JOURNAL OF VIROLOGY, Aug. 2007, p. 8101–8111 Vol. 81, No. 15
0022-538X/07/$08.00+0 doi:10.1128/JVI.00127-07

The Neutralizing Activity of Anti-Hepatitis C Virus Antibodies Is Modulated by Specific Glycans on the E2 Envelope Protein▽

François Helle,[1]† Anne Goffard,[1,2]† Virginie Morel,[1,3] Gilles Duverlie,[1,3] Jane McKeating,[4] Zhen-Yong Keck,[5] Steven Foung,[5] François Penin,[6] Jean Dubuisson,[1]* and Cécile Voisset[1]

Institut de Biologie de Lille (UMR8161), CNRS, Université Lille I & II and Institut Pasteur de Lille, Lille, France[1]; Service de Virologie/UPRES EA3610, Faculté de Médecine, Université Lille II, Lille, France[2]; Laboratoire de Virologie, Centre Hospitalier Universitaire d'Amiens, Amiens, France[3]; Institute of Biomedical Research, University of Birmingham, Birmingham, United Kingdom[4]; Department of Pathology, Stanford University School of Medicine, Stanford, California[5]; and Institut de Biologie et Chimie des Protéines, UMR5086, CNRS, Université de Lyon, IFR128 BioSciences Lyon-Gerland, 69367 Lyon, France[6]

Received 19 January 2007/Accepted 13 May 2007

Hepatitis C virus (HCV) envelope glycoproteins are highly glycosylated, with up to 5 and 11 N-linked glycans on E1 and E2, respectively. Most of the glycosylation sites on HCV envelope glycoproteins are conserved, and some of the glycans associated with these proteins have been shown to play an essential role in protein folding and HCV entry. Such a high level of glycosylation suggests that these glycans can limit the immunogenicity of HCV envelope proteins and restrict the binding of some antibodies to their epitopes. Here, we investigated whether these glycans can modulate the neutralizing activity of anti-HCV antibodies. HCV pseudoparticles (HCVpp) bearing wild-type glycoproteins or mutants at individual glycosylation sites were evaluated for their sensitivity to neutralization by antibodies from the sera of infected patients and anti-E2 monoclonal antibodies. While we did not find any evidence that N-linked glycans of E1 contribute to the masking of neutralizing epitopes, our data demonstrate that at least three glycans on E2 (denoted E2N1, E2N6, and E2N11) reduce the sensitivity of HCVpp to antibody neutralization. Importantly, these three glycans also reduced the access of CD81 to its E2 binding site, as shown by using a soluble form of the extracellular loop of CD81 in inhibition of entry. These data suggest that glycans E2N1, E2N6, and E2N11 are close to the binding site of CD81 and modulate both CD81 and neutralizing antibody binding to E2. In conclusion, this work indicates that HCV glycans contribute to the evasion of HCV from the humoral immune response.

More than 170 million people worldwide are seropositive for hepatitis C virus (HCV) (65). Despite induction of effective immune responses, 80% of HCV-infected individuals progress from acute to chronic hepatitis, which can lead to cirrhosis and hepatocellular carcinoma (42). Escape strategies may be operating for both the innate and the adaptive immune systems, but the exact mechanisms whereby HCV establishes and maintains its persistence have not yet been determined (59). It is known that an immune response composed of both cellular ($CD4^+$ and $CD8^+$ T cells) and humoral (antibodies produced by B cells) immune responses is present during acute and chronic infections (40). Typically, HCV infection results in production of antibodies to various HCV proteins in the majority of chronically infected people. Moreover, neutralizing antibodies have been detected in sera of HCV-infected patients (2, 3, 19, 39, 41, 44, 69), but the role of these antibodies in host protection has been questioned since reinfection in both humans and chimpanzees has been described (18, 38). Investigations of HCV-neutralizing antibodies have long been hampered by difficulties in propagating HCV in cell culture, but the recent development of HCV pseudoparticles (HCVpp) (3, 15, 31), consisting of the native HCV envelope glycoproteins, E1 and E2, assembled onto retroviral core particles, offered new opportunities in this field (2, 3, 31, 39, 41, 44, 52, 69).

The ability of HCV to persist in its host in the presence of neutralizing antibodies remains unexplained. Several mechanisms by which HCV could evade the host humoral immune response have been proposed. It is suggested that the high variability of its genomic RNA represents a first escape strategy. Typically, the presence of different but closely related viral variants within the same individual, commonly defined as quasispecies, may allow the virus to circumvent the immune response (6, 26, 32, 59, 63). In particular, the infection outcome in humans was predicted by sequence changes in hypervariable region 1 (HVR1) of the E2 envelope glycoprotein, a major target for the antibody response (20). Furthermore, high-density lipoproteins have recently been shown to attenuate the neutralization of HCVpp by antibodies from HCV-infected patients by accelerating HCV entry (4, 13, 62).

The HCV envelope glycoproteins E1 and E2, present at the surface of the viral particles, are the potential targets of neutralizing antibodies (48). These glycoproteins form a heterodimer which interacts with (co)receptors on target cells (10). The CD81 tetraspanin is the best-characterized entry factor for HCV. Indeed, it interacts with HCV glycoprotein E2 (54), and HCVpp show a restricted tropism for human hepatic cell lines expressing CD81 (5, 12, 31). Furthermore, anti-CD81

* Corresponding author. Mailing address: Hepatitis C Laboratory, CNRS-UMR8161, Institut de Biologie de Lille, 1 rue Calmette, BP447, 59021 Lille cedex, France. Phone: (33) 3 20 87 11 60. Fax: (33) 3 20 87 12 01. E-mail: jean.dubuisson@ibl.fr.

† F.H. and A.G. contributed equally to this work.

▽ Published ahead of print on 23 May 2007.

monoclonal antibodies (MAbs), as well as a recombinant soluble form of the large extracellular loop of CD81, inhibit HCV entry (for a review, see reference 10). Interestingly, the lectin cyanovirin-N binds to glycans on HCV particles and inhibits virus entry by blocking the interaction between E2 and CD81 (29). Based on studies with blocking MAbs or E2 deletion mutants, several regions of E2 have been proposed to be critical for CD81 binding (for a review, see reference 10). Recent analyses using mutagenesis in the context of HCVpp have provided more-accurate data on the specific residues involved in contacts with CD81 (14, 49). This allowed the identification of at least three discontinuous sequence segments (see Fig. 1). In addition, one cannot exclude the involvement of additional residues in another region of E2 (56).

The ectodomains of HCV envelope glycoproteins are highly glycosylated. Indeed, 4 or 5 potential glycosylation sites on E1 and up to 11 sites on E2 are modified by N-linked glycosylation (24, 25). Some of these glycans have been shown to play an essential role in protein folding and/or HCV entry (9, 24). Furthermore, the high level of glycosylation suggests that these glycans may also modulate the immunogenicity of HCV envelope glycoproteins and restrict the binding of certain antibodies to their epitopes on the virion surface, as observed for human immunodeficiency virus (HIV) (53, 66, 67). In this report, we sought to determine whether glycans present on HCV envelope glycoproteins allow the virus to escape recognition by host neutralizing antibodies. Our data suggest a protective role for HCV envelope proteins glycans against antibody-dependent neutralization. Indeed, specific E2 glycans contribute to reduce the sensitivity of HCVpp to antibody neutralization. Importantly, these glycans also reduced the access of CD81 to its E2 binding site, as shown by using a soluble form of the extracellular loop of CD81 in testing for inhibition of entry. These data suggest that these glycans are close to the binding site of CD81 and modulate both CD81 and neutralizing antibody binding to E2.

MATERIALS AND METHODS

Cell culture. 293T human embryo kidney cells (HEK293T) and Huh-7 human hepatoma cells (45) were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

Antibodies and reagents. Anti-HCV MAbs A4 (anti-E1) (16); 9/27, 3/11, and 1/39 (anti-E2) (22); and H48 and H54 (anti-E2) (48) and anti-murine leukemia virus capsid (anti-CA; ATCC CRL1912) were produced in vitro by using a MiniPerm apparatus (Heraeus) as recommended by the manufacturer. Anti-E2 human MAbs CBH-5 and CBH-7 have been described previously (27). MAb 3C7 was kindly provided by M. Mondelli (IRCCS Policlinico San Matteo, Pavia, Italy) (8). The soluble recombinant form of the CD81 large extracellular loop (CD81-LEL) was produced as a glutathione *S*-transferase fusion protein as described previously (30). Purified cyanovirin-N was kindly provided by K. Gustafson (National Institutes of Health, National Cancer Institute, Frederick, MD) (29).

Serum samples. Sera of 25 HCV-positive patients chronically infected with genotype 1a HCV were selected for this study (Table 1 and data not shown). HCV RNA was detected and quantified using HCV RNA Amplicor Monitor tests (Roche Diagnostic Systems, Inc., Branchburg, NJ), and results were reported in $\log_{10}$ of international units per ml. Determination of HCV genotypes was obtained using the InnoLIPA HCV 2.0 test (Bayer Diagnostics, Emeryville, CA) and confirmed by sequence analysis. Serum samples were used for E1E2 sequencing and antibody purification. Sera of 10 HCV-negative individuals were used as negative controls. Total antibodies were purified using the NAb protein G spin purification kit (Pierce, Rockford, IL).

Study of the conservation of potential glycosylation sites. HCV E1 and E2 sequences available from the European HCV database (euHCVdb; http://euhcvdb.ibcp.fr) (11), which contains all HCV sequences deposited in public databases, were collected and aligned with ClustalW software (60) using website facilities available at the Institut de Biologie et Chimie des Protéines. The repertoire of residues at each amino acid position and their frequencies observed in natural sequence variants were computed by using a program developed at the Institut de Biologie et Chimie des Protéines (F. Dorkeld, C. Combet, F. Penin, and G. Deleage, unpublished data). The consensus sequence for N-linked glycosylation is defined as Asn-X-Ser/Thr-X, where X is any amino acid except Pro (36), and we looked for the presence or absence of this consensus sequence for all HCV E1 and E2 sequences. We retrieved 1,393 sequences for E1 and 451 for E2 from the genotypes represented in the database. The positions of the glycosylation sites are indicated by a number corresponding to the positions in the polyprotein of reference strain H (EMBL access number AF 009606) (37). The glycosylation sites of genotype 1a HCV E1 and E2 that are glycosylated are named with an N followed by a number related to the position of the glycosylation site (e.g., N1, N2, etc.; see Fig. 1).

TABLE 1. Patients of the study (genotype 1a)

Patient	RNA level ($\log_{10}$)	EC_{50} (μg/ml)	Accession no.[a]
1	5.94	5	EF207585
2	1.6–2.8	20	EF207586
3	4.96	20	EF207587
4	3.23	15	EF207588
5	6.80	4	EF207589
6	2.95	5	EF207590
7	6.56	9	EF207591
8	4.88	10	EF207592
9	5.29	15	EF207593
10	3.23	1	EF207594
11	5.66	10	EF207595
12	6.14	5	EF207596
13	7.11	10	EF207597
14	6.39	5	EF207598
15	1.6–2.8	8	EF207599
16	6.10	14	EF207600
17	1.6–2.8	10	EF207601
18	4.94	30	EF207602
19	5.98	15	EF207603

[a] Accession numbers of E1E2 sequences of HCV isolates.

Amplification and sequencing of E1 and E2 genes. RNA was extracted from 140 μl serum using the QIAmp viral RNA minikit (QIAGEN). Ten microliters of the RNA was used for reverse transcription using SuperScript II RNase H reverse transcriptase (Invitrogen) with a specific primer (p7/HCV/AS, 5′ GGG RACCCACACKTGCA 3′; nucleotides 2927 to 2911). The cDNA obtained was used to amplify different fragments with Platinum *Taq* high-fidelity DNA polymerase (Invitrogen). Two methods were alternatively performed: (i) a PCR amplifying the E1-E2 gene region from nucleotide 879 to 2927, followed if necessary by two heminested PCRs amplifying fragments from nucleotides 879 to 1484 and 1290 to 2927, respectively, or (ii) one PCR amplifying the E1 gene region from nucleotide 895 to 1484 and one PCR amplifying the E2 gene region from nucleotide 1290 to 2612. Outer primers for E1-E2 gene PCR were N753 (5′ GCCCTGCTCTCTTGCCTGA 3′) and p7/HCV/AS. Inner primers were N753 and N754 (5′ GACGCCGGCAAATAACAGC 3′) and HV1 (5′ CGCATGGC TTGGGATATGATGAT 3′) and p7/HCV/AS. Primers for the E1 PCR were S895 (5′ TGACTGTGCCCGCTTCAGCCT 3′) and N754. Primers for the E2 PCR were HV1 and A2612 (5′ TGCTGCATTGAGTATTACGA 3′).

Direct sequencing of each strand of the PCR products was then performed after purification, using the cycle sequencing method with the Applied Biosystems BigDye Terminator v1.1 kit, according to the manufacturer's instructions. Sequences obtained were purified and read by the ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequencing primers were the following: N753, S895, HV1, A1327 (5′ GTAGGGGACCAGTTCATCATCA 3′), N754, S1788 (5′ CGCCCCTAYTGCTGGCACTAC 3′), N490 (5′ AAGGACGAAGACATC CCGT 3′), A2320 (5′ GACCTGTCCCTGTCTTCCAGA 3′), A2612, and p7/HCV/AS. Nucleotide positions are numbered according to the H77 sequence, and the E1E2 genes are within positions 915 to 2579. Electropherograms were interpreted using the Sequence Navigator and AutoAssembler 2.1 software (Applied Biosystems). Multiple nucleotide and amino acid sequence alignments were carried out with Clustal X software.

TABLE 2. Percent conservation of the potential glycosylation sites in HCV envelope protein E1 in the most represented genotypes

Genotype	n^a	% Conservation at site:					
		196 (N1)	209 (N2)	234 (N3)	250	299	305 (N4)
1	1,119	98.2	98.7	97.0	29.4	0	99.1
1a	768	98.7	98.8	96.5	0	0	99.2
1b	345	97.1	98.6	98.0	95.4	0	98.8
2	74	100	100	95.9	0	51.4	98.6
2a	22	100	100	86.4	0	0	100
2b	39	100	100	100	0	97.4	97.4
3	98	100	100	100	0	0	100
3a	75	100	100	100	0	0	100
4	26	100	100	100	0	0	100
4a	12	100	100	100	0	0	100
5	21	100	100	100	0	0	100
6	55	98.2	94.5	100	96.4	0	98.2
6a	19	100	100	100	94.7	0	100
Total	1,393	98.5	98.8	97.3	27.4	2.7	99.1

[a] *n*, number of sequences.

Production of HCVpp and neutralization assay. HCVpp were produced as described previously (3, 48) with plasmids kindly provided by B. Bartosch and F. L. Cosset (INSERM U758, Lyon, France). Plasmids encoding native or mutated HCV envelope glycoproteins of genotype 1a (H strain) were used to produce HCVpp (24). Our H strain sequence corresponds to accession number AAB67037 with the following amino acid changes: R564C, V566A, and G650E. Supernatants containing the pseudotyped particles were harvested 48 h after transfection and filtered through 0.45-μm-pore-size membranes. Neutralization assays were performed by preincubating HCVpp and antibodies for 2 h at 37°C before contact with target cells. After 3 h of contact with HCVpp, the cells were further incubated for 48 h with Dulbecco's modified essential medium containing 10% fetal bovine serum before measuring the luciferase activities as indicated by the manufacturer (Promega). Student's *t* test was used to compare percentages of neutralizing activities between wild-type and mutant HCVpp. It was found that, by using the antibodies purified from infected patients at concentrations indicated in Table 1, the neutralizing activities on the wild type ranged between 36 and 59%. The significance threshold *P* was set to 0.01. The results were also confirmed using the nonparametric test of Mann-Whitney.

RESULTS

Conservation of the potential glycosylation sites in HCV envelope glycoproteins. We have previously reported that the potential glycosylation sites in the HCV envelope proteins are highly conserved (25). Since the number of HCV envelope protein sequences has recently increased, we updated our analysis. As previously observed, E1 contains conserved potential N-glycosylation sites at positions 196 (N1), 209 (N2), 234 (N3), and 305 (N4) on the polyprotein (Table 2, Fig. 1). All of the conserved sites have been shown to be occupied by glycans (43). An additional potential glycosylation site was observed at position 250 or 299 in E1 from a limited number of HCV genotypes. Indeed, the glycosylation site at position 250 was present in E1 from genotypes 1b and 6, whereas the site at position 299 was present only within E1 from genotype 2b. The site at position 299, which has been reported in another recent study (71), was not identified in our previous study due to the small number of sequences available for genotype 2b at the time of our analysis. The role of the glycosylation sites 250 and 299 in E1 from their specific genotypes remains to be determined. As previously observed for E2, 9 of the 11 glycosylation sites were conserved in E1 from all the genotypes analyzed (positions 417 [N1], 423 [N2], 430 [N3], 448 [N4], 532 [N6], 556 [N8], 576 [N9], 623 [N10] and 645 [N11] on the polyprotein; Table 3, Fig. 1). The site at position 476 (N5) was less conserved and was absent in some subtypes; however, the small number of sequences available does not allow a definitive conclusion. In agreement with our previous study, the site at position 540 (N7) was absent in genotype 3-encoded and the majority of genotype 6-encoded protein sequences. It is worth noting that the 11 glycosylation sites on E2 have been shown to be occupied by glycans (24). Altogether, these results demonstrate the conserved nature of most potential glycosylation sites in the HCV envelope glycoproteins, suggesting that the glycans have an important function(s) in the HCV life cycle.

Mutation of specific N-linked glycans in E2 increases the sensitivity of HCVpp to neutralization by antibodies from HCV-seropositive patients. Several viruses have been reported

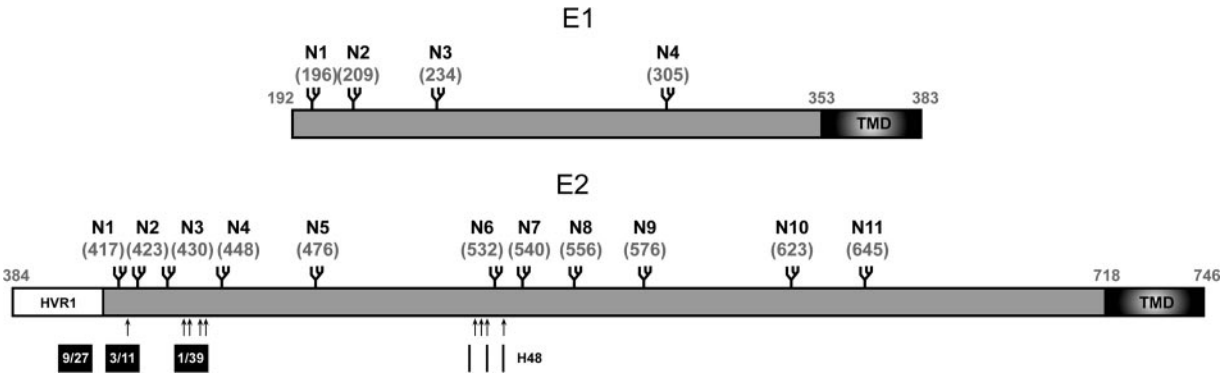

FIG. 1. Schematic representation of N-glycosylation sites in HCV glycoproteins E1 and E2. The mutants are named with an N followed by a number relating to the position of the glycosylation site in the sequence. The numbers in parentheses correspond to the positions of the glycosylation sites in the polyprotein of reference strain H (GenBank accession no. AF009606). Epitopes recognized by MAbs 9/27, 3/11, and 1/39 are indicated as dark boxes. Amino acid residues 523, 530, and 535, involved in the formation of the conformation-dependent epitope of H48, are indicated as black bars. Residues 420, 437, 438, 441, 442, 527, 529, 530, and 535, involved in CD81 binding (14, 49), are indicated by arrows. TMD, transmembrane domain.

TABLE 3. Percent conservation of the potential glycosylation sites in HCV envelope protein E2 in the most represented genotypes

Genotype	n^a	% Conservation at site:										
		417 (N1)	423 (N2)	430 (N3)	448 (N4)	476 (N5)	532 (N6)	540 (N7)	556 (N8)	576 (N9)	623 (N10)	645 (N11)
1	294	99.7	100	98.3	98.0	39.1	97.6	98.3	99.3	98.6	99.7	99.7
1a	77	100	100	98.7	98.7	97.4	90.9	100	100	100	100	100
1b	213	99.5	100	98.1	97.7	17.8	100	97.7	99.5	98.6	99.5	99.5
2	51	100	100	98.0	100	88.2	100	100	96.1	100	100	98.0
2a	21	100	100	95.2	100	90.5	100	100	95.2	100	100	100
2b	25	100	100	100	100	92.0	100	100	96.0	100	100	96.0
3	68	100	98.5	100	100	98.5	97.1	0	86.8	98.5	98.5	100
3a	66	100	98.5	100	100	100	97.0	0	86.4	98.5	98.5	100
4	9	100	100	100	100	88.9	100	100	100	100	100	100
4a	8	100	100	100	100	87.5	100	100	100	100	100	100
5	2	100	100	100	100	0	100	50	100	100	100	100
6	27	100	100	96.3	100	85.2	100	3.7	100	96.3	100	100
6a	15	100	100	100	100	100	100	0	100	100	100	100
Total	451	99.8	99.8	98.4	98.7	57.2	98.0	77.8	97.1	98.7	99.6	99.6

[a] *n*, number of sequences.

to evade the immune system by masking immunodominant epitopes by glycosylation (1, 17, 53, 55, 58, 66). Here, we wanted to analyze whether a similar mechanism is observed for HCV. To this end, we purified antibodies from the sera of individuals chronically infected with HCV genotype 1a (Table 1) and evaluated their neutralizing activities for wild-type and mutant HCVpp (strain H). Serum antibodies from 10 uninfected individuals were used as controls. As expected, antibodies purified from uninfected individuals had no effect on HCVpp infectivity (data not shown). To identify differences in the neutralizing activity of antibodies between wild-type and mutant HCVpp, we determined the concentration of antibody required to neutralize HCVpp infectivity by approximately 50% (EC_{50}). Antibodies from 19 patients neutralized HCVpp with an EC_{50} of between 1 and 30 μg/ml (Table 1). These neutralizing antibodies were screened for their ability to neutralize HCVpp expressing wild-type or variant envelope glycoproteins containing mutations at individual glycosylation sites. Indeed, if a glycan reduces the access of a neutralizing antibody to its epitope, a mutant lacking this glycan is expected to be more sensitive to neutralization by this antibody. The relative infectivities of the various glycosylation mutants have been reported previously (24) (see the Fig. 2 legend). All mutants showing a level of infectivity of at least 15% of that of the wild type were used in these experiments. Since E2N2, E2N4, E2N8, and E2N10 mutants were noninfectious (24), we could not evaluate their phenotype in this assay. To compare the neutralizing activities of antibodies for wild-type and mutant HCVpp, we determined the ratio of the percentage of neutralization of HCVpp mutants to that of the wild type.

The average ratios of neutralization were 1.06, 1.05, 0.89, and 0.94 for mutants E1N1, E1N2, E1N3, and E1N4, respectively (Fig. 2A and Table 4), indicating that there is no significant increase in neutralizing activities between E1 mutant and wild-type HCVpp. As shown in Fig. 2B, incorporation of E1, E2, and CA proteins into HCVpp was similar to what has been previously observed for these mutants (24). Indeed, the levels of incorporation of E2 and CA into HCVpp were similar for each mutant, whereas E1 incorporation into HCVpp was dramatically reduced for the E1N1 mutant and was reduced to a lesser extent for the E1N4 mutant. The very low level of incorporation of E1N1 into HCVpp suggests that the density of functional E1 on pseudoparticles that is required for infectivity is rather low. Together, these data suggest that the N-linked glycans of E1 do not contribute to the masking of neutralizing epitopes.

In contrast to what was found for the E1 mutants, differences in neutralizing activities of antibodies between mutant and wild-type HCVpp were observed for some E2 mutants (Fig. 2C and Table 4). Compared to that for wild-type particles, the average ratios of neutralization were 1.60, 1.50, and 1.61 for E2N1, E2N6, and E2N11 mutants, respectively, indicating that HCVpp containing glycosylation mutants E2N1, E2N6, or E2N11 were significantly more sensitive to neutralization by all the serum antibodies tested. Mutant E2N9 was also significantly more sensitive to neutralization, but the effect was less pronounced. It is worth noting that, when analyzed individually, all the patients' antibodies had similar neutralizing activities against each of the mutants E2N1, E2N6, and E2N11. Dose-response curves confirmed the increased sensitivity to neutralization for mutants E2N1, E2N6, and E2N11 compared to that for the wild type (Fig. 2E, patient 12). These data suggest that glycans at positions E2N1, E2N6, and E2N11 reduce the accessibility of antibodies to some neutralizing epitopes on the E2 glycoprotein. Interestingly, mutants E2N5 and E2N7 were significantly more resistant to neutralization (Fig. 2C and Table 4). This effect is likely due to a local conformational change which reduces the affinity of neutralizing antibodies for the E2 glycoprotein. As described previously (24), similar amounts of E2 glycoproteins incorporated into HCVpp were observed for each infectious mutant except for mutant E2N7 (Fig. 2D), indicating that the increase of sensitivity to neutralization was not due to a difference in the amount of antigen available. Together, these results indicate

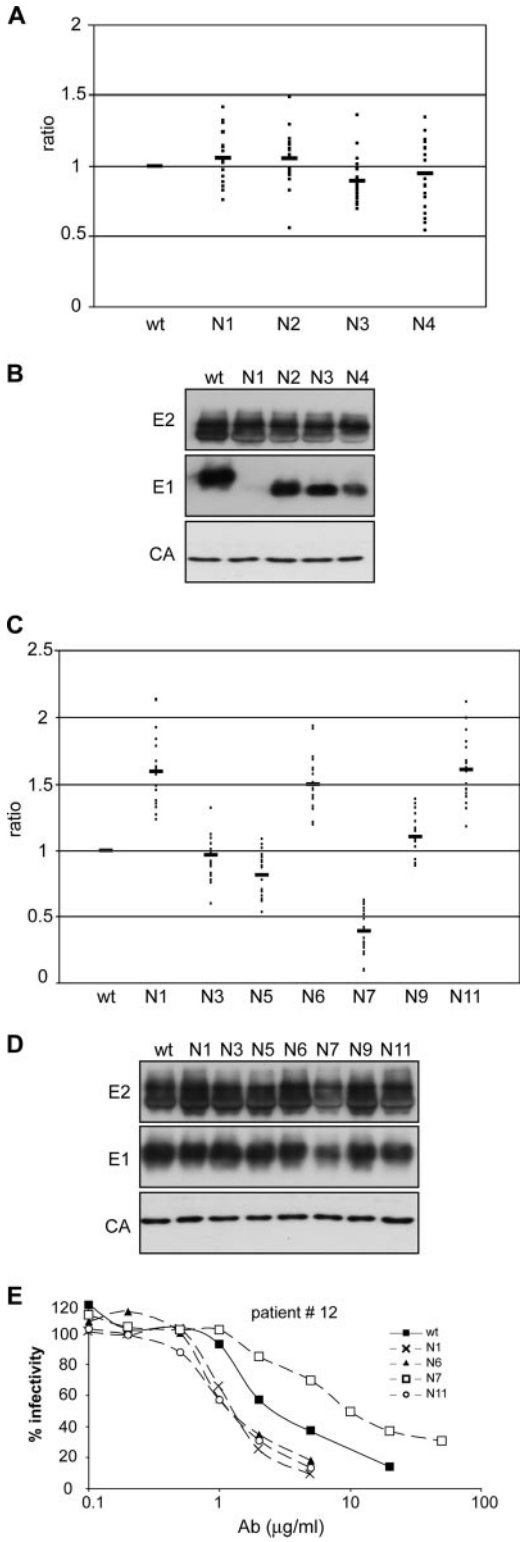

FIG. 2. Effect of the mutation of N-linked glycans of HCV envelope glycoproteins on the sensitivity of HCVpp to neutralization by antibodies from genotype 1a HCV-seropositive patients. (A and C) Neutralization assays were performed by preincubating HCVpp glycosylation mutants or wild-type HCVpp (wt) with antibodies purified from patient sera at a concentration which inhibits wild-type HCVpp infectivity by approximately 50% (see Table 1) for 2 h at 37°C before incubation with target cells. After 3 h of contact with pseudoparticles, cells were further incubated for 48 h before measuring luciferase activity. Results are expressed as the ratios of the percentages of neutralization of HCVpp mutants to that of the wild type. Each point corresponds to the ratio for one batch of neutralizing antibodies. The black bars correspond to the mean ratios. In a representative experiment, infectivities of the glycosylation mutants in relative light units were 31.8×10^3 (wt), 6.4×10^3 (E1N1), 4.6×10^3 (E1N2), 33.2×10^3 (E1N3), 4.7×10^3 (E1N4), 17.7×10^3 (E2N1), 27.8×10^3 (E2N3), 29.0×10^3 (E2N5), 21.1×10^3 (E2N6), 20.3×10^3 (E2N7), 28.9×10^3 (E2N9), and 8.9×10^3 (E2N11). (B and D) Particles were pelleted through 30% sucrose cushions and analyzed by Western blotting using anti-E1 (A4), anti-E2 (3/11), and anti-CA to check the amount of each incorporated protein. (E) Neutralization assays were performed with various concentrations of antibodies (Ab) from a representative patient (patient 12). Results are expressed as percentages of infectivity compared to infection in the absence of antibodies.

TABLE 4. Effect of the mutations of N-linked glycans of HCV envelope glycoproteins on the sensitivity of HCVpp to neutralization by antibodies from genotype 1a HCV-seropositive patients

Mutant	Ratio[a]	SD	P^b
E1N1	1.06	0.19	0.221
E1N2	1.05	0.19	0.253
E1N3	0.89	0.17	0.007
E1N4	0.94	0.25	0.309
E2N1	1.60	0.24	<0.0001
E2N3	0.96	0.17	0.330
E2N5	0.82	0.17	<0.0001
E2N6	1.50	0.21	<0.0001
E2N7	0.39	0.17	<0.0001
E2N9	1.12	0.15	0.002
E2N11	1.61	0.24	<0.0001

[a] Average of ratios (percentage of neutralization of mutant/percentage of neutralization of wild type).
[b] Student's *t* test was used to compare percentages of neutralizing activities between wild-type and mutant HCVpp.

that E2N1, E2N6, and E2N11 glycans contribute to the masking of neutralizing epitopes on E2. Since these three glycans are not close to each other on the primary sequence, they may partially mask several conserved neutralizing epitopes located on different regions of E2. Alternatively, it is possible that, on the folded E2 protein, these three glycans are located in the same region.

Mutation of glycosylation sites at positions 417 (N1), 532 (N6), and 645 (N11) in E2 increases the sensitivity of HCVpp to MAb neutralization. To investigate whether the glycans at positions 417 (N1), 532 (N6), and 645 (N11) contribute to the masking of several distinct epitopes, we tested the neutralizing activities of a series of MAbs (9/27, 3/11, 1/39, H48, H54, CBH-5, and CBH-7) against HCVpp bearing E2 glycosylation mutants. Epitopes recognized by MAbs 9/27, 3/11, and 1/39 are located at positions 396 to 407 (i.e., HVR1), 412 to 423, and 432 to 443, respectively (22, 31) (Fig. 1), whereas MAbs H48, H54, CBH-5, and CBH-7 recognize conformation-dependent epitopes (27, 48). CBH-5 and CBH-7 are specific to epitopes representing two distinct immunogenic domains (33, 34), and amino acid residues 523, 530, and 535 have been shown to be involved in the formation of the H48 epitope (49) (Fig. 1). As observed with antibodies from HCV-infected patients, HCVpp

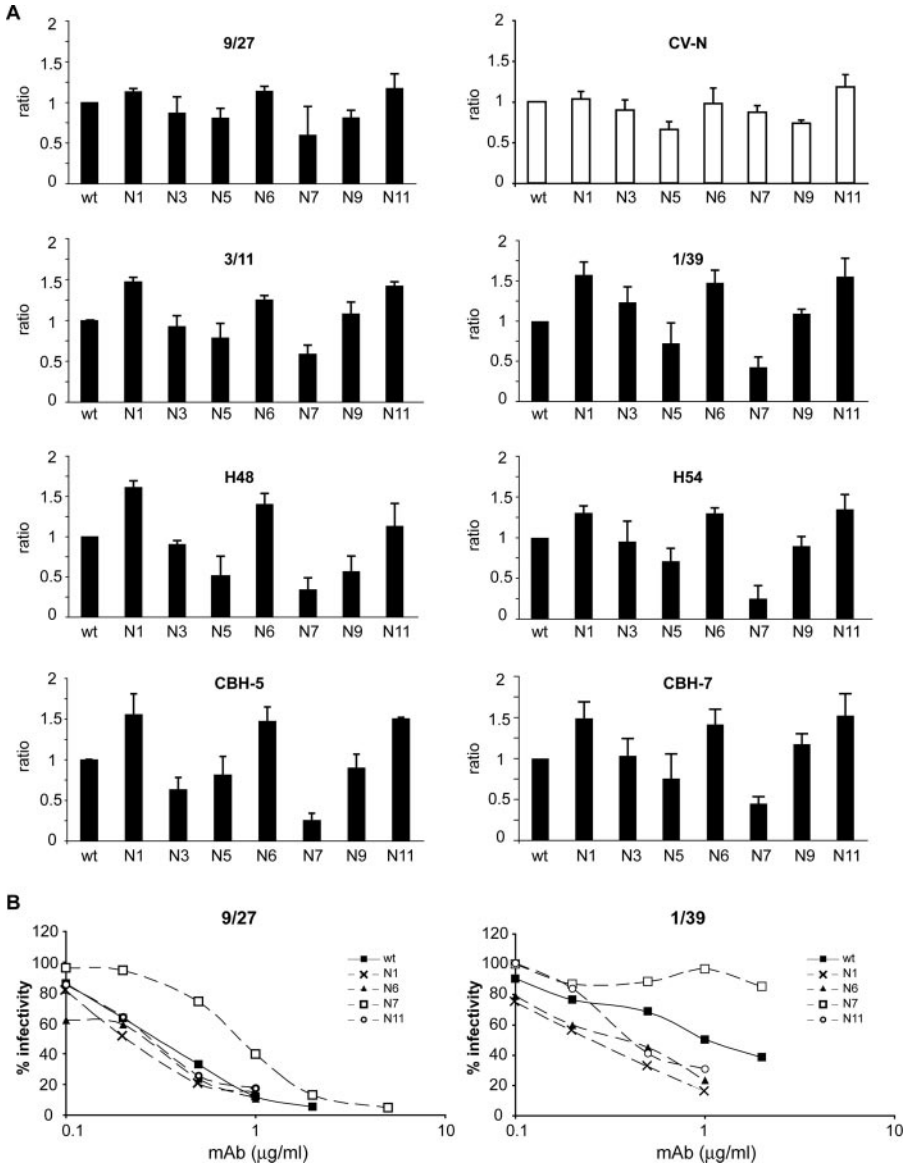

FIG. 3. Effect of the mutation of N-linked glycans of HCV envelope glycoproteins on the sensitivity of HCVpp to neutralization by MAbs. (A) Neutralization assays were performed by preincubating HCVpp glycosylation mutants or wild-type HCVpp (wt) with MAb 9/27, 3/11, 1/39, H48, H54, CBH-5, or CBH-7 (black histograms) at a concentration which inhibits wild-type HCVpp infectivity by approximately 50% (0.3, 5.5, 6.5, 0.3, 0.6, 30, and 10 μg/ml for 9/27, 3/11, 1/39, H48, H54, CBH-5, and CBH-7, respectively) for 2 h at 37°C before contact with target cells. After 3 h of contact with pseudoparticles, the cells were further incubated for 48 h before measuring luciferase activity. In a parallel experiment, inhibition of entry was tested with cyanovirin-N (CV-N; 0.05 μg/ml) (white histogram), which was used as a control to determine the sensitivity of the glycosylation mutants to another inhibitor of virus entry. Results are expressed as the ratios of the percentages of neutralization of HCVpp mutants to that of the wild type and are reported as the means ± standard deviations of three independent experiments. (B) Neutralization assays were performed with various concentrations of MAbs 9/27 and 1/39. Results are expressed as percentages of infectivity compared to infection in the absence of antibodies and are reported as the means of three independent experiments.

containing glycosylation mutant E2N1, E2N6, or E2N11 were more sensitive to neutralization by MAbs 3/11, 1/39, H48, H54, CBH-5, and CBH-7 (Fig. 3A). These results were confirmed in dose-response curve analyses (Fig. 3B, MAb 1/39). Similarly to what was observed with antibodies from HCV-positive sera, an ~1.5-fold increase in percentages of neutralization was obtained when MAbs 3/11, 1/39, CBH-5, and CBH-7 were tested against E2N1, E2N6, and E2N11 mutants. The effect was less pronounced for MAb H54, indicating that this antibody might be less affected by these glycans. Interestingly, MAb H48 had an effect similar to that of MAbs 3/11, 1/39, CBH-5, and CBH-7 on E2N1 and E2N6 mutants; however, the effect was less pronounced for the E2N11 mutant. This suggests that the H48 epitope might be closer to E2N1 and E2N6 glycosylation sites than the E2N11 site. The lectin cyanovirin-N, which binds to glycans on HCV particles and inhibits virus entry (29), was used as a positive control in our neutralization experiments. It is worth noting that the lack of glycan at position E2N1, E2N6,

or E2N11 had no effect on the inhibition of HCVpp entry by cyanovirin-N (Fig. 3A), confirming that the observed effect of these mutations on the neutralizing activity of the tested MAbs was not due to differences in the levels of E2 incorporation into pseudoparticles (Fig. 2D). MAb 9/27 had almost the same neutralizing activity against E2N1, E2N6, and E2N11 mutants as against wild-type HCVpp (Fig. 3A and B), and similar results were obtained with MAb 3C7, another neutralizing MAb directed against HVR1 (data not shown). These results indicate that the epitopes recognized by MAbs 3/11, 1/39, H48, H54, CBH-5, and CBH-7 are similarly affected by glycans at positions E2N1, E2N6, and E2N11, suggesting that they are located in the same region of the tridimensional structure of E2. In contrast, epitopes of 9/27 and 3C7, which are located in HVR1, are likely located outside of the region delimited by the glycans at positions E2N1, E2N6, and E2N11. As observed with antibodies from HCV-infected patients, HCVpp containing glycosylation mutant E2N7 were more resistant to neutralization by all the MAbs tested (Fig. 3A). This observation reinforces the hypothesis of a local conformational change induced by this mutation, which reduces the affinity of neutralizing antibodies for the E2 glycoprotein. This hypothesis may also explain the lower level of neutralization of mutant E2N5, as observed for some MAbs. Together, these data indicate that mutation of glycosylation sites at positions 417 (N1), 532 (N6), and 645 (N11) in E2 increases the sensitivity of HCVpp to neutralization by MAbs whose epitopes are located outside of HVR1.

Mutation of glycosylation sites at positions 417 (N1), 532 (N6), and 645 (N11) in E2 increases the sensitivity of HCVpp to inhibition by CD81-LEL. In contrast to MAb 9/27, MAbs 3/11, 1/39, H48, H54, CBH-5, and CBH-7 have been reported to inhibit E2 binding to a soluble form of the extracellular loop of CD81 (CD81-LEL) (13, 22, 27, 31), suggesting that their epitopes are close to the CD81-binding domain on E2. We therefore wanted to investigate whether glycans at positions E2N1, E2N6, and E2N11 can also affect the interaction between HCVpp and CD81. Since HCVpp infectivity can be inhibited by CD81-LEL (31), we used the same approach as above to determine the effect of glycans on the E2-CD81 interaction. Indeed, if a glycan reduces the access of CD81 to its binding region on E2, a mutant lacking the corresponding glycan is expected to be more sensitive to inhibition by CD81-LEL. As observed with MAbs 3/11, 1/39, H48, H54, CBH-5, and CBH-7, HCVpp containing glycosylation mutant E2N1, E2N6, or E2N11 were more sensitive to inhibition by CD81-LEL (Fig. 4A and B). The lower level of inhibition of mutants E2N3, E2N5, and E2N7 might be explained by a local conformational change induced by the mutations, as discussed above. Altogether, our data suggest that, in the context of HCVpp, the glycans at positions E2N1, E2N6, and E2N11 modulate both CD81 and neutralizing antibody binding to E2.

Identification of conserved amino acids close to glycosylation sites E2N1, E2N6, and E2N11. The first neutralizing epitopes on HCV envelope glycoproteins described were found within HVR1 (21). However, HVR1-specific neutralizing antibodies are isolate specific and are hardly effective against more than one inoculum. On the other hand, some studies pointed out the probable existence of additional neutralizing epitopes elsewhere in the E2 glycoprotein by describing antibodies with a broader neutralizing activity (2, 35, 44). Our results suggest that a neutralizing region outside of HVR1 may be partially masked by glycans at positions E2N1, E2N6, and E2N11. To extend the above findings, we investigated the variability of neighborhoods of glycosylation sites E2N1, E2N6, and E2N11 by using multiple alignment of sequences to identify conserved amino acids likely essential for the structure and/or function of E2. Based on these alignments, we derived the amino acid repertoire shown in Fig. 5, which lists the various amino acids observed at every position of the E2 sequence in decreasing order of frequency. It is worth noting that the sequences derived from the HCV infecting the 19 patients in this study were derived by PCR amplification and direct sequencing of PCR products and thus will provide only the sequence of the most frequently observed quasispecies. The comparison of the amino acid repertoire of the main quasispecies of the 19 patients studied in this report with that of 447 sequences of genotype 1a available in the euHCVdb database allowed us to establish a consensus hydropathic pattern, which highlights the conservation of the physicochemical features of each sequence position despite the variability of residues

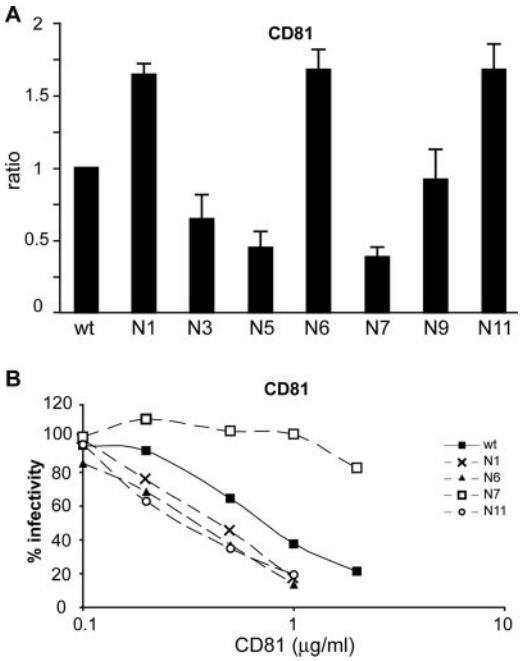

FIG. 4. Effect of the mutation of N-linked glycans of HCV envelope glycoproteins on the sensitivity of HCVpp to inhibition by CD81-LEL. (A) Inhibition assays were performed by preincubating HCVpp glycosylation mutants or wild-type HCVpp (wt) with CD81-LEL at a concentration which inhibits wild-type HCVpp infectivity by approximately 50% (0.75 μg/ml) for 2 h at 37°C, before contact with target cells. After 3 h of contact with pseudoparticles, the cells were further incubated for 48 h before measuring the luciferase activity. Results are expressed as the ratios of the percentages of inhibition of HCVpp mutants to that of the wild type and are reported as the means ± standard deviations of three independent experiments. (B) Inhibition assay performed with various concentrations of CD81-LEL. Results are expressed as percentages of infectivity compared to infection in the absence of antibodies and are reported as the means of three independent experiments.

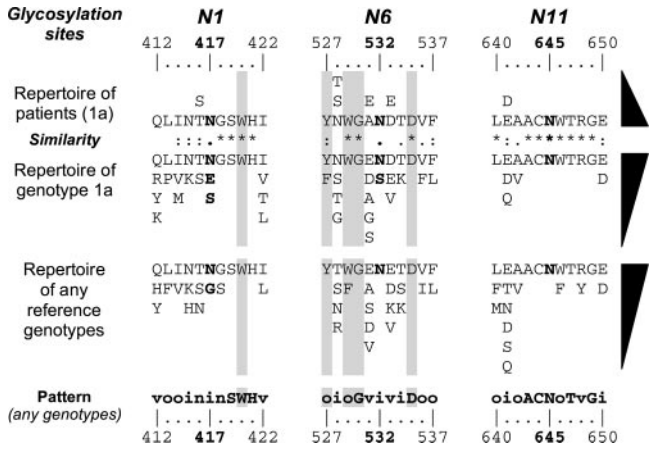

FIG. 5. Conservation of sequences close to glycosylation sites E2N1, E2N6, and E2N11. The amino acid repertoires of E2 sequence segments including glycosylation sites N1 (417), N6 (532), and N11 (645), deduced from the multiple alignment sequences from the various patients studied in this report (19 sequences, top), 447 sequences from genotype 1a extracted from the euHCVdb database (middle), and 26 representative sequences from confirmed HCV genotypes/subtypes (listed in reference 57) (bottom) are shown. Amino acids are listed in decreasing order of observed frequencies (symbolized with black triangles), from bottom to top for the 19 patient sequences and from top to bottom for the 447 sequences from genotype 1a and the 26 representative sequences from confirmed HCV genotypes/subtypes. The undetermined residues (denoted "X" in the patient sequences) were not reported in the corresponding repertoire. The consensus hydropathic pattern (50) for genotypes deduced from these repertoires (bottom of the panel) is indicated as follows: o, hydrophobic residue (F, I, W, Y, L, V, M, P, C, A); n, neutral residue (G, T, S); i, hydrophilic residue (K, Q, N, H, E, D, R); v, variable position (i.e., when the three classes of residues are observed at a given position). The fully conserved residues are indicated by their single-letter code. For sequences from genotype 1a, the degree of conservation is also highlighted by the similarity index according to Clustal W convention (asterisk, invariant; colon, highly similar; dot, similar) (60). For the repertoire of 447 sequences from genotype 1a, the least frequently observed residues at each position (i.e., less than twice) were not reported, as they may be due to PCR and/or sequencing errors and/or sequencing of defective viruses. The positions 420, 527, 529, 530, 535, recently reported to be critical for CD81 binding (49), are shaded in gray.

observed (50). Each position was classified as hydrophobic, hydrophilic, neutral, or variable, according to the nature of residues observed at this position (see the Fig. 5 legend for details). Figure 5 highlights the full conservation of specific residues adjacent to the potential E2N1, E2N6, and E2N11 glycosylation sites. This conservation can be extended to the corresponding positions in E2 encoded by HCV of any genotype for some residues, as shown by the hydropathic patterns (S419-W420-H421 around site E2N1; G530 and D535 around site E2N6; and A643-C644-N645, T647, and G649 around site E2N11). Importantly, some of these residues have been reported to be essential for CD81 binding (Fig. 5). Such conservation across genotypes supports the essential role of these residues for E2 structure and/or function.

DISCUSSION

N-linked glycosylation is the major modification of a nascent protein targeted to the secretory pathway. In the early secretory pathway, glycans play a role in protein folding, quality control, and certain sorting events. Viral envelope proteins usually contain N-linked glycans that can play a major role in their folding, in their entry functions, or in modulating the immune response (28, 46, 47, 61, 64, 66). HCV envelope proteins E1 and E2 are highly glycosylated, and some glycans present on these proteins have been shown to play an essential role in protein folding and/or HCV entry (9, 24). However, their role in modulating the neutralizing antibody response has never been studied to date. Here, we investigated whether the glycans associated with HCV envelope glycoproteins modulate the neutralizing activity of anti-HCV antibodies. Our data demonstrate that at least three glycans on E2 (denoted E2N1, E2N6, and E2N11) reduce the sensitivity of HCVpp to antibody neutralization. Furthermore, these three glycans reduced the access of CD81 to its E2 binding site. Together, these data indicate that glycans E2N1, E2N6, and E2N11 are close to the binding site of CD81 and indicate that this region is a major target of neutralizing antibodies.

Modulation of the humoral immune response by glycans has been observed for HIV gp120, another highly glycosylated envelope protein. Interestingly, it has been shown that the appearance and repositioning of glycans on gp120 limit recognition by neutralizing antibodies and allow the generation of escape variants (66). In their paper, Wei et al. have shown that, individually, mutations of glycosylation sites on the HIV envelope glycoprotein could modify the EC_{50} of neutralizing antibodies by 1.2- to 2.6-fold. Similar effects were observed for HCV mutants lacking a glycan at position E2N1, E2N6, or E2N11. In addition, for HIV, simultaneous deletions of several glycans could modify the EC_{50} of neutralizing antibodies by more than 100-fold (66). Unfortunately, in contrast to HIV, deletion of several glycans strongly affected the infectivity of HCVpp. For this reason, double-glycosylation mutants E2N1N6, E2N1N11, and E2N6N11 could not be used in neutralization studies (F. Helle et al., unpublished data). In contrast to what has been observed for HIV, potential glycosylation sites on HCV envelope glycoproteins are highly conserved and shifting sites are seldom observed (71) (Tables 2 and 3). In particular, glycosylation sites E2N1, E2N6, and E2N11, which protect the CD81 binding site from neutralization, are highly conserved (99.8%, 98.0%, and 99.6%, respectively; Table 3). This high level of conservation is likely due to other roles played by these glycans. The high level of conservation of most other glycans on HCV envelope proteins is also likely due to their role in folding and/or entry (e.g., E2N2, E2N4, E2N8, and E2N10) (24).

Regions of envelope proteins interacting with a receptor or coreceptor represent an Achilles' heel for a virus because, due to their accessibility for interaction with a cellular partner, they are also potentially more exposed to the humoral immune response. To circumvent this problem, viruses have adapted by reducing the effect(s) of neutralizing antibodies without modulating their interaction(s) with cellular receptors or coreceptors. HCV envelope glycoproteins present at least two regions that are accessible to neutralizing antibodies: HVR1 and the CD81 binding region. Due to their role in HCV entry, both regions need to be accessible for interactions with cellular partners (7, 10). To avoid being eliminated too rapidly by neutralizing antibodies, HCV seems to have adapted two ways

depending on the region involved. In the case of HVR1, due to the low level of sequence constraints in this region, the virus can escape neutralizing antibodies by the rapid selection of mutants. For the CD81 binding site, our data indicate that maintaining glycans close to this region is another way for HCV to avoid being eliminated too rapidly. However, the latter strategy may have a cost for the virus because it reduces the accessibility of E2 to CD81. It is indeed interesting that an adaptive mutation of the JFH-1 isolate to cell culture is the loss of the glycan at position E2N6 (D. Delgrange, A. Pillez, S. Castelain, L. Cocquerel, Y. Rouillé, J. Dubuisson, T. Wakita, G. Duverlie, and C. Wychowski, unpublished data). However, while the presence of a glycan may decrease the kinetics of receptor binding, its intrinsic flexibility is not expected to prevent this binding. Finally, the presence of glycans surrounding the CD81 binding site may explain how the lectin cyanovirin-N inhibits HCV entry (29).

In the absence of a three-dimensional structure, it is difficult to have a clear idea of the organization of functional domains on the surface of E2. Although a predictive model has been proposed for E2 (68), this model does not appear to be reliable. Typically, the glycosylation sites which were buried in this model (E2N2, E2N7, and E2N11) are actually modified by N-linked glycans (24). Our data suggest that the CD81 binding region is close to glycosylation sites E2N1, E2N6, and E2N11. In agreement with this hypothesis, a recent study aimed at identifying conserved residues involved in CD81 interaction showed that amino acids W420, Y527, W529, G530, and D535 are critical for CD81 binding (49). Indeed, glycosylation sites E2N1 (at position 417) and E2N6 (at position 532) are close to amino acid W420 and to amino acids W529, G530, and D535, respectively. In addition, the observation that the glycan at position E2N11 affects CD81 binding suggests that other residues of E2 located close to this glycosylation site may also be involved in the interaction with CD81. The full conservation of some amino acids close to these glycosylation sites, as observed in Fig. 5, points to an essential role of these residues for the HCV life cycle, potentially for its binding to CD81.

Glycans associated with HCV envelope proteins reduce the accessibility of the protein moiety. Indeed, one-third of the molecular weight of the E1E2 heterodimer corresponds to glycans. In addition, if these envelope proteins have a folding pattern similar to class II fusion proteins, as currently thought (51), the proteins should lie flat on the surface of the particle and the glycans should be concentrated on the upper face of the protein. In contrast to HCV, HIV gp120 forms protruding spikes (70), and folding into spikes leads to the exposure of a larger surface to accommodate the presence of a large number of glycans. Interestingly, it has been proposed that gp120 presents an immunologically "silent face," which consists of heavily glycosylated regions of gp120 that may appear as self to the immune system (67). Taking into account the size of one glycan, one can also suppose that the presence of a large concentration of glycans on the surface of an HCV particle could limit the immunogenicity of the envelope proteins. This may explain why it is difficult to elicit an antibody response to E1 when immunizing mice with E1E2 heterodimers (A. Pillez and J. Dubuisson, unpublished data). This is in keeping with the observation that mutation of the fourth glycosylation site of E1 (N4) enhances the anti-E1 humoral response in terms of both seroconversion rates and antibody titers (23). Together, these data suggest that, as observed for gp120, HCV envelope glycoproteins contain immunologically silent regions.

Since HCV is a virus that is well adapted to its human host, one can assume that the high level of glycosylation of the envelope glycoproteins is likely the result of a long period of selection to reach a compromise between receptor binding site conservation and limitation of the immunogenicity of the receptor binding site(s). Two mechanisms have already been proposed to explain the ability of HCV to persist in the presence of neutralizing antibodies, i.e., a rapid evolution through point mutations (6, 20, 26, 32, 59, 63) and an attenuation of neutralization due to an acceleration of entry by high-density lipoproteins (4, 13, 62). Here, we show that glycans on E2 reduce the sensitivity of HCVpp to antibody neutralization. This new mechanism likely represents an additional strategy for HCV to evade the humoral immune response. Interestingly, this mechanism could be exploited for the development of new antiviral drugs targeting HCV entry, as suggested by the observation that the lectin cyanovirin-N inhibits HCV entry by blocking the interaction between E2 and CD81 (29).

ACKNOWLEDGMENTS

We thank Angéline Bilheu and Sophana Ung for their technical assistance. Thanks are also due to Yann Ciczora for his helpful advice and critical comments on the manuscript. We are grateful to B. Bartosch, F. L. Cosset, M. Mondelli, and K. Gustafson for providing us with reagents.

This work was supported by the Centre National de la Recherche Scientifique and by the Agence Nationale de Recherche sur le Sida et les Hépatites virales (ANRS) and in part by NIH grants HL079381 and AI47355 to S.F. F.H. and C.V. were supported by fellowships from the French Ministry of Research and the ANRS, respectively. J.D. is an international scholar of the Howard Hughes Medical Institute.

REFERENCES

1. **Aguilar, H. C., K. A. Matreyek, C. M. Filone, S. T. Hashimi, E. L. Levroney, O. A. Negrete, A. Bertolotti-Ciarlet, D. Y. Choi, I. McHardy, J. A. Fulcher, S. V. Su, M. C. Wolf, L. Kohatsu, L. G. Baum, and B. Lee.** 2006. N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. J. Virol. **80:**4878–4889.
2. **Bartosch, B., J. Bukh, J. C. Meunier, C. Granier, R. E. Engle, W. C. Blackwelder, S. U. Emerson, F. L. Cosset, and R. H. Purcell.** 2003. In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes. Proc. Natl. Acad. Sci. USA **100:**14199–14204.
3. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C pseudo-particles containing functional E1E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
4. **Bartosch, B., G. Verney, M. Dreux, P. Donot, Y. Morice, F. Penin, J. M. Pawlotsky, D. Lavillette, and F. L. Cosset.** 2005. An interplay between the hypervariable region 1 of the HCV E2 glycoprotein, the scavenger receptor BI and HDL promotes both enhancement of infection and protection against neutralizing antibodies. J. Virol. **79:**8217–8229.
5. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624–41630.
6. **Bowen, D. G., and C. M. Walker.** 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature **436:**946–952.
7. **Callens, N., Y. Ciczora, B. Bartosch, N. Vu-Dac, F. L. Cosset, J. M. Pawlotsky, F. Penin, and J. Dubuisson.** 2005. Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. J. Virol. **79:**15331–15341.
8. **Cerino, A., A. Meola, L. Segagni, M. Furione, S. Marciano, M. Triyatni, T. J. Liang, A. Nicosia, and M. U. Mondelli.** 2001. Monoclonal antibodies with broad specificity for hepatitis C virus hypervariable region 1 variants can recognize viral particles. J. Immunol. **167:**3878–3886.
9. **Choukhi, A., S. Ung, C. Wychowski, and J. Dubuisson.** 1998. Involvement of endoplasmic reticulum chaperones in folding of hepatitis C virus glycoproteins. J. Virol. **72:**3851–3858.
10. **Cocquerel, L., C. Voisset, and J. Dubuisson.** 2006. Hepatitis C virus entry:

potential receptors and their biological functions. J. Gen. Virol. **87:**1075–1084.
11. **Combet, C., N. Garnier, C. Charavay, D. Grando, D. Crisan, J. Lopez, A. Dehne-Garcia, C. Geourjon, E. Bettler, C. Hulo, P. Le Mercier, R. Bartenschlager, H. Diepolder, D. Moradpour, J. M. Pawlotsky, C. M. Rice, C. Trepo, F. Penin, and G. Deleage.** 2007. euHCVdb: the European hepatitis C virus database. Nucleic Acids Res. **35:**D363–D366.
12. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **101:**7270–7274.
13. **Dreux, M., T. Pietschmann, C. Granier, C. Voisset, S. Ricard-Blum, P. E. Mangeot, Z. Keck, S. Foung, N. Vu-Dac, J. Dubuisson, R. Bartenschlager, D. Lavillette, and F. L. Cosset.** 2006. High density lipoprotein inhibits hepatitis C virus-neutralizing antibodies by stimulating cell entry via activation of the scavenger receptor BI. J. Biol. Chem. **281:**18285–18295.
14. **Drummer, H. E., I. Boo, A. L. Maerz, and P. Poumbourios.** 2006. A conserved Gly436-Trp-Leu-Ala-Gly-Leu-Phe-Tyr motif in hepatitis C virus glycoprotein E2 is a determinant of CD81 binding and viral entry. J. Virol. **80:**7844–7853.
15. **Drummer, H. E., A. Maerz, and P. Poumbourios.** 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. FEBS Lett. **546:**385–390.
16. **Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:**6147–6160.
17. **Dyall-Smith, M. L., I. Lazdins, G. W. Tregear, and I. H. Holmes.** 1986. Location of the major antigenic sites involved in rotavirus serotype-specific neutralization. Proc. Natl. Acad. Sci. USA **83:**3465–3468.
18. **Farci, P., H. J. Alter, S. Govindarajan, D. C. Wong, R. Engle, R. R. Lesniewski, I. K. Mushahwar, S. M. Desai, R. H. Miller, N. Ogata, et al.** 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science **258:**135–140.
19. **Farci, P., H. J. Alter, D. C. Wong, R. H. Miller, S. Govindarajan, R. Engle, M. Shapiro, and R. H. Purcell.** 1994. Prevention of hepatitis C virus infection in chimpanzees after antibody-mediated in vitro neutralization. Proc. Natl. Acad. Sci. USA **91:**7792–7796.
20. **Farci, P., A. Shimoda, A. Coiana, G. Diaz, G. Peddis, J. C. Melpolder, A. Strazzera, D. Y. Chien, S. J. Munoz, A. Balestrieri, R. H. Purcell, and H. J. Alter.** 2000. The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science **288:**339–344.
21. **Farci, P., A. Shimoda, D. Wong, T. Cabezon, D. De Gioannis, A. Strazzera, Y. Shimizu, M. Shapiro, H. J. Alter, and R. H. Purcell.** 1996. Prevention of hepatitis C virus infection in chimpanzees by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc. Natl. Acad. Sci. USA **93:**15394–15399.
22. **Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating.** 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J. Virol. **73:**6235–6244.
23. **Fournillier, A., C. Wychowski, D. Boucreux, T. F. Baumert, J. C. Meunier, D. Jacobs, S. Muguet, E. Depla, and G. Inchauspe.** 2001. Induction of hepatitis C virus E1 envelope protein-specific immune response can be enhanced by mutation of N-glycosylation sites. J. Virol. **75:**12088–12097.
24. **Goffard, A., N. Callens, B. Bartosch, C. Wychowski, F. L. Cosset, C. Montpellier-Pala, and J. Dubuisson.** 2005. Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. J. Virol. **79:**8400–8409.
25. **Goffard, A., and J. Dubuisson.** 2003. Glycosylation of hepatitis C virus envelope proteins. Biochimie **85:**295–301.
26. **Gremion, C., and A. Cerny.** 2005. Hepatitis C virus and the immune system: a concise review. Rev. Med. Virol. **15:**235–268.
27. **Hadlock, K. G., R. E. Lanford, S. Perkins, J. Rowe, Q. Yang, S. Levy, P. Pileri, S. Abrignani, and S. K. Foung.** 2000. Human monoclonal antibodies that inhibit binding of hepatitis C virus E2 protein to CD81 and recognize conserved conformational epitopes. J. Virol. **74:**10407–10416.
28. **Hebert, D. N., J. X. Zhang, W. Chen, B. Foellmer, and A. Helenius.** 1997. The number and location of glycans on influenza hemagglutinin determine folding and association with calnexin and calreticulin. J. Cell Biol. **139:**613–623.
29. **Helle, F., C. Wychowski, N. Vu-Dac, K. R. Gustafson, C. Voisset, and J. Dubuisson.** 2006. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. J. Biol. Chem. **281:**25177–25183.
30. **Higginbottom, A., E. R. Quinn, C. C. Kuo, M. Flint, L. H. Wilson, E. Bianchi, A. Nicosia, P. N. Monk, J. A. McKeating, and S. Levy.** 2000. Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. J. Virol. **74:**3642–3649.
31. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100:**7271–7276.
32. **Kanto, T., and N. Hayashi.** 2006. Immunopathogenesis of hepatitis C virus infection: multifaceted strategies subverting innate and adaptive immunity. Intern. Med. **45:**183–191.
33. **Keck, Z. Y., T. K. Li, J. Xia, B. Bartosch, F. L. Cosset, J. Dubuisson, and S. K. Foung.** 2005. Analysis of a highly flexible conformational immunogenic domain A in hepatitis C virus E2. J. Virol. **79:**13199–13208.
34. **Keck, Z. Y., A. Op De Beeck, K. G. Hadlock, J. Xia, T. K. Li, J. Dubuisson, and S. K. Foung.** 2004. Hepatitis C virus E2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. J. Virol. **78:**9224–9232.
35. **Keck, Z. Y., J. Xia, Z. Cai, T. K. Li, A. M. Owsianka, A. H. Patel, G. Luo, and S. K. Foung.** 2007. Immunogenic and functional organization of hepatitis C virus (HCV) glycoprotein e2 on infectious HCV virions. J. Virol. **81:**1043–1047.
36. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
37. **Kuiken, C., C. Combet, J. Bukh, I. T. Shin, G. Deleage, M. Mizokami, R. Richardson, E. Sablon, K. Yusim, J. M. Pawlotsky, and P. Simmonds.** 2006. A comprehensive system for consistent numbering of HCV sequences, proteins and epitopes. Hepatology **44:**1355–1361.
38. **Lai, M. E., A. P. Mazzoleni, F. Argiolu, S. De Virgilis, A. Balestrieri, R. H. Purcell, A. Cao, and P. Farci.** 1994. Hepatitis C virus in multiple episodes of acute hepatitis in polytransfused thalassaemic children. Lancet **343:**388–390.
39. **Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset.** 2005. Characterization of host-range and cell entry properties of hepatitis C virus of major genotypes and subtypes. Hepatology **41:**265–274.
40. **Lechner, F., D. K. Wong, P. R. Dunbar, R. Chapman, R. T. Chung, P. Dohrenwend, G. Robbins, R. Phillips, P. Klenerman, and B. D. Walker.** 2000. Analysis of successful immune responses in persons infected with hepatitis C virus. J. Exp. Med. **191:**1499–1512.
41. **Logvinoff, C., M. E. Major, D. Oldach, S. Heyward, A. Talal, P. Balfe, S. M. Feinstone, H. Alter, C. M. Rice, and J. A. McKeating.** 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. Proc. Natl. Acad. Sci. USA **101:**10149–10154.
42. **Major, M. E., B. Rehermann, and S. M. Feinstone.** 2001. Hepatitis C viruses, p. 1127–1162. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology. Lippincott Williams & Wilkins, Philadelphia, PA.
43. **Meunier, J.-C., A. Fournillier, A. Choukhi, A. Cahour, L. Cocquerel, J. Dubuisson, and C. Wychowski.** 1999. Analysis of the glycosylation sites of hepatitis C virus (HCV) glycoprotein E1 and the influence of E1 glycans on the formation of the HCV glycoprotein complex. J. Gen. Virol. **80:**887–896.
44. **Meunier, J. C., R. E. Engle, K. Faulk, M. Zhao, B. Bartosch, H. Alter, S. U. Emerson, F. L. Cosset, R. H. Purcell, and J. Bukh.** 2005. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. Proc. Natl. Acad. Sci. USA **102:**4560–4565.
45. **Nakabayashi, H., K. Taketa, K. Miyano, T. Yamane, and J. Sato.** 1982. Growth of human hepatoma cells lines with differentiated functions in chemically defined medium. Cancer Res. **42:**3858–3863.
46. **Ohuchi, M., R. Ohuchi, A. Feldmann, and H. D. Klenk.** 1997. Regulation of receptor binding affinity of influenza virus hemagglutinin by its carbohydrate moiety. J. Virol. **71:**8377–8384.
47. **Ohuchi, R., M. Ohuchi, W. Garten, and H. D. Klenk.** 1997. Oligosaccharides in the stem region maintain the influenza virus hemagglutinin in the metastable form required for fusion activity. J. Virol. **71:**3719–3725.
48. **Op De Beeck, A., C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F. L. Cosset, and J. Dubuisson.** 2004. Characterization of functional hepatitis C virus envelope glycoproteins. J. Virol. **78:**2994–3002.
49. **Owsianka, A. M., J. M. Timms, A. W. Tarr, R. J. Brown, T. P. Hickling, A. Szwejk, K. Bienkowska-Szewczyk, B. J. Thomson, A. H. Patel, and J. K. Ball.** 2006. Identification of conserved residues in the E2 envelope glycoprotein of the hepatitis C virus that are critical for CD81 binding. J. Virol. **80:**8695–8704.
50. **Penin, F., C. Combet, G. Germanidis, P. O. Frainais, G. Deléage, and J. M. Pawlotsky.** 2001. Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. J. Virol. **75:**5703–5710.
51. **Penin, F., J. Dubuisson, F. A. Rey, D. Moradpour, and J. M. Pawlotsky.** 2004. Structural biology of hepatitis C virus. Hepatology **39:**5–19.
52. **Pestka, J. M., M. B. Zeisel, E. Blaser, P. Schurmann, B. Bartosch, F. L. Cosset, A. H. Patel, H. Meisel, J. Baumert, S. Viazov, K. Rispeter, H. E. Blum, M. Roggendorf, and T. F. Baumert.** 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. Proc. Natl. Acad. Sci. USA **104:**6025–6030.
53. **Pikora, C. A.** 2004. Glycosylation of the ENV spike of primate immunodeficiency viruses and antibody neutralization. Curr. HIV Res. **2:**243–254.
54. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282:**938–941.
55. **Reitter, J. N., R. E. Means, and R. C. Desrosiers.** 1998. A role for carbohydrates in immune evasion in AIDS. Nat. Med. **4:**679–684.
56. **Roccasecca, R., H. Ansuini, A. Vitelli, A. Meola, E. Scarselli, S. Acali, M. Pezzanera, B. B. Ercole, J. McKeating, A. Yagnik, A. Lahm, A. Tramontano, R. Cortese, and A. Nicosia.** 2003. Binding of the hepatitis C virus E2 glyco-

protein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. J. Virol. **77:**1856–1867.

57. **Simmonds, P., J. Bukh, C. Combet, G. Deleage, N. Enomoto, S. Feinstone, P. Halfon, G. Inchauspe, C. Kuiken, G. Maertens, M. Mizokami, D. G. Murphy, H. Okamoto, J. M. Pawlotsky, F. Penin, E. Sablon, I. T. Shin, L. J. Stuyver, H. J. Thiel, S. Viazov, A. J. Weiner, and A. Widell.** 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology **42:**962–973.
58. **Skehel, J. J., D. J. Stevens, R. S. Daniels, A. R. Douglas, M. Knossow, I. A. Wilson, and D. C. Wiley.** 1984. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. Proc. Natl. Acad. Sci. USA **81:**1779–1783.
59. **Thimme, R., V. Lohmann, and F. Weber.** 2006. A target on the move: innate and adaptive immune escape strategies of hepatitis C virus. Antivir. Res. **69:**129–141.
60. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
61. **van Kooyk, Y., and T. B. Geijtenbeek.** 2003. DC-SIGN: escape mechanism for pathogens. Nat. Rev. Immunol. **3:**697–709.
62. **Voisset, C., A. Op De Beeck, P. Horellou, M. Dreux, T. Gustot, G. Duverlie, F. L. Cosset, N. Vu-Dac, and J. Dubuisson.** 2006. High-density lipoproteins reduce the neutralizing effect of hepatitis C virus (HCV)-infected patient antibodies by promoting HCV entry. J. Gen. Virol. **87:**2577–2581.
63. **von Hahn, T., J. C. Yoon, H. Alter, C. M. Rice, B. Rehermann, P. Balfe, and J. A. McKeating.** 2007. Hepatitis C virus continuously escapes from neutralizing antibody and T-cell responses during chronic infection in vivo. Gastroenterology **132:**667–678.
64. **von Messling, V., and R. Cattaneo.** 2003. N-linked glycans with similar location in the fusion protein head modulate paramyxovirus fusion. J. Virol. **77:**10202–10212.
65. **Wasley, A., and M. J. Alter.** 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. **20:**1–16.
66. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422:**307–312.
67. **Wyatt, R., P. D. Kwong, E. Desjardins, R. W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski.** 1998. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature **393:**705–711.
68. **Yagnik, A. T., A. Lahm, A. Meola, R. M. Roccasecca, B. B. Ercole, A. Nicosia, and A. Tramontano.** 2000. A model for the hepatitis C virus envelope glycoprotein E2. Proteins **40:**355–366.
69. **Yu, M. Y., B. Bartosch, P. Zhang, Z. P. Guo, P. M. Renzi, L. M. Shen, C. Granier, S. M. Feinstone, F. L. Cosset, and R. H. Purcell.** 2004. Neutralizing antibodies to hepatitis C virus (HCV) in immune globulins derived from anti-HCV-positive plasma. Proc. Natl. Acad. Sci. USA **101:**7705–7710.
70. **Zanetti, G., J. A. Briggs, K. Grunewald, Q. J. Sattentau, and S. D. Fuller.** 2006. Cryo-electron tomographic structure of an immunodeficiency virus envelope complex in situ. PLoS Pathog. **2:**e83.
71. **Zhang, M., B. Gaschen, W. Blay, B. Foley, N. Haigwood, C. Kuiken, and B. Korber.** 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology **14:**1229–1246.