet over time, these isolate-specific antibodies drive the emergence of new viral variants that the concurrent immune response poorly recognizes. A study of sequential HCV isolates obtained from a patient, H, who was meticulously followed for a 26-year period starting 3 weeks after exposure to the virus, showed that the serial HCV variants were poorly neutralized by the concurrent serum antibodies (52). Escape was associated in part with mutations in HVR1 leading to decreased binding and neutralization by monoclonal antibodies (MAbs) to HVR1 that were produced against the first isolate obtained from this patient.

Broadly neutralizing antibodies are usually directed against conformational epitopes within E2 (2, 8, 13, 14, 44). We previously described a panel of neutralizing and nonneutralizing human MAbs (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 (23–25). All nonneutralizing antibodies fell within one cluster, designated domain A (24). Neutralizing HMAbs segregated into two clusters, designated domains B and C, with domain B HMAbs having greater potency than domain C HMAbs in blocking infection with the strain JFH1 genotype 2a HCV (23, 25).

The epitopes of increasing numbers of anti-HCV E2 neutralizing antibodies include residues that are also critical for binding of E2 to CD81. All of our domain B HMAbs inhibit binding of E2 to CD81. Alanine scanning mutagenesis of E2 domains implicated in binding to CD81 identified two highly conserved residues, G530 and D535, that are needed for all domain B antibodies, with a subset also requiring W529 (25, 26, 36). Other laboratories have isolated similar neutralizing antibodies to epitopes containing these residues (20, 32, 38). A similar panel of E2 mutants was previously used to identify five amino acid residues, W420, Y527, W529, G530, and D535, that affect CD81 binding and virus entry. The conserved nature of these mutations on E2 at a considerable distance from CD81 binding is unlikely that they are part of the domain B HMAb or the AP33 epitopes. Site-directed substitutions at these mutations restored neutralization sensitivity to all antibodies and CD81 dependency.

MATERIALS AND METHODS

Cell culture, antibodies, and reagents. HEK-293T cells were obtained from the ATCC. HuH7.5 cells (6) were grown in Dulbecco’s modified essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Sigma-Aldrich Co., St. Louis, MO) and 2 mM glutamine. HMAbs CBH-2, CBH-5, CBH-6, CBH-11, HC-2, and HC-11 and MAb AP33 against HCV E2 were generated as described previously (14, 23, 35). MAb against human CD81 clone JS-81 was purchased from BD Bioscience (San Jose, CA). A molecular clone encoding the HCV large extracellular loop fused to glutathione X-transferee was generously provided by Shoshana 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). A series of plasmids encoding H77c E1E2 variants derived from patient H sera obtained between 3 weeks postinfection and throughout the subsequent 26 years thereafter were constructed as described previously (52). The plasmids were designated aa.bb, where aa denotes the year of serum sample collection and bb denotes the clone number. For example, 02.E10 represents HCV RNA that was isolated from a plasma sample collected in 2002 and the E10 clone represents the consensus sequence among multiple clones obtained from 2002.

HCVpp production, infection, and neutralization assay. HCVpp were produced as described previously (17, 24) by cotransfection of 293T cells with plasmids containing the env-defective 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 or phosphate-buffered saline instead of the antibody plus an infectivity control plus 4 μg/ml polybrene at 37°C for 40 min. The HCVpp-antibody mixture was added to preseeded Huh7.5 cells (8 × 10^3 cells/well) in a white nontransparent 96-well plate. For blocking of virus entry by anti-CD81, the cells with preincubated for 1 h with the mouse antibody prior to the addition of HCVpp. The plate was centrifuged at 730 × g for 2 h at room temperature before being placed in a humidified cell culture chamber containing 5% CO2, at 37°C. Unbound virus was replaced with fresh complete medium, and then the plates were incubated for a total of 72 h. After addition of 100 μl of reconstituted Bright-Glo (Promega) to each well, followed by mixing for 2 min at room temperature, luciferase activity was measured by a Veritas microplate luminometer (Turner Biosystems). The virus neutralization activity of an antibody was determined by the percent reduction of luciferase activity compared with the level for the phosphate-buffered saline control. For HCVpp infectivity studies, the virus-containing extracellular medium was normalized for human immunodeficiency virus p24 expression by using a QuickTiter lentivirus titer kit (Cell Biolabs, San Diego, CA) in accordance with the manufacturer’s instructions. The transfected 293T cells were lysed with a buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 20 mM iodoacetamide, and protease inhibitors. The lysates were used as the source of E2 antigen at approximately 225 ng/ml in the immunoassays as described below.

Quantitative enzyme-linked immunoassays. Microtiter plates were prepared by coating each well with 500 ng of Galanthus nivalis lectin (GNA; Sigma, St. Louis, MO), followed by blocking of the wells with BLOTTO, consisting of 2.5% nonfat dry milk and 2.5% normal goat serum in TBST (20 mM Tris-HCl, pH 7.5, 0.1% Tween 20, 150 mM NaCl).
TABLE 1. Neutralization and antibody binding affinity for sequential patient H variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>CBH-11</th>
<th>CBH-2</th>
<th>CBH-8C</th>
<th>CBH-5</th>
<th>HC-2</th>
<th>HC-11</th>
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<td>Kᵣ</td>
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<td>77.19</td>
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<td>77.20</td>
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<td>NR</td>
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<td>3.5</td>
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<td>NB</td>
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<td>47.6</td>
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<tr>
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<td>NB</td>
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<td>71.4</td>
<td>17.2</td>
<td>20.3</td>
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<tr>
<td>95.C5</td>
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<td>NB</td>
<td>79.9</td>
<td>148.5</td>
<td>15.5</td>
<td>59</td>
<td>14.9</td>
</tr>
<tr>
<td>02.E10</td>
<td>NR</td>
<td>NB</td>
<td>216.5</td>
<td>106.3</td>
<td>21.6</td>
<td>59</td>
<td>131.5</td>
</tr>
</tbody>
</table>

* Plasmids encoding HCV variant E2 glycoproteins are designated aa.bb, where aa denotes the year of serum sample collection and bb denotes the clone number (52). IC₅₀ and Kᵣ are in μg/ml, and Kᵣ is in nM. NR, not reaching 50% neutralization; NB, no binding.

150 mM NaCl, 0.1% Tween 20). For antibody affinity analysis, lysates of 293 cells expressing HCV E1E2 as described above were first captured on the GNA-coated plates. The bound antigens were then incubated with HMAb to HCV E2 at concentrations of 0, 0.05, 0.2, 1, 5, 20, 100, and 200 μg/ml. After a wash, the bound antibodies were then visualized with AP-conjugated goat anti-human immunoglobulin G (IgG; Santa Cruz Biotechnologies, Santa Cruz, CA) and developed using p-nitrophenyl phosphate disodium hexahydrate. Absorbance was measured at 405 nm and 570 nm using a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA). The data were analyzed by nonlinear regression to measure the antibody dissociation constant (Kᵣ) and the maximum binding level (Bₘₐₓ; optical density at 405 nm), using Prism software (GraphPad).

For CD81 binding analysis, 20% sucrose cushion-pelleted HCVpp were re-suspended in NTE buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) and captured on the GNA-coated plates. Bound antigen was first incubated with purified CD81 at concentrations of 0, 0.5, 1.0, 2.0, 10, 20, 50, 100, 200, and 500 μg/ml. The bound CD81 was visualized with AP-conjugated mouse anti-human CD81 (Santa Cruz Biotechnologies, Santa Cruz, CA). Color development and absorbance measurement were performed as described above.

In vitro 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 02.E10 variant (GenBank accession no. DQ979514) E1E2-encoding sequence was used as the template for initial single mutations and then as the template for introduction of double mutations. The triple mutations were constructed sequentially in the context of the double mutations. 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 mutated constructs were designated X#Y, where # is the residue located in H77c (GenBank accession no. AF009606), X denotes the single-letter code for the H77c or 02.E10 amino acid, and Y denotes the altered amino acid.

RESULTS

Patient H, followed for a 26-year period starting 3 weeks after exposure to HCV, has had a clinical course of mild and nonprogressive chronic hepatitis typical of most patients with HCV infection (52). Earlier studies showed that his chronic infection continuously generated quasispecies that were poorly neutralized by the concurrent serum antibodies. While the persistent viremia can be explained in part by escape from neutralizing antibodies to HVR1, cross-neutralizing serum antibodies were observed beginning at week 111 after infection in patient H. This raises questions regarding the extent to which chronic infection leads to quasispecies that are able to evade broadly neutralizing antibodies and the identity of the mechanisms that contribute to such escape.

Profiling of binding of neutralizing antibody to E2 variants. Six neutralizing HMAbs to overlapping epitopes within E2 domain B were selected to test for binding to sequential E2 samples obtained from patient H (52). This was performed using lysates from transfected cells expressing wild-type H77c and variant E1E2 polyproteins that were captured onto GNA-coated enzyme-linked immunosorbent assay (ELISA) plates. Each antibody was tested at dilutions ranging from 0.1 to 100 μg/ml (Table 1). R04, a human Ig isotype control HMAb to human cytomegalovirus, showed no binding to either H77c or the other H variants. Of the six anti-E2 antibodies, only three, CBH-5, HC-2, and HC-11, bound to H77c E2. Interestingly, all six antibodies showed low-minimum binding (HC-11 > HC-2 > CBH-8C > CBH-5 > CBH-2) to no binding (CBH-11) to 02.E10 E2, a dominant variant isolated at the 26th year after acute infection. With 02.E10 set aside, four binding patterns emerged. The first pattern, represented by CBH-11, was failure to bind to H77c and all of the variants. The second binding pattern, displayed by CBH-2 and CBH-8C, showed binding to the sequential variants, beginning with those collected in 1991. The antibody Kᵣs for these variants ranged from 63.4 to 270.9 nM and from 40.5 to 71.4 nM, respectively. The third binding pattern, represented by CBH-5, showed binding to all variants but had lower Kᵣs for the 1977 isolates (131.3 to 424.7 nM) than for the subsequent sequential variants (6.9 to 20.3 nM). The fourth binding pattern, represented by HC-2 and HC-11, showed binding to all variants, including H77c, and had a tighter range of Kᵣs, which were from 4.4 to 23.4 nM and 9.9 to 44.3 nM, respectively. Taken together, the variants showing altered binding affinities for individual antibodies at different years from 1977 to 1995 suggest that the virus might continuously mutate within E2 regions as defined by these domain B antibodies. However, the 02.E10 variant isolated at the last sampling point showed low to no binding by all domain B antibodies tested. This variant, in comparison to H77c, also showed reduced binding by MAb AP33 (see Fig. 5B), which is in agreement with earlier findings with MAb 3/11 recognizing a similar epitope on E2 (11, 35, 50, 52). In addition, these two murine antibodies exhibit no cross-competition with any domain B antibodies (data not shown). The findings suggest that the 02.E10 variant might have undergone a structural change that negatively modulates binding by the antibodies to overlapping conformational epitopes within domain B as well as to two nonoverlapping linear epitopes on E2. These antibodies
share a common mechanism of virus neutralization by blocking virus binding to CD81.

Neutralization of the sequential HCV variants. To assess whether the binding patterns of the tested antibodies correlated with neutralization, antibody dose-dependent studies were performed using HCVpp consisting of E1E2 variants assembled onto retroviral core particles. Four neutralization patterns that paralleled the antibody binding patterns were observed. As shown in Fig. 1 and summarized in Table 1, H77c and all of the variants escaped from CBH-11 neutralization in the first pattern. Low neutralizing activity against 02.E10 was observed, but without a dose-dependent effect. In the second pattern, the 1977 isolates failed to be neutralized by CBH-2 and CBH-8C. Neutralization was observed for CBH-8C starting with the 1991 variants and the subsequent variants through 1995, but not for CBH-2, where decreased neutralizing activity was observed for the 1992 variant. The 50% inhibitory concentrations (IC50s) ranged from 0 to 95.3 μg/ml for CBH-2 and from 9.0 to 98.0 μg/ml for CBH-8C. CBH-5 showed a third neutralization pattern, with lower neutralization potency for H77c (IC50 at 82.5 μg/ml) and no neutralization with one of the 1977 isolates but high activities against the 1991-to-1995 variants (IC50 at 3.5 to 19.8 μg/ml). HC-2 and HC-11 displayed a fourth pattern, with relatively sustained neutralization activities against H77c and all of the variants from 1977 to 1995. The IC50s ranged from 1.2 to 27.0 μg/ml for HC-2 and from 2.1 to 35.8 μg/ml for HC-11. The different neutralization patterns confirm our previous observation that the six domain B antibodies recognize overlapping but distinct epitopes. Each of these epitopes contains residues that are also involved in binding of E2 to CD81 and other antibody-specific residues that are discontinuous from the CD81 binding region (25–27).

Neutralization studies with each antibody against 02.E10 HCVpp showed no dose-dependent effect and produced generally weak responses, with a maximum of 40% neutralization. The 02.E10 variant was resistant to CBH-5, HC-2, and HC-11 in comparison to H77c HCVpp and also resistant to CBH-2 and CBH-8C in comparison to the 95.C8 HCVpp. These findings are consistent with the loss in binding to the 02.E10 variant by all domain B antibodies. In the course of these studies, we noted that the 02.E10 HCVpp infectivity was very low, which contributed to unreliable measurements of neutralization, as shown by the lack of a dose-dependent effect with CBH-8C and CBH-11. The breadth of antibody escape by variant 02.E10 implies that the location of the mutation or mutations caused a change in the conformational structure of E2 in relationship to the epitopes identified by this group of neutralizing antibodies.

Sequence analysis of E2-carrying variants. To assess the sequence variation associated with the broad neutralization escape and low infectivity shown by the 02.E10 variant, the E2 sequences from all of the H variants were compared (Table 2).
based on the published sequences (52). Analysis revealed 25 amino acid changes that are outside the HVR1 region among the variants in relationship to the reference H77c isolate (GenBank accession no. AF009606). The mutations can be divided into four groups on the basis of what year they were identified in and whether the mutation was maintained throughout the remaining years of observation. The first group (type 1) contains mutants that emerged by 1991 and were fixed for the remaining years (mutations E431D, T435A, A466D, S528N, A531E, V538I, L580R, D610H, V636I, and A713T). In the second group (type 2), mutations were introduced in various isolates but not in the 02.E10 variant (at residues N415, L438, P453, D481, Y489, F537, I622, and V720) or were in 02.E10 and also other variants (at residues L480 and I626). The third group (type 3) contains mutations that are similar in pattern to the first group and were present in 1991 but maintained only through 1995, and then two new mutations were found in 2002 (Q444H and K446G). Finally, the fourth group (type 4) contains 2002 variant-specific mutations (E482Q, S501N, and V506A). Since we observed previously that S501 and V506 are located in a region that is crucial in maintaining a native E2 structure (18), we reasoned that the amino acid substitutions of the type 4 group most likely contribute to the observed phenotypes of the 02.E10 variant, followed by type 3 substitutions.

Mutations responsible for reduced 02.E10 variant infectivity. To assess which of the five type 3 and 4 amino acid changes in the 02.E10 variant led to reduced infectivity and/or the neutralization-resistant phenotype, we introduced substitutions at each site either individually or jointly in the 02.E10 clone (Fig. 2A). Each substitution was based on the corresponding residue in an earlier H variant or in H77c. The mutant 02.E10 E1E2 was then incorporated into HCVpp, and its infectivity was compared to that for H77c. HCVpp infection of Huh7.5 cells was quantified by a luciferase assay. As shown in Fig. 2A, substitutions Y444H, Y444Q, R446K, R446G, Q482E, and N501S in 02.E10 were unable to significantly increase 02.E10 infectivity, indicating that they were not responsible for the reduced infectivity of the 02.E10 variant. In contrast, A506V substitution restored 50% infectivity compared to the level for H77c. The mutation at residue 501, being in close proximity to residue 506, could compound the structural effects of a mutation at residue 506, which prompted us to introduce the double mutations (N501S/A506V) into HCVpp 02.E10 E2. Indeed, infectivity was restored to the levels of H77c HCVpp. Taken together, the substitutions at residues 444, 446, and 482 in 02.E10 have no role in viral infectivity, but introduction of substitutions at residues 501 and 506 restored the infectivity of the 02.E10 variant. It is probable that the proximity of this pair of mutations is a contributing factor in their effect on the E2 structure. To confirm these findings, alanine substitutions at these five residues were introduced

### Table 2: Sequence analysis of amino acid differences in the HCV E2 glycoproteins of H77c and variants outside HVR1

<table>
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<tr>
<th>A.A. Location</th>
<th>H77c</th>
<th>H77 319</th>
<th>H77 320</th>
<th>H91 64</th>
<th>H91 A6</th>
<th>H91 A11</th>
<th>H91 B1</th>
<th>H92 C1</th>
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*Type 1 (yellow), 2002 residues that are identical, with H91, H92, and H95 variants, but with an exception in one 1991 variant (A/T) at position 713; type 2 (purple), mutations in specific variants but not in the 2002 variant or mutations in both 2002 and other variants; type 3 (green), 2002-specific mutations, but with H77c being different from H91, H92, and H95 variants at residues 444 and 446; type 4 (red), 2002-specific mutations.
individually in the H77c construct (Fig. 2B). We observed that substitutions at residues 444, 446, and 501 decreased H77c HCVpp infectivity modestly and that substitution at residue 482 slightly increased infectivity, but H77c HCVpp bearing V506A decreased infectivity by more than 90%.

**Mutations at residues 501 and 506 modulate E2-CD81 interaction.** As the S501N/V506A mutations had a significant impact on 02.E10 HCVpp infectivity, we tested whether these mutations adversely affected the virus interaction with CD81. This in turn could account for the inefficient cell entry as displayed by the 02.E10 variant, relative to the levels for H77c and the other H variants (Fig. 2A). We investigated the effect of the double mutations on the binding of the virus to CD81. This was assessed by evaluating the ability of anti-CD81 to inhibit variant and mutant HCVpp entry and by determining the binding of the mutant E2 to CD81. As shown in Fig. 3A, pretreatment of Hu7.5 cells with anti-CD81 antibody inhibited H77c HCVpp infection by 90% but only 20% inhibition was observed for the 02.E10 HCVpp. A control without antibody was used as the baseline for each variant, again showing low infectivity for the 02.E10 HCVpp, as observed in Fig. 2A. Interestingly, anti-CD81 antibody inhibited infection by 02.E10 HCVpp bearing N501S/A506V mutations as efficiently as H77c, indicating that residues 501 and 506 in combination may contribute to the maintenance of the structural conformation for E2 that is optimum for its interaction with CD81. Isotype-matched control antibodies did not specifically reduce HCVpp infection (data not shown).

We next analyzed the binding disassociation constants of various E2 variants to CD81 by ELISA. As shown in Fig. 3B, C, and D, CD81 bound approximately fourfold less to 02.E10 E2 than to both H77c E2 and the N501S/A506V-02.E10 E2 variant, with $K_d$ of 6.32, 1.53, and 1.72 μM, respectively. But the $B_{max}$ values for binding of CD81 to all three E2 variants were relatively consistent, with values of 0.41 to 0.54, suggesting that the incorporations of envelope proteins were nearly equal among these variants. The observation that the binding affinity of 02.E10 E2 for CD81 was decreased but that the $B_{max}$ was similar to that for binding of CD81 to H77c and to N501S/A506V-02.E10 E2 is consistent with the view that substitutions at residues 501 and 506 caused a structural alteration of the E2-CD81 binding region. To ensure that 02.E10 HCVpp entry was occurring, since this variant showed a low luciferase (infectivity) reading, maximum blocking of entry with anti-CD81 was performed. Anti-CD81 blocked 02.E10 HCVpp entry more than 25% compared to the level for a vector control (data not shown). These studies collectively showed that the mechanism of escape by the 02.E10 variant from neutralizing antibodies is by negatively modulating the virus interaction with CD81.

**FIG. 3.** Substitutions S501N/V506A in the context of the 02.E10 variant compromise binding of HCVpp to CD81. (A) Blocking of HCVpp infectivity by anti-CD81. Huh7.5 cells were preincubated with 5 μg/ml of anti-CD81 prior to infection with HCVpp bearing H77c, 02.E10, and 02.E10-N501S/A506V E2 envelope proteins. The control for each variant was without anti-CD81. The results are expressed as percentages of neutralization relative to the level for a no-antibody control for each variant or mutant, as measured by luciferase activity. N501S/A506V 02.E10 envelope protein to CD81 by ELISA. The binding measurements were performed using 20% sucrose cushion pellet HCVpp. GNA-captured HCVpp were incubated with a range (0.01 to 500 μg/ml) of CD81 (x axis), and the bound receptor was detected using an anti-CD81 MAb. The y axis shows the mean optical density (OD) values for triplicate wells representing bound CD81. Error bars represent 1 standard deviation from the mean. The saturation binding curves were fit by nonlinear regression. $K_d$ (μM) and $B_{max}$ values were calculated using GraphPad Prism software, and Scatchard plots of the equilibrium binding data are shown in the insets.
Effect of S501N/V506A mutations on antibody-mediated neutralization. Since N501S/A506V restored 02.E10 infectivity by restoring E2-CD81 binding to a level similar to that for H77c, we next investigated whether mutations S501N/V506A are also responsible for the ability of the 02.E10 variant to escape from neutralizing antibodies (Fig. 4 and 5B). First, five domain B HMAbs and MAb AP33 were assessed for their binding affinities for 02.E10 E2 carrying N501S/A506V substitutions. CBH-11 was not included, because it failed to bind and neutralize H77c and all of the sequential variants (Fig. 1). Interestingly, CBH-5 and HC-11 had lower $K_d$ s for N501S/A506V-02.E10 E2 (6.2 and 7.8 nM, respectively) than for H77c (131.3 and 12.1 nM, respectively) or for 02.E10 E2 (131.5 and 37.3 nM, respectively), as summarized in Fig. 5B. In the cases of CBH-2 and CBH-8C, reduced $K_d$ s (71.1 and 49.4 nM, respectively) were also obtained in comparison to those for their binding to either 95.C8 E2 (148.5 and 59 nM, respectively) (Table 1) or 02.E10 E2 (216.5 and 106.3 nM, respectively). Note that these two antibodies do not bind to H77c. HC-2 had a binding affinity for 02.E10 E2 carrying N501S/A506V substitutions ($K_d$, 11.9 nM) that was lower than that for H77c ($K_d$, 4.4 nM) but higher than that for 02.E10 E2 ($K_d$, 55.7 nM). The AP33 binding disassociation constant remained similar to those for 02.E10 E2 and 02.E10 E2 carrying N501S/A506V substitutions ($K_d$s of 5.0 and 5.5 nM, respectively), which were higher than that for H77c ($K_d$, 1.7 nM). Taken together, the binding affinities of the antibodies for the E2 conformational epitopes CBH-2, CBH-8C, CBH-5, HC-2, and HC-11 were improved 3.0-, 2.1-, 21.1-, 4.7-, and 4.8-fold, respectively, by N501S/A506V substitutions in the 02.E10 variant. As residues 501 and 506 are not known to be part of the epitopes identified by these antibodies, the findings are consistent with the view that residues 501 and 506 affect the E2 conformational structure required for these antibodies to bind to E2 as well as for E2 to bind to CD81.

Next, we assessed whether these antibodies showed im-
proved neutralizing activities against 02.E10 HCVpp bearing N501S/A506V E2. H77c or 95.C8 HCVpp was used for comparison against appropriate antibodies. As shown in Fig. 4, N501S/A506V-02.E10 HCVpp exhibited dose-dependent neutralization by all antibodies tested. In comparison, 02.E10 HCVpp showed no consistent dose-dependent neutralization. However, recovery of neutralization activities against N501S/A506V-02.E10 HCVpp was only 30 to 40% for all five domain B HMAbs, even at an antibody concentration of 100 μg/ml, compared to the level for H77c or the 95.C8 variant. In the case of AP33, the IC_{50} for wild-type H77c HCVpp was 0.7 μg/ml (Fig. 5B). For 02.E10 HCVpp, only 20% neutralization was observed at 100 μg/ml (Fig. 4), and for the N501S/A506V-02.E10 HCVpp, the IC_{50} was 8.8 μg/ml (Fig. 5B). Although N501S/A506V restored >90% AP33 neutralization at 100 μg/ml compared to the level for H77c, the IC_{50} indicates only partial restoration. While substitutions S501N/V506A are responsible for the loss in the binding of domain B antibodies to the 02.E10 variant, they can account for only partial restoration of the neutralization activities of these antibodies to conformational epitopes. The findings suggest that other residues are likely involved in forming the binding pockets for domain B antibodies to effectively block E2-CD81 interaction.

Effects of other mutations on modulating neutralizing antibody potency. The unexpected low neutralization potency in association with fully recovered binding affinity prompted us to introduce additional substitutions. Each individual substitution (Y444Q, Y444H, R446K, R446G, or Q482E) (Table 2) in the context of N501S/A506V-02.E10 E2 was tested to determine whether additional residues beyond 501 and 506 could affect virus neutralization. Although residues Y444, R446, and Q482 are 02.E10 specific but were judged not to contribute to restoration of 02.E10 infectivity (Fig. 2), we speculated that they could, in concert with residues 501/506, adversely affect domain B neutralizing antibodies. The substitutions at each of these residues were based on the corresponding residue located in either H77c or a later H variant. Five domain B antibodies and AP33 were assessed for their abilities to neu-
tralize the five N501S/A506V-02.E10 HCVpp bearing Q482E, Y444Q, Y444H, R446K, or R446G, as summarized in Fig. 5B. The 02.E10 HCVpp bearing N501S/A506V E2 was used as the control. The neutralization activities of CBH-2 and HC-11 are shown as representatives of domain B antibodies in Fig. 5A. Variant 95.C8 was used as a baseline to assess the neutralization potencies of CBH-2, CBH-8C, and CBH-5. As shown in Fig. 5A, H77c HCVpp was neutralized by HC-11, with the IC_{50} at 2.1 μg/ml (Fig. 5B). N501S/A506V-02.E10 HCVpp showed 40% neutralization by HC-11 at 100 μg/ml, whereas the parent, 02.E10, was resistant to neutralization by this antibody. In contrast, four of the five substitutions, Y444H, Y444Q, R446G, and R446K of 02.E10 HCVpp bearing N501S/A506V E2, showed dose-dependent HC-11 neutralizations, with IC_{50} of 16.9, 3.3, 65.6, and 15 μg/ml, respectively. Introduction of Q482E to N501S/A506V-02.E10 HCVpp did not result in any improvement in HC-11 neutralization potency, which remained at 45% neutralization at an antibody concentration of 100 μg/ml. In the case of CBH-2, the IC_{50} for variant 95.C8 was 79.5 μg/ml (Table 1). N501S/A506V-02.E10 HCVpp was neutralized 36% at an antibody concentration of 100 μg/ml, and CBH-2 was unable to neutralize 02.E10. Similar to what was found for HC-11, four of the five substitutions, Y444H, Y444Q, R446G, and Q482E, showed dose-dependent CBH-2 neutralization in 02.E10 HCVpp bearing N501S/A506V E2, with IC_{50} of 26.7, 4.8, 80.3, and 7.7 μg/ml, respectively. Introduction of R446K into N501S/A506V-02.E10 HCVpp did not show any improvement with CBH-2 neutralization potency, which remained at approximately 40% neutralization at an antibody concentration of 100 μg/ml. Similar effects on neutralization potency were observed for CBH-8C, CBH-5, and HC-2, as summarized in Fig. 5B. Collectively, substitutions Y444(H/O), R446(G/K), and Q482E improved neutralization by these antibodies, with the exception of Q482E and R446K, which showed no changes for HC-11- and CBH-2-mediated neutralizations, respectively. Substitution at residue 444 showed greater improvement in neutralization potency than the substitutions at residues 446 and 482. Among the substituted amino acids, replacement with glutamine showed higher neutralization potency than replacement with histidine. Substitution Y444Q in combination with N501S/A506V led to a near-complete restoration of 02.E10 sensitivity to neutralization by the tested antibodies. The infectivity of this mutant was also similar to the level for H77c. These findings indicate that only in concert with mutations at residues 501 and 506 can a mutation at residue 444, 446, or 482 negatively modulate antibody-mediated neutralization by a mechanism independent of the epitopes, as identified by these antibodies.

This possibility was further tested with AP33 against the Y444H and Y444Q 02.E10 HCVpp bearing N501S/A506V E2 (Fig. 5B). The introduction of Y444Q to HCVpp bearing N501S/A506V-02.E10 E2 restored AP33 neutralization potency nearly to the same level as that observed with H77c. No effect was observed with the Y444H mutant. Their IC_{50} were 1.8 and 9.7 μg/ml, respectively, compared to the IC_{50} of 8.8 μg/ml for 02.E10 HCVpp bearing S501N/V506A E2. These findings confirm that residue 444 is able to negatively modulate neutralizing antibodies independently of the locations of their epitopes. As seen in the case of domain B conformational epitopes, replacing tyrosine with glutamine resulted in greater improvement than replacing tyrosine with histidine. Although the mechanism is unclear, these residues in concert with the mutations at residues 501 and 506 resulted in an alteration of the E2 structure that affected the potency of neutralizing antibodies without affecting E2-CD81.

**DISCUSSION**

A majority of broadly neutralizing anti-HCV antibodies identified to date seem to exert their inhibitory effect by blocking E2-CD81 interaction. In this respect, the epitopes of our neutralizing domain B HMABs include E2 amino acids W529, G530, and D535, residues that are also critical for the interaction of E2 with CD81 (37). These residues are also crucial for recognition by broadly neutralizing human antibodies isolated in other laboratories (20, 32, 38). Indeed, some of these antibodies have been shown to prevent infection with heterologous viruses in the human liver chimeric mouse model (32). Antibody cross-competition studies show that these residues collectively form a distinct cluster of overlapping epitopes, with various degrees of conservation among different HCV genotypes and subtypes (14, 25, 32). Furthermore, removal of an N-glycan by N532A substitution in E2 leads to enhanced neutralization by a domain B HMAB and sera from individuals infected with different viral genotypes, indicating that antibodies to domain B epitopes are commonly found during chronic HCV infection (15). Collectively, these observations confirm that domain B is a highly immunogenic region on HCV E2, containing multiple overlapping neutralization epitopes.

If broadly neutralizing antibodies are commonly found during chronic infection, what are the mechanisms by which the virus avoids immune containment? One possibility is that virion components such as glycans (as mentioned above) or lipoproteins may provide a protective shield. In the infected host, HCV can be found as lipoid-coated virions and antibody-complexed virions. Virions primarily associated with low-density lipoproteins display higher infectivity than higher-density virions associated with antibodies (16, 48). Furthermore, lower-density virions during chronic infection are associated with IgG, IgM, and apolipoprotein B (3), suggesting a modulating effect of antibody-mediated neutralization. Binding of lipoprotein-coated virions to the cellular low-density-lipoprotein receptor is a candidate pathway of virus entry (1). In this study, we found different patterns of domain B epitope evolution in the 26 years of HCV infection in patient H, providing evidence that immune pressure by domain B-like antibodies drives the evolution of this virus, leading to greater viral diversity and different mechanisms of escape. First, the 1977 variants were isolated from the serum of patient H within 3 weeks of exposure to HCV and prior to the development of serum antibodies to HCV (52). The emergence of early CBH-2, CBH-8C, and CBH-11 neutralization-resistant variants could be attributed to the presence of CBH-2-, CBH-8C-, and CBH-11-like serum antibodies in the original donor of the HCV-contaminated blood that was transfused to patient H. Of these three antibodies, CBH-2 has the epitope evolution pattern most analogous to the waxing and waning escape patterns associated with antibodies to HVR1 (52). It is possible that this
reflects CBH-2-like serum antibodies in patient H selecting resistant autologous quasispecies but able to neutralize heterologous isolates. The CBH-2 contact residues G530 and D535 are conserved among all HCV isolates, which explains the broad neutralizing profile of this antibody against different genotypes and subtypes (36). But the epitope also includes a site of variability at residue 431 on E2 that can lead to escape variants (26). The escape patterns associated with these three antibodies provide evidence that some antibodies to conformational epitopes that are more conserved among different genotypes and subtypes do drive a greater level of diversity in viral variants. Selection of escape variants can occur with a single-amino-acid substitution within these epitopes, leading to a high level of neutralization escape without apparent loss of replication fitness. This is analogous to neutralizing antibodies directed at HVR1 and explains in part the coexistence of persistent viremia despite the presence of broadly neutralizing antibodies in patients with chronic HCV infection. Nonetheless, two of the domain B antibodies, HC-2 and HC-11, showed sustained neutralization of all of patient H isolates from 1977 to 1995. In addition, CBH-5 neutralized all of the isolates except for one of the two 1977 variants. These sustained neutralization patterns raise the possibility that their epitopes could contain residues that are less susceptible to sequence variation because of their functional role in receptor binding or other associated steric constraints. If this is the case, the inclusion of these epitopes will be important in vaccine development since the likelihood of inducing escape mutations is reduced. It is also possible that the CBH-5, HC-2, and HC-11 epitopes are not immunogenic, leading to the lack of elicitation of these antibodies in this patient during chronic infection. However, this is unlikely, since domain B epitopes are highly immunogenic and commonly found during chronic HCV infection. The patient H sera from which the sequential variants were isolated are unfortunately no longer available for further testing.

As shown with the analysis of the 02.E10 isolate, HCV uses additional and complex mechanisms to escape from neutralizing antibodies to conserved epitopes, but with a cost in replication fitness. This variant was resistant to neutralization by all domain B antibodies as well as AP33, a broadly neutralizing antibody recognizing a linear epitope on E2 (35). However, 02.E10 HCVpp infectivity was reduced by over 90% compared with the level for H77c. Of the five unique 02.E10 mutations at residues 444, 446, 482, 501, and 506 in E2, in comparison to what was found for H77c and earlier H variants, the mutations at residues 501 and 506 were found to synergistically affect infectivity. With N501S and A506V substitutions in E2 of the 02.E10 variant, the HCVpp infectivity was nearly restored to the same level as that for H77c HCVpp. An alignment of these five amino acids on E2 with other HCV sequences in a national database (http://hcv.lanl.gov/) indicated that residue 506 is at 100% conservation in genotypes 1a, 2a, 3, 4, 5, and 6 but has 0.3% (V to M) and 3.7% (V to F) variations in genotypes 1b and 2b, respectively. As expected when V was replaced by F, H77c HCVpp bearing V506F E2 dramatically decreased its infectivity but V506M retained its infectivity (data not shown). The analysis showed no conservation at residues 444, 446, 482, and 501. Of note, restoration of infectivity correlated with improvement of E2 interaction with CD81. The binding affinity of 02.E10 E2 for CD81 was fourfold less than that of 02.E10 N501S/A506V E2 for CD81, which was similar to that of H77c E2 for CD81. In summary, mutations at residues 501/506 induce a structural change that compromises virus interaction with CD81, leading to inefficient virus entry. In contrast to restoring efficiency of binding to CD81 and infectivity with N501S/A506V, neutralization by domain B antibodies was restored to levels comparable to those for H77c. This residue is not part of domain B epitopes, since Q444A substitution in H77c E2 retained normal binding with domain B antibodies and MAb AP33 (data not shown). This implies that a mutation at residue 444 alone will not affect broadly neutralizing antibodies. Nevertheless, in conjunction with mutations at residues 501 and 506, their combined effect leads to complete escape from many, if not all, neutralizing antibodies.

The improved AP33 neutralizing activity observed with the 02.E10 HCVpp bearing N501S/A506V E2 is not associated with an improved antibody binding affinity but is associated with an improved binding of N501S/A506V E2 to CD81. Restoring a more effective entry pathway via CD81 restored the functional effect of AP33 binding with neutralization. The lack of improved binding is consistent with the view that residues 501 and 506 are not part of the AP33 epitope. The fact that the antibody binding affinity of AP33 for 02.E10 is significantly less than that for H77c suggests that the epitope of this antibody contains a conformational component. This is consistent with the observation that residue 655 on E2 could be involved with the AP33 epitope (12).

Recently, evidence for direct cell-to-cell transmission was described as an entry pathway for HCV that occurs in the presence of neutralizing antibodies in a CD81-independent manner (43, 49, 51, 54). This can serve as yet another mechanism by which the virus may escape from neutralizing antibodies. Studies are under way to assess the impact of mutations at residues 501 and 506 in the HCVcc system for cell-to-cell transmission. This should provide additional insights into whether residue 501/506 mutations decreasing entry via receptor-mediated endocytosis also affect cell-to-cell spread. In conclusion, the findings in this study suggest that the location of the mutation at residue 506 in the 02.E10 variant caused a structural change on E2 that distorts the binding of neutralizing antibodies to a cluster of overlapping conformational epitopes, domain B, containing residues that are also contact residues for binding of HCV E2 to CD81. A mutation at this residue could then exacerbate the effects of other mutations, e.g., at residue 444, that by themselves would have minimum or no effect on this cluster of conformational epitopes. But at the same time, the disadvantage is a lowered efficiency of virus binding to CD81 that compromises the virus entry, leading to lower infectivity. One can speculate from these analyses that during the chronic phase of HCV infection and in the presence of broadly neutralizing serum antibodies, a mutation at residue 506 and other mutations that are not part of an antibody binding epitope work in concert to provide a new mechanism of escape from broadly neutralizing antibodies to a highly immunogenic region on HCV E2 containing...
multiple overlapping neutralization epitopes, but at a substantial cost in viral fitness. While the chronology of the population rather than time-dependent selection.

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