Additional Glycosylation Within a Specific Hypervariable Region of Subtype 3a of Hepatitis C Virus Protects Against Virus Neutralization

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Background. The envelope glycoprotein E2 of hepatitis C virus (HCV) contains several hypervariable regions. Interestingly, 2 regions of intragenotypic hypervariability within E2 have been described as being specific to HCV subtype 3a. Based on their amino acid position in E2, they were named HVR495 and HVR575. Here, we further investigated these regions in order to better understand their role in HCV infection.

Methods. Sequences of HCV envelope glycoproteins from Pakistani patients infected with subtype 3a were cloned and compared with other subtype 3a sequences. The entry functions and the sensitivity to antibody neutralization of selected HCV glycoprotein sequences were tested in the HCV pseudotyped particles (HCVpp) system. In addition, the cell-cultured HCV system (HCVcc) was also used to confirm some of the data obtained with the HCVpp system.

Results. We observed interesting new features within HVR495 and HVR575 for several subtype 3a isolates. Indeed, changes in glycosylation sites were observed with the appearance of a new glycosylation site within HVR495. Importantly, HCVpp and HCVcc that contained this new HVR495 glycosylation site were less sensitive to antibody neutralization.

Conclusions. We identified a new glycosylation site within the HVR495 region of HCV subtype 3a that has a protective effect against antibody neutralization.

Keywords. hepacivirus; hepatitis C virus; viral glycoproteins; neutralizing antibodies; sequence variability.

Hepatitis C virus (HCV) is a major infectious agent that has a tendency to establish a persistent infection in the human liver. Worldwide, approximately 160 million people are infected by this virus and are at risk of developing chronic inflammation of the liver, cirrhosis, and hepatocellular carcinoma [1]. In the past, antiviral therapy against HCV consisted of a bitherapy of pegylated interferon and ribavirin [2]. Recently, new direct-acting antivirals that target HCV NS3-4A protease, telaprevir and boceprevir, have been added to the therapy. This has resulted in an increase in the sustained virological response in patients infected with HCV genotype 1 of up to 70% [3]. However, this treatment remains relatively toxic and expensive, which limits its potential use in many developing countries. For such countries, the development of an efficient vaccine remains a priority. However, the development of a vaccine that is protective against HCV has proven...
to be an extremely challenging task. Extensive research in this area suggests that a successful HCV vaccine will need to stimulate the production of neutralizing antibodies and potent HCV-specific T-cell responses. To this end, it is crucial to define all neutralizing determinants in HCV envelope glycoproteins and particularly conserved structural features that could be targeted to obtain cross-neutralization among different virus genotypes and to minimize the likelihood of immunological escape. In addition, it is important to understand the molecular basis of HCV neutralization resistance.

The HCV genome encodes 2 envelope glycoproteins, E1 and E2. These 2 proteins assemble as noncovalent heterodimers within infected cells and as large disulfide-linked oligomers on the surface of HCV particles [4]. Since they are present on the virion, HCV envelope glycoproteins are recognized by neutralizing antibodies, with E2 being the major target [5]. This protein is highly glycosylated with up to 11 N-glycosylation sites [6]. Interestingly, at least 5 of these glycans reduce the sensitivity of HCV to neutralization, indicating that glycans can limit the recognition of neutralizing epitopes at the surface of E2 [6, 7].

There is no high-resolution structure of HCV envelope glycoprotein E2. However, based on a comparison with other fusion proteins from viruses belonging to the same family as well as the identification of E2 disulfide bonds, a model of the E2 ectodomain, consisting of 3 separate domains, has recently been proposed [8]. Furthermore, located within the E2 ectodomain are 3 highly variable sequences: hypervariable region 1 (HVR1), HVR2, and the intergenotypic variable region (igVR) [9]. Interestingly, 2 regions of intragenotypic hypervariability within the envelope protein E2 have also been described as being specific to subtype 3a [10, 11]. Based on their amino acid position in E2, they were named HVR495 and HVR575. It is worth noting that HVR575 is located within the intergenotypic variable region igVR [9]. Importantly, longitudinal analysis of patients with acute HCV subtype 3a infection showed that positively selected mutations within HVR495 and HVR575 arose early during primary infection, suggesting an influence of the host immune response in the variability of these regions [10, 11]. Here, we further investigated these regions in order to better understand their role in HCV infection. To this aim, we analyzed E2 sequences from subtype 3a. We observed interesting new features within HVR495 and HVR575 for these isolates, with the appearance of a new glycosylation site within HVR495. Our data show that this new glycosylation site plays a major role in protection against antibody neutralization.

**MATERIALS AND METHODS**

**Patients**

HCV-positive patients were enrolled for this study under approval of the Internal Review Board of the National University of Sciences and Technology, Atta ur Rahan School of Applied Biosciences, Pakistan; a patient consent form was duly signed for each patient. HCV patients selected for this study were under interferon and ribavirin therapy and had completed 3–4 months of initial treatment. Their characteristics are presented in Supplementary Table 1. HCV RNA was quantified with a HCV RNA quantification kit (RoboGene; A) Roboscreen) according to the manufacturer’s instructions.

**Amplification, Cloning, and Sequencing of E1 and E2 Genes**

Viral RNA was extracted from serum using an RNA extraction kit (Qiagen) according to the manufacturer’s protocol. Extracted RNA was used as the template for cDNA synthesis, and the HCV core-NS2 region was amplified with a high-fidelity Taq DNA polymerase (Roche). The sequences of primers were as follows: 5′-AAAGAATTCGCCACCATGTATGATGGCGGA ATACGTCGATGCCC-3′ (sense) and 5′-AAAGCGGCCGCCTCAC CCCAGGTGAGCTTGGATTCCC-3′ (antisense). Polymerase chain reaction (PCR) products were cloned into PCRII TOPO cloning vector (Invitrogen). Selected clones were subjected to sequencing and were used to clone full-length E1/E2 into pCDNA3.1 expression vector (Invitrogen). Mutations were introduced into the E2 sequence by sequential PCR steps as described [12] using a PCR system (Expand High Fidelity PLUS, Roche). Mutated clones were confirmed by sequencing.

**Sequence Analyses and Structure Predictions**

Sequence analyses were performed using the Web site tools of the European HCV database [13] and network protein sequence analysis [14], available at the Institut de Biologie et Chimie des Protéines. Multiple sequence alignments and amino acid conservations were carried out with the ClustalW program using default parameters [15]. Amino acids are numbered with respect to the polyprotein of HCV strain H77 consensus sequence that is used as a reference strain [16].

**Antibodies**

Polyclonal antibodies were purified from a pool of 20 Pakistani patients infected with HCV 3a subtype. Purified immunoglobulins from uninfected patients were used as a negative control. Monoclonal antibodies (Mabs) 3/11 (anti-E2) [17], JS81 (anti-CD81; BD-Pharmingen), R187 (anti-murine leukemia virus capsid; American Type Culture Collection [AATC]-CRL1912), and C167 (anti-scavenger receptor B1 [SRB1]) [18] were used in this work. Anti-claudin-1 (CLDN1) Mab has been previously described [19]. Anti-ApoE antibody was from EMD Millipore.

**Cell Culture**

Huh-7 human hepatoma cells [20] and human embryonic kidney 293T cells (ATCC number CRL-11268) were grown in Dulbecco’s modified essential medium (Invitrogen) supplemented with 10% fetal calf serum.
Figure 1. Sequence analyses of E2 of subtype 3a Pakistani patients. Multiple alignments of E2 sequences were performed with ClustalW [15]. A–D, Consensus sequences of patients (this study) are compared with consensus sequences of patients 99 and 301 [11] exhibiting an additional glycosylation site in HVR4 region. Consensus sequences were deduced from quasispecies sequences reported in Supplementary Figure 1. Sequence of the UKN3A-1.28 synthetic clone used for functional studies in this work is indicated here as genotype 3a reference sequence (accession number: AY734984). Amino acids are numbered with respect to the polyprotein of hepatitis C virus strain H77 consensus sequence used as a general reference [16] (top row). To highlight the variability at each position, amino acids identical to the UKN3A-1.28 sequence are indicated by dots. The degree of amino acid physicochemical conservation at each position relative to the UKN3A-1.28 sequence is indicated with the similarity index according to ClustalW convention (asterisk, invariant; colon, highly similar; dot, similar) [15]. Expected glycosylation sites are labeled (bottom row) and highlighted in gray. Additional and defective glycosylation sites are highlighted by a gray box and an open box, respectively. Patient sequences have been submitted to GenBank; the accession numbers are indicated in Supplementary Figure 1.
Additional glycosylation sites were observed in subtype 3a, except position N7, which is totally absent in subtype 3a [6]. In addition, 140 sequences also showed the absence or addition of another glycosylation site (Figure 1 and Supplementary Figures 1 and 2). Although mutations abolishing a glycosylation site are observed for all glycosylation positions, they are particularly frequent at positions N3, N6, and N9 but rare at positions N4 and N5 (Table 1). The presence of glycosylation mutations at positions N8 or N10 is more surprising since the absence of an N-glycan at these 2 positions affects protein folding and viral particle assembly in subtypes 1a and 2a [6, 7, 27, 28]. However, these natural mutations are not exactly the same as those introduced in subtypes 1a and 2a.

Interestingly, in comparison with previously published sequences, an insertion of 2 additional amino acids was observed in HVR575 of all the quasispecies of patient PD, which increased HVR575 length to 11 residues instead of 9 (Figure 1). Importantly, this 2-amino acid insertion was never found in other subtype 3a sequences exhibiting a glycosylation site in this region. Also, a 3-amino acid insertion coupled to the absence of the N9 glycosylation site was reported for 5 sequences of patient P288 [11] (Supplementary Figure 2). It is also worth noting that the glycosylation site N9 that is present in HVR575 is absent in the consensus sequence of patient PC (Figure 1) and 1 sequence of patient PA (Supplementary Figure 2). Analysis of all 3a sequences present in the databases shows that this glycosylation site is absent in 41 sequences from 8 patients (including patients PA, PC, P288; Table 1 and Supplementary Figure 2). Another major feature that we observed for all quasispecies of patient PC is the presence of a new N-glycosylation site in the HVR495 segment, which has also been observed before for 2 patients (P99 and P301) [11] (Figure 1 and Supplementary Figures 1 and 2). It is worth noting that an additional HVR495 glycosylation site together with the absence of the N5 glycosylation site is observed in subtype 3b sequences reported by Chaudhuri et al [29] (Figure 1). However, the HVR495 glycosylation site was never found in other genotypes (data not shown).

Effect of New HVR495 Glycosylation Site on the Entry Functions of HCV Envelope Proteins

To gain further information on the role of HVR495 glycosylation in E2 function, we produced HCVpp with the sequences from 2 quasispecies of patient PC (PC2 and PC3; Supplementary Figure 1). Previously described functional HCVpp from a subtype 3a (UKN3A-1.28) were used as a positive control of infectivity [23]. As shown in Figure 2, HCVpp containing the PC2 or PC3 envelope proteins were infectious. However, the level of infectivity was lower than what was observed for our positive control. This observation is not unexpected since HCVpp of genotype 3 are known to be less infectious as compared with other genotypes [23].

### RESULTS

**Characterization of E2 Sequences From Pakistani Patients**

To gain further information on variable regions specifically observed in subtype 3a, we sequenced several E2 clones from Pakistani patients because this subtype is highly prevalent in Pakistan. Four different clones were analyzed from each patient; their consensus sequences are presented in Figure 1 (sequence details are presented in Supplementary Figure 1). In addition, these sequences were compared with subtype 3a sequences available in the databases (Table 1 and Supplementary Figure 2). Among 476 nonredundant sequences from 71 patients available for E2, the large majority had potential glycosylation sites located at the same positions as those described for subtype 1a, except position N7, which is totally absent in subtype 3a [6]. In addition, 140 sequences also showed the absence or addition of another glycosylation site (Figure 1 and Supplementary Figures 1 and 2). Although mutations abolishing a glycosylation site are observed for all glycosylation positions, they are particularly frequent at positions N3, N6, and N9 but rare at positions N4 and N5 (Table 1). The presence of glycosylation mutations at positions N8 or N10 is more surprising since the absence of an N-glycan at these 2 positions affects protein folding and viral particle assembly in subtypes 1a and 2a [6, 7, 27, 28]. However, these natural mutations are not exactly the same as those introduced in subtypes 1a and 2a.

**Production of Viruses**

The luciferase-based HCV pseudotyped retroviral particles (HCVpp) [21] were produced as previously described [22]. The UKN3A-1.28 (AY734984) clone was used as a genotype 3a reference isolate for HCVpp experiments [23]. For experiments with cell-cultured HCV (HCVcc), a modified JFH1 virus [24], intergenotypic HCV chimeras H77/JFH1 [25], and GT3a(452)/JFH1 [26] were used.

**Neutralization and CD81 Inhibition Assays**

Neutralization and CD81 inhibition experiments were performed as previously described [7, 27]. Student t test was used to compare the percentages of infectivity.

### Table 1. Statistics of E2 Sequences of Subtype 3a Exhibiting Defective or Additional Glycosylation Sites

<table>
<thead>
<tr>
<th>Glycosylation Sites</th>
<th>Sequences</th>
<th>Patients</th>
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<tbody>
<tr>
<td>Defective sites</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>N6</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>N7 (. . .) (. . .) (. . .) (. . .)</td>
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<td>4</td>
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<tr>
<td>N8</td>
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<td>5</td>
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<tr>
<td>N11</td>
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Additional sites

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<table>
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</tr>
<tr>
<td>658</td>
<td>1</td>
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</tr>
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</table>

Data deduced from the analysis of 476 nonredundant full-length E2 sequences of subtype 3a from 71 patients available in euHCVdb [13]. Sequence fragments exhibiting defective or additional glycosylation sites are reported in Supplementary Figure 2.
To gain more information on the potential effect of the HVR495 glycosylation site in PC isolate, we replaced the asparagine residue in HVR495 of PC2 and PC3 isolates with a proline residue, which occupies the same position in the UKN3A-1.28 clone. This mutation improved HCVpp infectivity by approximately 6 fold (Figure 2A and data not shown). To more specifically determine the effect of a glycosylation site within HVR495, we used site-directed mutagenesis to engineer a glycosylation site in this region in the context of the UKN3A-1.28 clone. As shown in Figure 2A, this single mutation reduced HCVpp infectivity by approximately 6 fold. Although there was some variation in the incorporation of the envelope glycoprotein E2 (Figure 2C), this did not correlate with the changes in infectivity. Indeed, UKN-mut495 showed a slightly higher level of incorporation into HCVpp, and infectivity was lower for this mutant (Figure 2A and C). Together, these data indicate that the selection of an additional glycosylation site within HVR495 reduces the efficiency of HCVpp entry into the hepatocyte.

Effect of New HVR575 Mutation on the Entry Functions of HCV Envelope Proteins

To gain additional information on the role of HVR575 in E2 function, we swapped 5 amino acids of HVR575 (DNNQA sequence) between PC2 and UKN3A-1.28 (KNESD sequence) clones. As shown in Figure 2B, this single mutation reduced HCVpp infectivity by approximately 6 fold. Although there was some variation in the incorporation of the envelope glycoprotein E2 (Figure 2C), this did not correlate with the changes in infectivity. Indeed, UKN-mut575 showed a slightly higher level of incorporation into HCVpp, and infectivity was lower for this mutant (Figure 2A and C). Together, these data indicate that the selection of an additional glycosylation site within HVR495 reduces the efficiency of HCVpp entry into the hepatocyte.
clones. We also deleted this 5–amino acid segment in both PC2 and UKN3A-1.28 clones. In addition to the change in amino acids, these mutations led to the removal of glycosylation site N9 in the UKN3A-1.28 clone when its HVR575 sequence was replaced by the equivalent sequence of PC2 as well as when this segment was deleted. On the contrary, this 5–amino acid swap in the context of PC2 isolate led to a gain in a glycosylation site at position N9. However, in contrast with the mutations in HVR495, none of these HVR575 mutations affected HCVpp infectivity (Figure 2C). It is worth noting that the mutations did not modify the level of incorporation of E2 into HCVpp (Figure 2D). Together, these data indicate that the loss of a glycosylation site at position N9 within HVR575 does not affect the efficiency of HCVpp entry into the hepatocyte.

HVR495 Glycosylation Site Protects Against Virus Neutralization

It has been reported that several highly conserved glycans present on E2 glycoprotein protect against antibody neutralization [6, 7, 28]. We therefore wondered whether the presence of an additional glycan in HVR495, as observed in the quasispecies of patient PC, could also affect HCV sensitivity to antibody neutralization. To this aim, we used a pool of purified antibodies from patients infected with HCV of subtype 3a. The neutralization experiments were then performed for each HCVpp mutant using antibodies at a concentration that corresponded to the half maximal effective concentration (EC50), as determined on wild-type UKN3A-1.28-HCVpp. Importantly, in these conditions, HCVpp containing the envelope proteins of PC2 or PC3 isolates were not neutralized by the anti-HCV antibody, whereas the mutants lacking the HVR495 glycosylation site were neutralized as efficiently as HCVpp containing the envelope proteins of the UKN3A-1.28 clone (Figure 3A). On the contrary, the addition of a glycosylation site in HVR495 of the UKN3A-1.28 clone blocked antibody neutralization. Neutralization experiments were also performed on HVR575 mutants. As shown in Figure 3B, HCVpp containing the envelope proteins of PC2 mutated in HVR575 were more sensitive to neutralization. However, in contrast to HVR495 mutants, none of these HVR575 mutations modulated HCVpp neutralization in the context of the control UKN3A-1.28 HCVpp (Figure 3B). Together, these results indicate that HVR495 glycans contributes to the masking of important neutralizing epitopes on E2 in the context of the HCVpp system.

Analysis of HVR495 Glycosylation Site in the Context of HCVcc

To further characterize the potential role of HVR495 in protection against neutralization, we engineered a new glycosylation site in the context of the HCVcc system. We also constructed another chimeric virus in which HVR575 mutation was introduced. Unfortunately, this latter mutant was not infectious and we could not use it in our neutralization experiments. The lack of infectivity of this mutant could be due to intersequence incompatibility within the igVR region as previously reported [30].

As shown in Figure 4A, the addition of a glycosylation site in HVR495 in the JFH1 virus or in GT3a(452)/JFH1 or H77/JFH1
chimeras did not modify HCVcc infectivity. The presence of an additional glycosylation site in these mutants was confirmed by sequencing, and the change in the migration profile of E2 in the context of the GT3a(452)/JFH1 chimera was confirmed by western blotting (Figure 4B). It should be noted that the mutation did not modify the density of the major peak of the infectious particles (Figure 4C) nor virus sensitivity to neutralization by anti-ApoE antibodies (Table 2). Importantly, HVR495 glycan reduced the sensitivity of the virus to antibody neutralization (Figure 5A). Indeed, the EC50 for neutralization was 3.0 µg/mL (± 0.06) for wild-type and 13.3 µg/mL (± 0.36) for mutant GT3a(452)/JFH1 (Table 2). These results indicate that HVR495 glycan masks important neutralizing epitopes on E2 in the context of the HCVcc system.

Since we previously reported that several conserved glycans reduce the accessibility of a soluble form of CD81 to its binding region on E2 [7], we also investigated whether HVR495 glycan affects the accessibility of CD81 to its binding region. To this end, we analyzed the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. As shown in Figure 5B, our results indicate that the presence of HVR495 glycan reduces the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. As shown in Figure 5B, our results indicate that the presence of HVR495 glycan reduces the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. As shown in Figure 5B, our results indicate that the presence of HVR495 glycan reduces the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. As shown in Figure 5B, our results indicate that the presence of HVR495 glycan reduces the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81. As shown in Figure 5B, our results indicate that the presence of HVR495 glycan reduces the sensitivity of GT3a(452)/JFH1 mutant to inhibition by a soluble form of CD81.
experiments with anti-CD81, anti-SRB1, and anti-CLDN1 antibodies were performed. As shown in Figure 5 and Table 2, viruses containing HVR495 glycan were less sensitive to neutralization by anti-CD81 and anti-SRB1 antibodies but not to anti-CLDN1 antibody. These results suggest that the presence of HVR495 glycan reduces the accessibility to CD81 and SRB1 but not to

<table>
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<th>3a/2a</th>
<th>495-3a/2a</th>
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<th>495-JFH1</th>
<th>1a/2a</th>
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<td>Soluble CD81</td>
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<td>8.8 ± 0.58**</td>
<td>2.2 ± 0.20</td>
<td>16.3 ± 1.47**</td>
<td>0.7 ± 0.07</td>
<td>3.7 ± 0.60*</td>
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<td>Anti-CD81</td>
<td>0.6 ± 0.05</td>
<td>3.7 ± 0.61*</td>
<td>0.6 ± 0.03</td>
<td>2.3 ± 0.10**</td>
<td>0.7 ± 0.10</td>
<td>4.4 ± 0.55***</td>
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<tr>
<td>Anti-SRB1</td>
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<td>3.0 ± 0.26***</td>
<td>1.1 ± 0.08</td>
<td>5.9 ± 0.73*</td>
<td>0.6 ± 0.06</td>
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</tr>
<tr>
<td>Anti-claudin-1</td>
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<td>Anti-ApoEAb</td>
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<td>0.00085 ± 0.00004</td>
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</table>

Abbreviation: ND, not determined.

EC_{50} values are expressed in μg/ml except for anti-ApoE Ab for which the values are expressed as dilution factors.

* P < .05, ** P < .01, and *** P < .001.

Figure 5. Effect of HVR495 glycan on cell-cultured hepatitis C virus (HCVcc) sensitivity to antibody neutralization and receptor usage. Inhibition experiments were performed by incubating mutant (495-2a/3a) or wild-type GT3a(452)/JFH1(2a/3a) with various concentrations of anti-HCV antibodies (3a HCV+ Abs) (A), CD81 large extracellular loop (LEL) fused to GST (GST-CD81-LEL) (B), anti-CD81 Mab JS81 (C), anti-SRB1 Mab C167 (D), or anti-CLDN1 Mab (E). At 72 hours post infection, infected cells were quantified by immunofluorescence. Values are the combined data from 2 independent experiments; error bars represent standard errors of the means.
CLDN1. However, the lower sensitivity of mutant GT3a(452)/JFH1 to both inhibition by soluble CD81 and anti-CD81 suggests a reduced dependence on CD81 for the entry of this virus.

**DISCUSSION**

Two regions of hypervariability within E2 in HCV subtype 3a chronically infected individuals have previously been identified, and it has been shown that these regions are subject to strong intrahost selective pressure that arises early during acute infection [11]. Here, we further investigated these regions by reanalyzing E2 sequences of subtype 3a. In our major observation was the characterization of a new glycosylation site within HVR495, which has a protective effect against antibody neutralization.

The presence of a glycosylation site within HVR495 reduces the efficiency of HCVpp entry into the hepatocyte. The ectodomains of HCV envelope glycoproteins are highly glycosylated, with most sites being highly conserved. However, a site at position 476 (E2N5) exists in most genotypes but is rarely present in sequences of genotype 1b, and another site at position 540 (E2N7) is absent in genotypes 3 and 6. This high level of conservation is due to the role of these glycans in the HCV life cycle [7, 27, 28]. Interestingly, some specific glycans associated with HCV envelope protein E2 can modulate the entry function of these proteins by modifying the affinity for receptor(s) or by affecting the fusion activity [7]. For instance, it has been observed that the loss of glycan at position E2N6 increases the infectivity of HCVcc due to better accessibility of the CD81-binding region on E2 [7, 28]. Furthermore, the emergence of adaptive mutations that lead to the loss of glycosylation site E2N6 has been observed in cell culture [31, 32]. In the case of HVR495 glycan, we observed a decrease in HCVpp entry in the presence of this glycosylation site. However, in the context of HCVcc, this effect was not observed. Differences between HCVpp and HCVcc have already been reported, which are likely due to differences in the assembly process of these 2 models [7].

The presence of a glycan in HVR495 protects against virus neutralization. A major function of the glycans associated with HCV envelope proteins is to protect them from neutralization by forming a glycan shield [33]. Indeed, at least 5 glycans on E2 (E2N1, E2N2, E2N4, E2N6, and E2N11) reduce the sensitivity of HCV to antibody neutralization, indicating that they limit the recognition of neutralizing epitopes at the surface of E2 [6, 7, 28]. Interestingly, these glycans modulate the neutralizing activity of antibodies directed against conserved epitopes. In addition, 4 of them (E2N1, E2N2, E2N4, and E2N6) reduce the accessibility to the CD81 binding site. It is also likely that the presence of glycans at the surface of HCV particles also limits the immunogenicity of the envelope proteins [33]. Here, we show that an additional glycan in domain DIII, supposedly located far away from previously identified sites [8], can also protect against neutralization. However, the observation that the HVR495 glycan protects against inhibition by CD81 suggests that HVR495 might be located more closely than expected to the CD81-binding region. As compared with CD81 and SRB1, there was no effect on CLDN1 usage. This is likely due to the fact that HCV envelope glycoproteins do not directly interact with CLDN1, whereas a direct contact has been demonstrated for CD81 and SRB1 (reviewed in [34]). One can wonder why the HVR495 glycosylation site is not observed in more than 4.2% of patients infected with subtype 3a (Table 1). The rather low frequency of the HVR495 glycosylation site does not seem to be due to a decrease in viral fitness since in vitro data do not show any decrease in infectivity. However, we cannot exclude that other mechanisms of evasion such as the masking of the CD81 binding region by HVR1 dominate the appearance of HVR495 glycosylation.

The presence of a glycan in HVR575 does not affect virus entry and does not protect against antibody neutralization. It is worth noting that the appearance of a glycosylation site in HVR495 might be associated with the disappearance of a glycosylation site within HVR575 located in igVR. The presence of the E2N9 glycan in igVR is highly conserved in all HCV genotypes, suggesting that it might play a role in HCV infection. However, this glycan does not affect HCV entry in subtypes 1a and 2a and it does not reduce HCV sensitivity to antibody neutralization in these same genotypes [6, 7, 27, 28]. In the context of patient PC, it is possible that the disappearance of E2N9 glycan is linked to the appearance of a new glycosylation site in HVR495. However, this could not be demonstrated in our experimental setting since restoring E2N9 glycan within HVR575 did not affect virus entry. This is in line with the lack of effect of E2N9 mutation in subtypes 1a and 2a [6, 7, 27, 28]. However, we cannot exclude that this potential glycosylation shift is important for maintaining some in vivo function(s) of this glycan.

In conclusion, our functional characterization of regions of hypervariability within E2 in HCV subtype 3a provides new information on the high level of plasticity of HCV envelope glycoprotein E2. Such data are important for the future design of HCV vaccines in countries that cannot afford the high cost of the current antiviral treatments.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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