JOURNAL OF VIROLOGY, Nov. 2005, p. 13199–13208 Vol. 79, No. 21
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.21.13199–13208.2005

Analysis of a Highly Flexible Conformational Immunogenic Domain A in Hepatitis C Virus E2

Zhen-Yong Keck,[1] Ta-Kai Li,[1] Jinming Xia,[1] Birke Bartosch,[2] François-Loïc Cosset,[2] Jean Dubuisson,[3] and Steven K. H. Foung[1]*

Department of Pathology, Stanford University School of Medicine, Stanford, California 94305[1]; Laboratoire de Vectorologie Rétrovirale et Thérapie Génique, INSERM U412, IFR 74, Ecole Normale Supérieure de Lyon, Lyon, France[2]; and CNRS-UPR2511, Institut de Biologie de Lille, and Institut Pasteur de Lille, Lille, France[3]

Received 18 May 2005/Accepted 8 August 2005

Hepatitis C (HCV) E2 glycoprotein is involved in virus attachment and entry, and its structural organization is largely unknown. Characterization of a panel of human monoclonal antibodies (HMAbs) to HCV by competition studies has led to an immunogenic organization model of E2 with three domains designated A, B, and C and epitopes in each domain having similar structural and functional properties. Domain A contains nonneutralizing epitopes, and domains B and C contain neutralizing epitopes. The isolation and characterization of three new HMAbs within domain A for a total of six provide support for this model. All six domain A HMAbs do not neutralize HCV retroviral pseudotype particle (HCVpp) infection on Huh-7 cells, and all six HMAbs have similar binding affinity and maximum binding, B_{max}, a relative indicator of epitope density, as other neutralizing HMAbs, suggesting that neutralization is epitope specific and not by binding to any surface epitope. The dose-dependent neutralizing activity of CBH-7, an HMAb to a domain C epitope in spatial proximity to domain A, and of CBH-5, a domain B HMAb to a more distant epitope, were tested in the presence and absence of each domain A HMAb. No enhancement or reduction in CBH-7 or CBH-5 neutralizing activity was observed, indicating that the potential induction of nonneutralizing antibodies should not be a central issue for HCV vaccine design. To assess whether domain A is involved in the structural changes as part of a pH-dependent virus envelope fusion process, changes in antibody binding patterns to normal pH and acid pH-treated HCVpp were measured. Antibody binding affinity of HMAbs to HCVpp was not affected by low pH. However, the B_{max} values for low-pH-treated HCVpp with antibodies to domain A increased 46%, for domain C (CBH-7) they increased 23%, and for domain B (CBH-5) there was a decrease of 12%. Collectively, the organization and function of HCV E2 antigenic domains are roughly analogous to the large envelope glycoprotein E organizational structure for other flaviviruses with three distinct structural and functional domains.

Infection with hepatitis C virus (HCV), leading to chronic hepatitis, cirrhosis, and hepatocellular carcinoma, is a major healthcare problem worldwide that affects over 170 million individuals (17). Although current therapy has led to clinical improvements in some patients, significant adverse effects and a high relapse rate when patients discontinue treatment are major limitations. Other strategies are needed to treat and prevent HCV infection. An effective vaccine will need detailed information on the structure and function of the viral envelope proteins. The virus is a member of the family *Flaviviridae*, composed of three structural proteins, capsid, two envelope proteins, E1 and E2, and six nonstructural proteins (15, 18, 24). Virus entry is thought to be mediated by the envelope proteins, which are responsible for virus attachment, and receptor-mediated endocytosis which, under a low-pH environment in the endosomes, triggers conformational structural changes leading to virus envelope fusion with the endosomal membrane (2, 10, 21). These early steps of infection have been difficult to study because of an inability to reliably grow the virus in vitro. A robust means to analyze functional envelope proteins involved in virus attachment and entry is infectious HCV retroviral pseudotype particles expressing E1E2 (HCVpp) (2, 3, 10). These HCVpp contain fully functional envelope glycoproteins that preferentially infect human hepatocytes and hepatocellular cell lines. The envelope proteins are at least in part noncovalent E1E2 heterodimers, and their recognition by a panel of human monoclonal antibodies (HMAbs) to conformational epitopes on E2 confirms the expression of native antigenic structures (21).

Vaccine studies with recombinant E1E2 proteins in chimpanzees showed some degree of protection against infection challenge with homologous virus that correlated with the neutralizing antibody titers to the E2 glycoprotein (5, 26). Subsequent binding studies with soluble E2 glycoprotein and cell surface molecules have identified candidate receptors, including CD81, a member of the tetraspannin family of proteins (14), the low-density lipoprotein receptor (1, 20), the scavenger receptor type B class I (2, 27), and two closely related membrane-associated C-type mannose binding lectins, DC-SIGN and L-SIGN (6, 16, 22). The functional and structural organization of the envelope E2 glycoprotein clearly are central to understanding the mechanisms of virus-cell interactions. But the structural organization of the E2 glycoprotein compared with other flaviviruses remains largely unknown, which is caused by difficulties in performing high-resolution crystal structural studies for HCV proteins hindered by an inability to purify HCV particles in sufficient amounts from infectious sources. An alternative approach to gain insights on properties

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

of HCV E2 is functional and biochemical studies with antigen-specific antibodies. Based on competition binding studies with a panel of HMAbs to conformational epitopes on HCV E2, three distinct antigenic domains containing conformational epitopes on E2 are proposed and designated as domains A, B, and C (11). Domain A contains conserved conformational epitopes among different HCV genotypes as originally defined by three nonneutralizing HMAbs. Domains B and C contain conformational epitopes that mediate virus neutralization and are either highly conserved or more restricted among different HCV genotypes. Domain A is felt to be in spatial proximity to domain C in that moderate cross-competition or binding enhancement is observed between antibodies to these two domains. This organization of HCV E2 antigenic domains has some similarity to the large envelope glycoprotein E organizational structure for other flaviviruses, such as tick-borne encephalitis virus (TBEV), and is consistent with the proposed three-dimensional structural model of HCV E2, having three domains based on fold recognition methods (23, 28). Detailed immunogenic, functional, and high-resolution crystal structural studies established an organizational structure of TBEV glycoprotein E into three distinct immunogenic and functional domains (DI, II, and III) with epitopes within each structural domain having similar structural and functional properties (23; review in reference 19). DI is a central domain containing type- and subtype-specific epitopes, and DII containing cross-reactive epitopes is responsible for dimerization. A conserved hydrophobic sequence among all flaviviruses is at the tip of DII and is felt to be responsible for the fusion activity. The low-pH-induced structural rearrangement affects DI and DII, with hinge-like motion leading to exposure of the fusion peptide and subsequent fusion. DIII resembles an immunoglobulin-like constant region, is least affected by low pH, and is felt to be responsible for receptor binding.

We report here the functional and structural role of an immunogenic domain, A, encoding predominately nonneutralizing epitopes, that supports the model of three distinct domains for HCV E2. Two related issues relevant to vaccine design are also addressed. First, whether nonneutralizing antibodies are to epitopes expressed on the surface of HCV virions was explored with six HMAbs to domain A using HCVpp as a model virus. For some viruses, such as the human immunodeficiency virus type 1 (HIV-1), the existence of virion surface epitopes not mediating virus neutralization is controversial, with some studies showing nonneutralizing antibody binding to virions (9) and other studies suggesting that nonneutralizing antibodies do not bind to surface native structures (8). Second, whether nonneutralizing antibodies can interfere with neutralizing antibodies was investigated by studying the functional relationship between nonneutralizing epitopes in domain A that are in spatial proximity to a neutralizing epitope in domain C and to a more distant epitope in domain B. Furthermore, in an effort to determine the functional role of domain A, its involvement in the low-pH-induced structural rearrangement of HCV glycoproteins leading to virus envelope fusion was studied by observing for changes in antibody binding patterns of domain A HMAbs to normal and low-pH-treated HCVpp.

MATERIALS AND METHODS

Cells and culture conditions. 293T cells were obtained from the American Type Culture Collection (CRL-11268), and Huh-7 cells were generously provided by Michael Lai (University of Southern California). The cells were grown in Dulbecco's modified minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Gemini Bioproducts Inc., Calabasas, CA) and 2 mM glutamine.

Monoclonal antibodies. Isolation of new HCV HMAbs from hybridomas produced from peripheral blood B cells of a chronically HCV-infected individual was as described elsewhere (7) using recombinant E2 protein (genotype 1b; GenBank accession no. AF348705) constitutively expressed in CHO cells (11) as the target antigen. The external domain of human CD4 (amino acids 1 to 371) was amplified from peripheral blood leukocyte cDNA and cloned into the same vector as the E2 gene (above) serving as a negative control for antibody identification. Monoclonality was confirmed by DNA sequencing (Megabace 1000; GE Healthcare, Piscataway, NJ) of the immunoglobulin G (IgG) genes from 10 individual cell clones derived from each hybridoma. Cloning and analysis of the V_L and V_H domains of these clones were performed as previously described (12). HCV HMAb production and purification were performed as described elsewhere (7), and biotinylation of the antibodies was carried out according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL).

Production and purification of HCV-retroviral pseudoparticles. HCVpp (genotype 1a) were produced as described elsewhere (3) and isolated by first passing supernatant containing HCVpp through a 0.45-μm filter. Concentrated HCVpp were obtained by processing 30 ml of filtered supernatant through a 20% sucrose cushion by ultracentrifugation using a Beckman Coulter SW 28 rotor (25,000 rpm, 2 h at 4°C). The cushion pellet was resuspended in 150 μl of NTE buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). For further purification, HCVpp cushion pellets were separated by sucrose density-equilibrium gradient centrifugation in 20 to 60% sucrose at 36,000 rpm for 18 h at 4°C using a Beckman SW 55Ti rotor. After ultracentrifugation, 250-μl fractions were collected from top to bottom of the gradient. E1E2 in the fractions was detected by Western blot analysis, and HCVpp infectivity was detected by a luciferase assay as described below for virus neutralization. Based on HCVpp infectivity peaks, fractions 10, 11, and 12 were pooled for the following studies as purified HCVpp.

Antibody affinity measurements. Antibody affinity measurements were performed with sucrose density-equilibrium gradient–purified HCVpp. Microtiter plates were prepared by coating each well with 500 ng of *Galanthus nivalis* lectin (GNA; Sigma, St. Louis, MO), followed by blocking of the wells with BLOTTO, consisting of 2.5% nonfat dry milk and 2.5% normal goat serum in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20). After blocking, HCVpp were captured on the GNA-coated plates and later bound by a range of 0.01 to 200 μg/ml of HMAbs. The bound HMAbs were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Promega, Madison, WI), followed by incubation with *p*-nitrophenyl phosphate disodium hexahydrate for color development. Absorbance was measured at 405 nm and 570 nm with an FL600 plate reader from Bio-Tek Instruments (Winooski, VT). The data were analyzed by nonlinear regression to measure the antibody disassociation constant, K_d, and maximum binding, B_{max} (optical density at 405 nm) using Prism software (GraphPad).

Acidic treatment of HCV retroviral pseudoparticles. Sucrose density-equilibrium gradient–purified HCVpp was diluted with 10% FBS-supplemented IMDM before ultracentrifugation with a 20% sucrose cushion to get sucrose-free HCVpp. The pelleted HCVpp was resuspended in NTE buffer and equally divided into two parts. One part was treated with 150 mM morpholinoethanesulfonic acid (MES; pH 5.5) at 37°C for 20 min, followed by neutralization with 150 mM triethanolamine (pH 7.5) at room temperature for 5 min. The second part was used for a normal pH control and adjusted with the same volume of NTE buffer following the same treatments as the acidified part. K_d and B_{max} values for HCVpp with or without acid treatment were determined as described above for antibody affinity measurements, with statistical analysis by paired *t* test using Prism software (GraphPad).

Competition assay. This test was performed essentially as previously described (11). Briefly, recombinant E2 protein was captured onto 96-well plates coated with GNA in phosphate-buffered saline (PBS) for 1 hour at 37°C. After washing and blocking, the competing antibodies at 20 μg/ml were added to E2 proteins for 30 min at room temperature, followed by adding the biotinylated test antibody at 2 μg/ml. After incubating for 1.5 h at room temperature, the test antibody was detected with alkaline phosphatase-conjugated streptavidin (Amersham-Pharmacia Biotech, Piscataway, NJ) followed by incubation with *p*-nitrophenyl phosphate disodium hexahydrate for color development. Absorbance was measured at 405 nm with a multiwell plate reader (BioTek Instruments, Winooski, VT). The mean optical density values measured with biotinylated test

A

Competing Antibody (20 ug/ml)	Biotinylated Test Antibody (2 ug/ml)		
	CBH-5*	CBH-7*	CBH-4D*
CBH-20	75	102	3
CBH-21	75	97	3
CBH-22	75	95	3

* Competition with itself was 99 -100%.

B

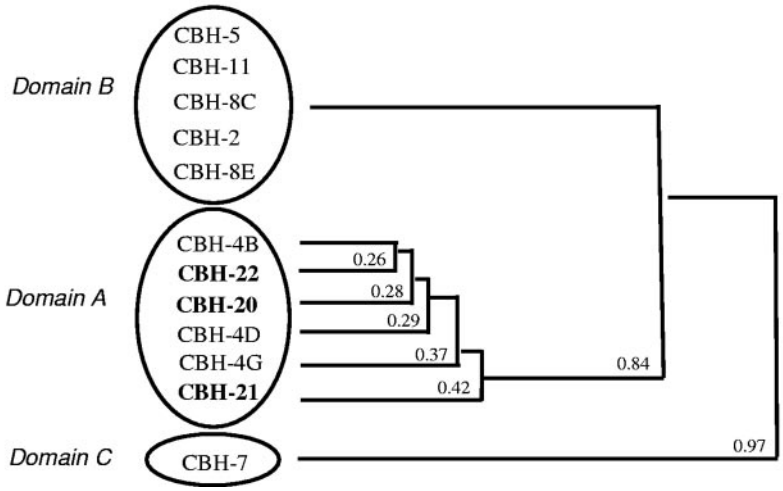


FIG. 1. Competition analysis of three new antibodies with existing HCV HMAbs (7). (A) Cross-competition matrix. (B) Phylogenetic grouping of HCV HMAbs based on the competitive binding assay. Solid lines with numbers indicate the relatedness of the two adjacent antibodies. Circles are clusters of antibodies in a specific domain. The three domains are as indicated on the left.

HMAb and E2 in the presence of competing antibody were divided by signals measured from biotinylated test HMAb and E2 without competing antibody, followed by multiplying by 100 to obtain the percentage of test antibody bound to E2.

Phylogenetic grouping of HCV HMAbs. Relatedness of the new HMAbs to other antibodies was found by competition studies and clusters within a phylogenetic group were identified using a modified approach of unweighted pair-group method using arithmetic averages (11). This method assumes the extent of bidirectional inhibition as the extent of epitope overlap by the competing antibodies. Unidirectional inhibition or enhancement is interpreted as proximal but not overlapping epitopes. If one antibody competed with another antibody, their epitopes were considered in close proximity and the antibodies were classified in the same cluster. In this analysis, the cross-competition percentages of any two antibodies were averaged and are expressed as a fraction (see Fig. 1B, below) (the smaller the fraction, the greater the cross-competition). A relationship matrix was generated, and the closest two antibodies were paired together. Their inhibition percentages against each of the other antibodies were averaged and added to the matrix in proximity to the original pair. This cycle was repeated until all HMAbs were assigned in this matrix.

Neutralization of HCV retroviral pseudoparticles. This assay was performed as described previously (3). Briefly, Huh-7 cells were seeded at 8×10^3 cells per well in a white nontransparent 96-well plate 24 h before infection. Filtered HCVpp-containing supernatants were added to Huh-7 cells as the infection medium. For the neutralization assay, the infection medium was incubated with various concentrations of HMAbs for 60 min at 37°C, with PBS and an isotype-matched HMAb, RO4, as controls. For the antibody competition assay, the infection medium was first incubated with 100 μg/ml of competing nonneutralizing antibody (e.g., an HMAb to domain A) for 30 min at room temperature followed by the addition of testing neutralizing HMAb (CBH-7 or CBH-5) and together incubated for an another hour before adding to Huh-7 cells. The cell culture plate was centrifuged at 1,000 × *g* for 2 hours at room temperature before placing in a humidified cell culture chamber containing 5% CO_2 at 37°C. After 15 h of incubation, the HCVpp medium was replaced with fresh complete medium and incubated for 72 h. After adding 100 μl of reconstituted Bright-Glo (Promega) to each well followed by 2 min of mixing, luciferase activity was measured by a Veritas microplate luminometer (Turner Biosystems). Virus neutralization activity of an antibody was determined by the percent reduction of luciferase activity compared with that with the infection medium containing PBS.

RESULTS

Identification of new domain A HMAbs. To follow up our previously proposed model of HCV E2 protein with three immunogenic domains containing conformational epitopes with distinct properties and biological functions (11), we reasoned that new antibodies to HCV E2 conformational epitopes

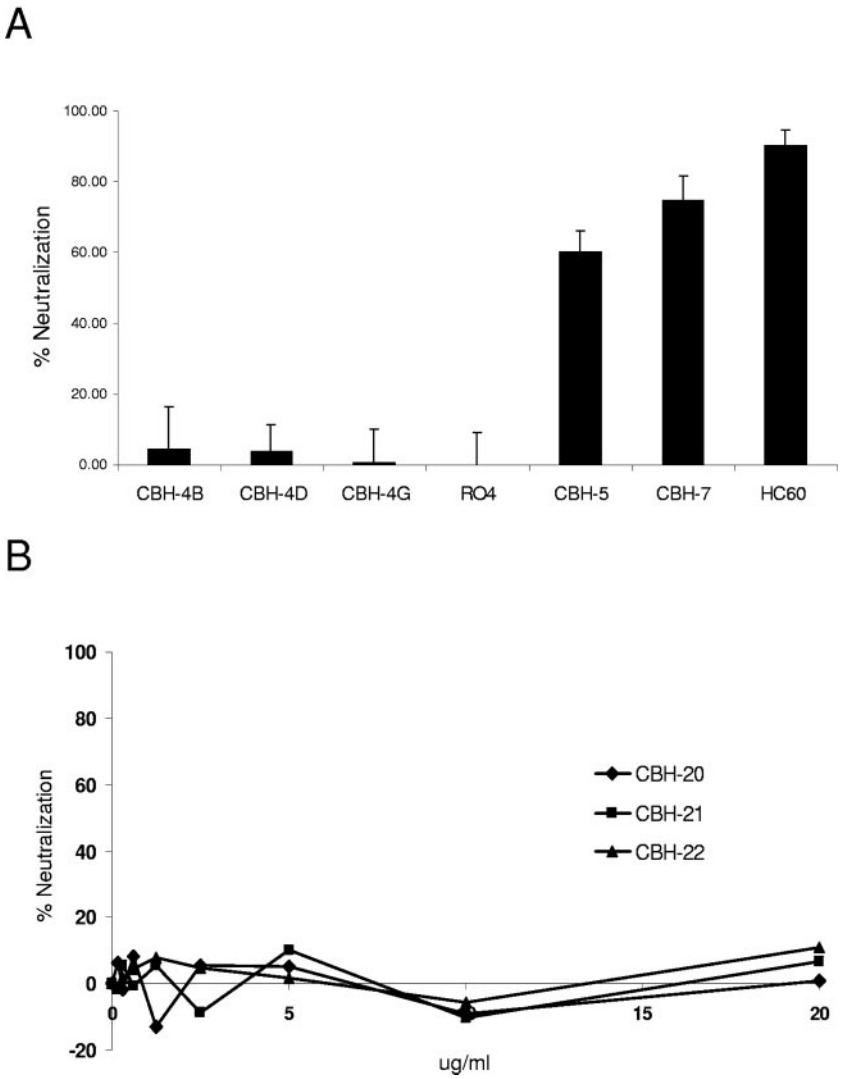
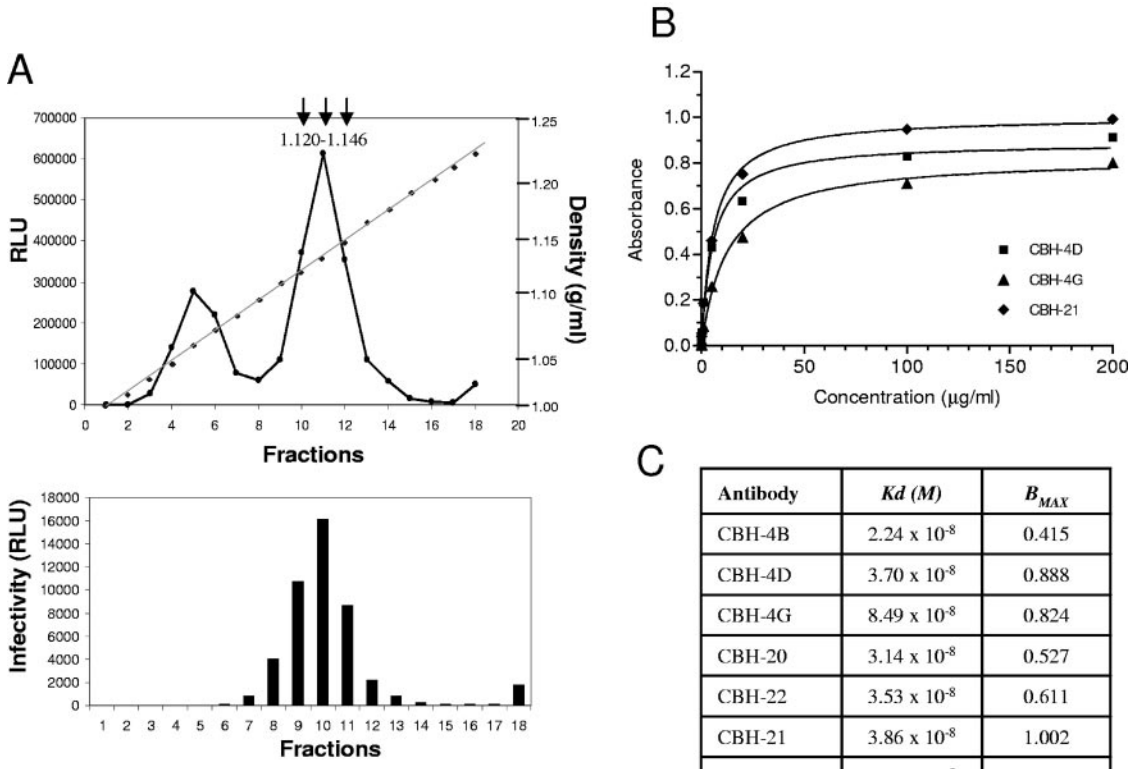


FIG. 2. HMAbs to domain A have no neutralization activities. (A) Neutralization assay with antibodies as indicated at 20 μg/ml. HC60, an HCV-positive human serum, was tested at 1:100 dilution. CBH-5 and -7 represent domain B and C HMAbs, respectively. RO4 is an isotype-matched HMAb to cytomegalovirus. (B) Neutralization assay with antibodies as indicated at various concentrations.

should fall into the three immunogenic domains having similar structural and functional properties. Peripheral blood B cells were isolated from a chronically HCV genotype 1b-infected individual with high-titer serum antibodies to E2 and high neutralization of binding activity. The B cells were activated by Epstein-Barr virus and used to produce human hybridomas as previously described (7, 26). Three hybridomas, labeled CBH-20, CBH-21, and CBH-22, were identified as secreting antibodies that bound to genotype 1a E2 protein by immunofluorescent assay. This screening emphasizes isolation of HMAbs to conserved epitopes, as shown previously (11). Monoclonality of the hybridomas was confirmed by sequencing the IgG genes isolated from 10 individual cell clones derived from each hybridoma. All three cell lines produced IgG1 antibodies with λ light chains and secreted 80 to 120 μg human IgG per ml in spent cultured supernatant. The three antibodies were able to immunoprecipitate E2 proteins but could not detect E2 proteins under reducing conditions by either enzyme-linked immunosorbent assay or Western blot analysis, suggesting that they are to conformational epitopes on the HCV E2 protein (data not shown). Sequence analysis of their Ig genes (V_L and V_H) showed that CBH-20, -21, and -22 were derived from independent B cells expressing a unique combination of heavy and light chain CDR1, -2, and -3 regions compared with the other CBH HMAbs to E2 protein (sequences deposited in GenBank as accession numbers DQ109964, QD109965, QD109966, QD109967, QD109968, and QD109969). Comparison with databank sequences allowed the assignment of CBH-20, -21, and -22 antibody germ line counterparts, respectively, for V_H domains VH3-21, VH3-21, and VH1-69 and for V_L domains VL1-1b, VL2-2e, and VL1-1b. To find out whether the new antibodies fell into one of the previously described immunogenic epitope clusters on E2 protein, competition analysis with selected biotin-labeled domain A (CBH-4D), B (CBH-5), and C (CBH-7) HMAbs was performed (Fig. 1). All three HMAbs showed either minimum or no competition with



C

Antibody	*Kd (M)*	B_{MAX}
CBH-4B	2.24×10^{-8}	0.415
CBH-4D	3.70×10^{-8}	0.888
CBH-4G	8.49×10^{-8}	0.824
CBH-20	3.14×10^{-8}	0.527
CBH-22	3.53×10^{-8}	0.611
CBH-21	3.86×10^{-8}	1.002
CBH-5	3.48×10^{-8}	0.630
CBH-7	3.10×10^{-9}	1.083

FIG. 3. (A) Sucrose density-equilibrium gradient purification of HCVpp. Extracellular supernatant was centrifuged through 20% sucrose. The pellet was then subjected to sucrose density-equilibrium gradient centrifugation in 20 to 60% sucrose. Fractions were collected, with fraction 1 referring to the top of the gradient. The sucrose gradient is shown as a dotted line by weight. HCVpp infectivity was analyzed on Huh-7 cells. Sedimentation densities of sucrose gradient fractions 10, 11, and 12 are indicated by arrows. (B) Representative saturation binding of HMAbs to purified 1a HCVpp. (C) Antibody binding affinity as represented by the disassociation constant, K_d, and maximum binding, B_{max}, of tested HMAbs.

domain B and C antibodies and 97% competition with CBH-4D (Fig. 1A), suggesting the epitopes recognized by these new antibodies are in domain A. The six antibodies to domain A were further cross-competed with each other, and their relatedness is shown as a phylogenetic group (Fig. 1B). In this analysis, the most closely related antibodies among the six domain A HMAbs were placed next to each other as determined by an algorithm as previously described (11), where the cross-competition percentages of two antibodies are averaged and a smaller fraction indicates greater cross-competition. After the two closest-related antibodies (CBH-4B and CBH-22) were placed next to each other, their cross-competition average was then used to identify the next-closest-related antibody (CBH-20) to this pair, until all antibodies were assigned (Fig. 1B).

Effect of new domain A HMAbs on HCVpp infection of Huh-7 cells. Earlier studies showed that genotype 1a HCVpp infection of Huh-7 cells could be blocked by domain B and C HMAbs but not by domain A HMAbs (21). The expectation is that CBH-20, -21, and -22 should have similar characteristics as the three other HMAbs (CBH-4B, -4D, and -4G), since all six HMAbs are clustered in the same domain. As shown in Fig. 2, the three domain A HMAbs (CBH-4B, -4D, and -4G) showed minimum or no virus neutralization activities at 20 μg/ml (Fig. 2A). The three new antibodies (CBH-20, -21, and -22) also showed no effect on 1a HCVpp infection of Huh-7 cells at a concentration of 0.1 to 20 μg/ml of HMAbs (Fig. 2B). All six domain A HMAbs were tested and showed no neutralization at higher concentrations of up to 200 μg/ml (data not shown). In contrast, domain B HMAb (CBH-5) or domain C HMAb (CBH-7) and HC-60 (a polyclonal HCV serum) showed greater than 70% neutralization against HCVpp infection of Huh-7 cells. No neutralization activity was detected with R04, an isotype control antibody (HMAb to a cytomegalovirus-specific protein). The six domain A antibodies showed the same nonneutralizing activity with genotype 1b HCVpp (data not shown). These results are consistent with the model of three segregated conformational immunogenic domains on the E2 protein having distinct functions.

The inability of HMAbs in domain A to neutralize HCVpp to Huh-7 cells could be caused by their epitopes not being on the surface of HCVpp or by inadequate domain A antibody binding to a critical number of sites on the surface of HCVpp. We previously showed that some HMAbs in domain A can immunoprecipitate HCVpp, suggesting that their epitopes are accessible on the surface of HCVpp (11). We measured antibody binding affinity and maximum binding, B_{max}, as a relative indicator of the number of binding sites of domain A epitopes

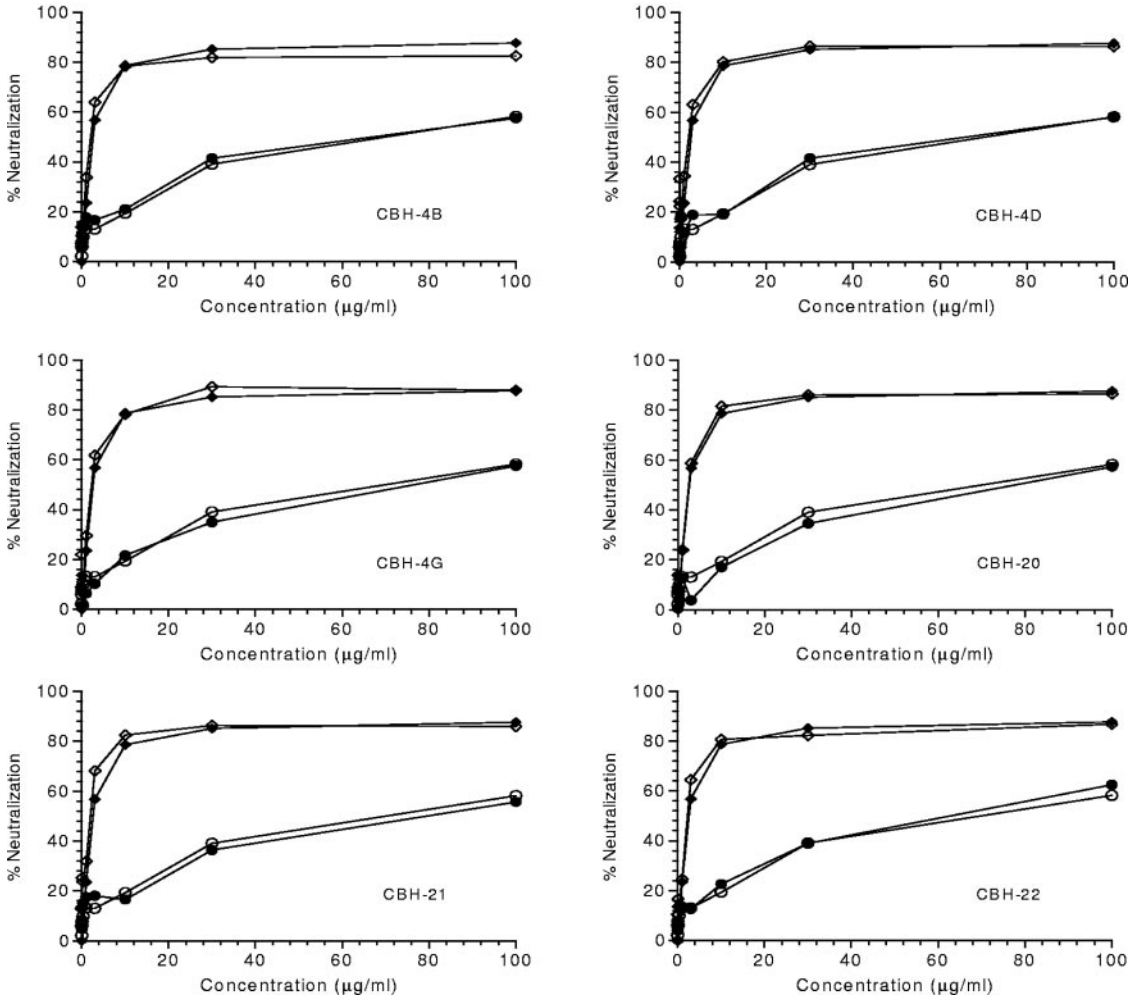


FIG. 4. HMAbs to domain A do not interfere with the function of neutralizing antibodies. Dose-dependent CBH-7 or CBH-5 neutralization of HCVpp on Huh-7 cells is shown for CBH-7 in the presence (◆) and absence (◇) of each HMAb to domain A and for CBH-5 in the presence (●) and absence (○) of each HMAb to domain A.

on the surface of HCVpp compared with epitopes in domains B and C, which have the capacity to mediate virus neutralization. To avoid contamination of unpackaged free E2 protein, HCVpp-containing supernatant was first passed through a sucrose cushion and the HCVpp pellet was further purified in a nonlinear sucrose density-equilibrium gradient from 20 to 60% (Fig. 3 A). As shown in Fig. 3A, peak infectivity of HCVpp sediment was as a distinct band with a density of 1.120 to 1.146 g/ml. Gradient-isolated HCVpp were used immediately for antibody affinity measurements (Fig. 3B). As summarized in Fig. 3C, each domain A antibody bound to genotype 1a HCVpp, confirming the expression of their epitopes on the surface of HCVpp and having moderate binding affinities of K_d at 10^{-8} M. The K_d values of domain A HMAbs are comparable to domain B HMAbs as represented by CBH-5 but lower than the single antibody to domain C, CBH-7. As for the density of HCVpp surface binding sites, CBH-4B, -20, and -22 have lower B_{max} values than CBH-5 and -7, whereas CBH-4D, -4G, and -21 have higher B_{max} values than CBH-5. If the six antibodies to domain A are taken as one group, their average B_{max} value (0.711) lies between the B_{max} for CBH-5 (0.630) and that for CBH-7 (1.083). The similar binding affinity and B_{max} values of domain A antibodies to epitopes on the surface of HCVpp compared to domain B and C antibodies to their respective epitopes suggest that neutralization is not merely caused by binding to any HCVpp surface epitope but is rather epitope specific.

Relationship between domain A and domains B and C. A potential concern in vaccine design is competition between inducing neutralizing and nonneutralizing antibodies. To find out whether nonneutralizing antibodies could interfere with the function of neutralizing antibodies by steric hindrance, the ability of each nonneutralizing domain A HMAb to modulate CBH-7 and CBH-5 (respective domain C and B HMAbs) neutralization of HCVpp infection to Huh-7 cells was studied (Fig. 4). Earlier competition studies showed that domain A is distant to domain B, but some bidirectional competition or enhancement of binding to E2 protein between HMAbs to domain A and domain C suggested that domains A and C are spatially in proximity to each other (11). As shown in Fig. 4, CBH-7 or CBH-5 dose-dependent neutralization of 1a HCVpp was measured in the presence and absence of each domain A HMAb at

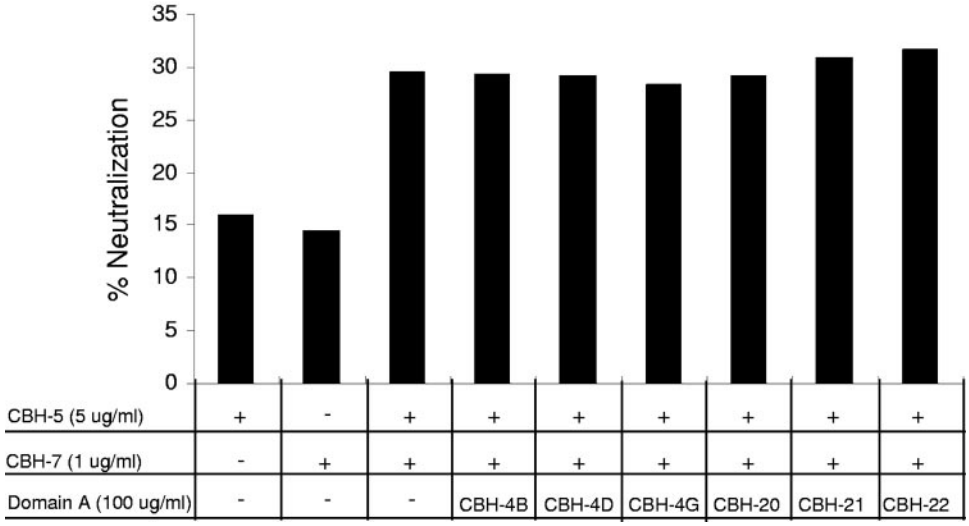


FIG. 5. HMAbs to domain A do not interfere with the functions of combined neutralizing HMAbs to domains B and C. Data shown are in the presence (+) and in the absence (-) of HMAbs, as indicated on the left.

an oversaturating antibody binding concentration of 200 μg/ml. Each nonneutralizing antibody was mixed with HCVpp for 30 min to maximize binding before adding CBH-7 or CBH-5. None of the domain A HMAbs either enhanced or inhibited the neutralizing activity of CBH-7 or CBH-5. The same results were obtained with various concentrations of nonneutralizing antibodies or when neutralizing and nonneutralizing antibodies were added simultaneously to HCVpp (data not shown). Identical experiments were performed with HCVpp genotype 1b to test whether this is a subtype-specific phenomenon. Similar results as with HCVpp 1a were noted (data not shown).

HCV infection induces a polyclonal antibody response in the susceptible host, where antibodies to all three HCV E2 immunogenic domains would be simultaneously present. We further studied whether nonneutralizing antibodies could interfere with the role of combined neutralizing antibodies to domains B and C (Fig. 5). Since CBH-7 is a log higher in antibody binding affinity over CBH-5, we used CBH-5 at 5 μg/ml, CBH-7 at 1 μg/ml, and each nonneutralizing domain A HMAb at 200 μg/ml. The nonneutralizing domain A HMAb was mixed with 1a HCVpp for 30 min to maximize binding before adding the combined CBH-7 and CBH-5. As shown in Fig. 5, CBH-5 and CBH-7 had respective neutralizing activities of 15.9% and 14.4%, and in combination it was 29.4%. The combined CBH-5 and -7 neutralizing activities remained essentially the same at 29.3%, 29.2%, 28.3%, 29.1%, 30.1%, and 30.7% in the presence of each domain A HMAb (CBH-4B, -4D, -4G, -20, -21, and -22, respectively). Collectively, nonneutralizing antibodies appear not to interfere with neutralizing antibodies to spatially related epitopes as with CBH-7, an antibody to domain C, or to more distant epitopes, as with CBH-5, an antibody to domain B. A more detailed analysis of the relationship between domains B and C will be discussed elsewhere (unpublished data).

Effects on domain A epitopes after low-pH treatment of HCVpp. A model of HCV entry involving low pH-induced envelope protein structural changes leading to fusion is supported by the observations that bafilomycin A1, which prevents vesicular acidification, will reduce HCVpp infectivity and that HCVpp infectivity is low-pH sensitive (2, 10). We reasoned that if the model for the three antigenic domains of the HCV E2 protein shared a similar organization as the structural domains of other flaviviruses, domain A might participate in the fusion process. To assess whether domain A undergoes conformational changes in such a pH-induced transition, domain A HMAbs binding to HCVpp under normal conditions and after low-pH treatment were analyzed (Fig. 6). Sucrose density-equilibrium gradient–purified 1a HCVpp, as shown in Fig. 3, was treated with morpholine ethanesulfonic acid and back neutralized. To ensure the integrity of HCVpp after low-pH treatment, the densities of HCVpp with and without low-pH treatment were compared using a sucrose density-equilibrium gradient. Similar sedimentation densities by sucrose gradient banding suggested that both forms of HCVpp remain intact as particles for this study. Antibody binding affinity to HCVpp with and without low-pH treatment was determined by a GNA-capture enzyme-linked immunosorbent assay with analysis using Prism software (GraphPad). Saturation binding profiles of HMAbs to domain A and B (CBH-5) and domain C (CBH-7) are shown in Fig. 6 and summarized in Table 1. The antibody disassociation constant, K_d, showed no significant changes with any HMAb to neutral or low pH-treated HCVpp, suggesting there were no detectable changes in the conformation of domain A epitopes under the treatment. In contrast, maximum binding, B_{max}, to low-pH-treated HCVpp with all antibodies to domain A was significantly increased in a range from 36.5 to 67.5%, with a P value of 0.0008. The average B_{max} increase for domain A was 46% and for domain C (CBH-7) the B_{max} increase was 23%. In contrast, binding of CBH-5 to domain B showed a slight decrease of 12%. A similar low-pH effect of B_{max} increase without a K_d change for domains C HMAbs is consistent with the spatial proximities of domain A and C on the surface of HCVpp. Low-pH-inducing irreversible conformational changes were confirmed by a 75% loss in HCVpp infectivity, consistent with previous observations by other investigators (10). To assure that the B_{max} increase observed with

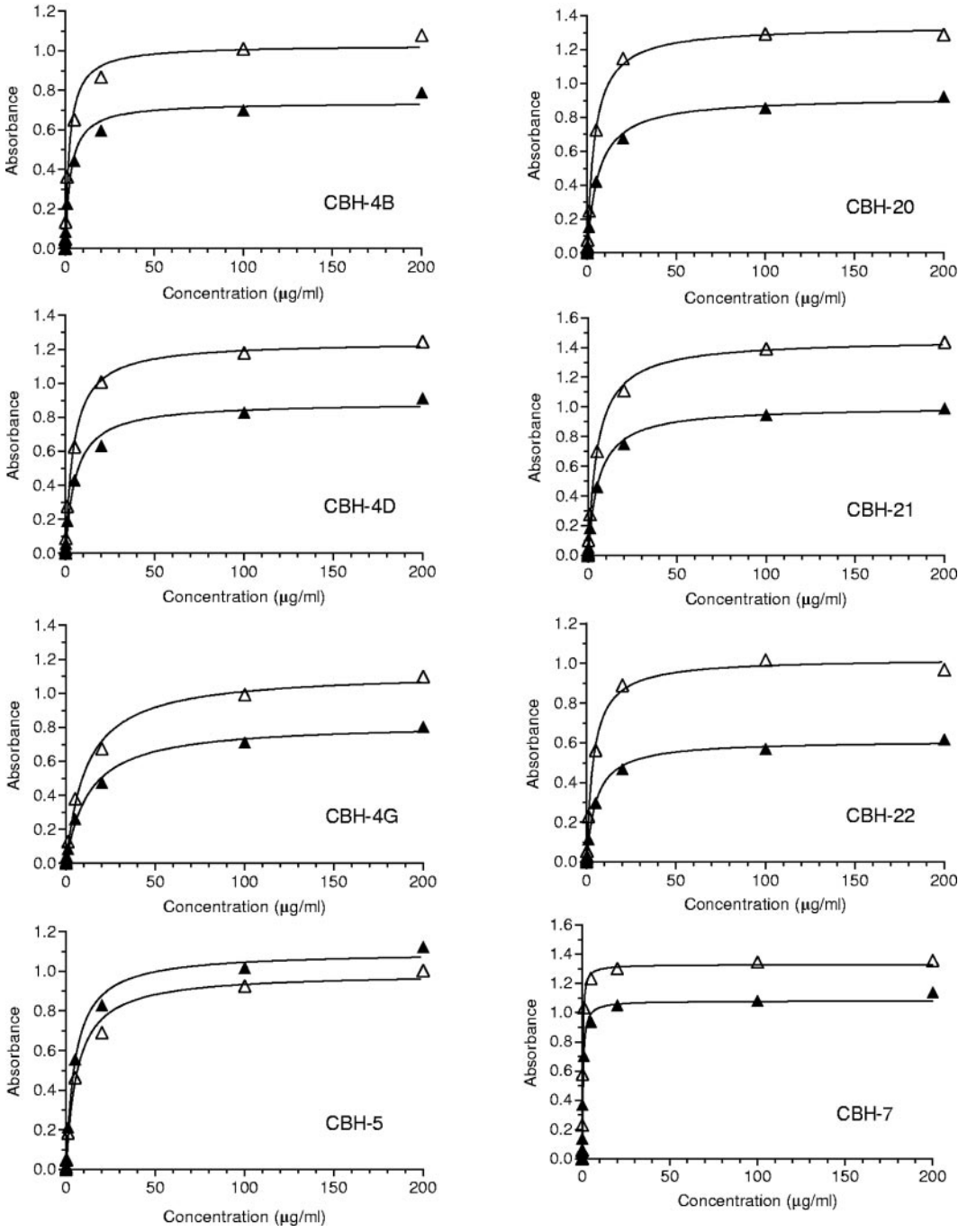


FIG. 6. Saturation binding of HMAbs to domain A on 1a HCV pp, with (△) and without (▲) low-pH treatment. CBH-5 and -7 represent domain B and C HMAbs, respectively, and were tested as controls.

domain A HMAbs was due to a low-pH-induced effect, binding to HCVpp in a pH 7.1 MES–triethanolamine buffer was compared to the pH 7.4 control NTE buffer. No significant difference in maximum binding was observed (data not shown). The significant rise in B_{max} for domain A epitopes without a change in antibody binding kinetics suggests some E2 glycoprotein structural rearrangement after exposure to low pH leading to a substantially higher number of domain A epitopes being more accessible without changing the conformation of domain A epitopes.

DISCUSSION

In this study, we showed that three new HMAbs, CBH-20, -21, and -22, are clustered in domain A by cross-competition studies with previously defined domain A, B, and C antibodies. The new antibodies and three other domain A HMAbs, CBH-

TABLE 1. Effect of acidic pH on HMAbs binding to 1a HCVpp domain A epitopes

Antibody	B_{max}			K_d (M)	
	Normal pH	Acidic pH[a]	% Delta[b]	Normal pH	Acidic pH
CBH-4B	0.415	0.579	39.4	2.24×10^{-8}	1.57×10^{-8}
CBH-4D	0.888	1.246	40.3	3.70×10^{-8}	3.00×10^{-8}
CBH-4G	0.824	1.125	36.5	8.49×10^{-8}	7.59×10^{-8}
CBH-20	0.497	0.732	47.2	3.65×10^{-8}	2.83×10^{-8}
CBH-22	0.611	1.024	67.5	3.53×10^{-8}	2.49×10^{-8}
CBH-21	1.002	1.456	45.3	3.86×10^{-8}	3.53×10^{-8}
CBH-5	0.630	0.552	−12.4	3.48×10^{-8}	4.12×10^{-8}
CBH-7	1.083	1.331	22.9	3.10×10^{-9}	1.80×10^{-9}

[a] Sucrose gradient-purified HCVpp was treated at pH 5.5.
[b] [Acidic pH/normal pH × 100] − 100%.

4B, -4D, and -4G bind to purified HCVpp, confirming the expression of these epitopes on the surface of HCVpp. Each HMAb has an antibody binding affinity of K_d in a range of 10^{-8} M and is not able to block HCVpp infection. Furthermore, all six HMAbs to domain A do not interfere with the virus neutralizing activity by CBH-7, a domain C HMAb whose epitope is in proximity to domain A or with CBH-5, a domain B HMAb to a more distant epitope. A lack of domain A effect was also observed on the combined neutralizing activity by CBH-5 and -7. The similarity in properties among domain A epitopes extends to low-pH-induced structural changes on the E2 glycoprotein. Each domain A HMAb has a significant increase in maximum binding, B_{max}, to low-pH-treated HCVpp compared with nontreated HCVpp. These findings are consistent with a proposed antigenic organization of HCV E2 glycoprotein with three immunogenic domains designated A, B, and C having similar structural and functional properties of the epitopes within each domain (11).

A controversial issue on mechanisms of virus neutralization and that is important for vaccine development is whether nonneutralizing antibodies could modulate the effects of neutralizing antibodies (8, 9). Antibodies to epitopes that are partially overlapping have been reported to be antagonistic, where a nonneutralizing HMAb to HIV-1 inhibited the activity of a neutralizing HMAb by competing for the binding site (9). Other studies showed the opposite or a lack of effect between overlapping nonneutralizing and neutralizing epitopes and questioned whether nonneutralizing antibodies actually bind to functional envelope complexes on the virion surface (8). For HCV, we showed that neutralizing (domains B and C) and nonneutralizing (domain A) HMAbs bind to E2 epitopes on the surface of HCVpp with similar binding affinity and B_{max} values, a relative indicator of epitope density. In addition, antibodies to domain A, which lies in spatial proximity to domain C, when tested at oversaturating concentrations did not interfere with the neutralizing activity of a domain C HMAb or a domain B HMAb to a more distant epitope. These findings are in contrast to an HIV-1 model in which inhibition of virus infection correlates with increasing antibody binding to virion surface sites and is independent of the epitopes recognized by the antibody. Neutralization is then the result of a critical number of binding sites being occupied and virus entry being prevented through steric hindrance (4). A model for HCV is more analogous to other flaviviruses, such as TBEV, where epitopes in an immunogenic domain share similar properties, such as virus neutralization, and where distinct domains are responsible for virus attachment and initiation of viral envelope fusion with the cellular membrane (23). Our studies suggest the potential induction of nonneutralizing antibodies should not be a central issue for HCV vaccine design.

What might be the functional and structural roles of domain A on HCV E2 glycoprotein? For TBEV, the major envelope glycoprotein E is organized in three antigenic domains that correspond to three structural domains, I, II, and III. Virus entry is through endocytosis, and in the low-pH endosomal environment the E protein undergoes an irreversible rearrangement. The low-pH-induced transition from a dimeric to trimeric form mostly involves domains I and II, as antibodies to conformational epitopes in these two regions are generally no longer recognized. This hinge-like motion leads to the exposure of the fusion protein, the "cd loop," that starts fusion with the endosomal membrane as part of the virus entry pathway (23, 25). Our results showed that domain A epitopes appear to be strongly affected after low-pH treatment of HCVpp. The maximum binding for domain A HMAbs rose on average 46%, which was observed to a lesser extent for domain C and not for domain B epitopes (Table 1). Sucrose gradient banding after low-pH treatment showed a similar HCVpp density as under normal pH, suggesting the pseudovirus particles are fundamentally intact. However, it remains possible that some E1E2 disassociation occurs, as suggested by a reduced amount of E1 coprecipitation after low-pH treatment (21). These observations show that E1E2 might reorganize under low pH with structural changes within E2 and/or between E1 and E2 glycoproteins (21). In addition, the change in antibody binding pattern to domain A from normal pH to acidified HCVpp suggests a region that is highly flexible with greater exposure or hinge-like as part of the low-pH-induced structural rearrangement. It is possible that domain A provides some structural protection to a fusion peptide region under normal pH. Whether domain A is part of the hinge region directly linked to a fusion peptide remains to be determined.

Although there are similarities in the immunogenic organization of HCV E2 glycoprotein with other flaviviruses, differences do exist, as suggested by the antibody binding profile to normal and low-pH-treated forms of envelope glycoproteins. A significant part of conformational epitopes in domains I and II are no longer recognized in TBEV under low pH, whereas an increase in the availability of epitopes in domain A and C of HCV E2 protein is observed, suggesting a different structural

rearrangement occurring in HCV during the fusion process. It remains possible that antibodies to pH-sensitive epitopes on HCV E2 protein will be identified, which would be more consistent with the conformational changes seen in DI and DII of TBEV. But unlike the TBEV E protein, HCV glycoprotein contains a heterodimer of E1 and E2 glycoproteins in its native form. Perhaps a second model for HCV is Semliki Forest virus (13), an alphavirus that has E1 and E2 glycoproteins as a heterodimer in its native form. Both flaviviruses and alphaviruses have class II pH-dependent fusion proteins and these viruses share a similar overall folding organization and probably descended from a common ancestor (23). Our study on the effect of low pH on the conformational structure of domain A shows that HCV E2 protein changes structurally when exposed to a low-pH environment, which hypothetically could lead to the exposure of a previously buried hydrophobic fusion peptide. We believe the results of domain A showing a changed binding pattern at low pH are the first direct evidence to support that the HCV E2 protein, like the TBEV E protein and Semliki Forest virus E1 protein, undergoes a structural rearrangement as part of an endocytosis entry pathway. A further understanding and detailed mapping for this highly flexible or hinge-like region will provide insight on the HCV fusion process as an important step of infection.

ACKNOWLEDGMENTS

We thank H. Y. Chen for technical assistance.

This work was supported in part by NIH grants HL079381 and AI47355 to S.K.H.F.

REFERENCES

1. **Agnello, V., G. Abel, M. Elfahal, G. V. Knight, and Q. X. Zhang.** 1999. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA **96:**12766–12771.
2. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cossett.** 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.
3. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C pseudoparticles containing functional E1E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
4. **Burton, D. R., E. Q. Saphire, and P. W. H. I. Parren.** 2001. A model for neutralization of viruses based on antibody coating of the virion surface. Curr. Top. Microbiol. Immunol. **260:**109–144.
5. **Choo, Q.-L., G. Kuo, R. Ralston, A. Weiner, D. Chien, G. Van Nest, J. Han, K. Berger, K. Thudium, C. Kuo, J. Kansopon, J. McFarland, A. Tabrizi, K. Ching, B. Moss, L. B. Cummins, M. Houghton, and E. Muchmore.** 1994. Vaccination of chimpanzees against infection by the hepatitis C virus. Proc. Natl. Acad. Sci. USA **91:**1294–1298.
6. **Gardner, J. P., R. J. Durso, R. R. Arrigale, G. P. Donovan, P. J. Maddon, T. Dragic, and W. C. Olson.** 2003. L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **100:**4498–4503.
7. **Hadlock, K. G., R. E. Lanford, S. Perkins, J. Rowe, Q. Yang, S. Levy, P. Pileri, S. Abrignani, and S. K. H. Foung.** 2000. Human monoclonal antibodies that inhibit the binding of hepatitis C virus E2 protein to CD81 and recognizing conserved conformational epitopes. J. Virol. **74:**10407–10416.
8. **Herrera, C., C. Spenlehauer, M. S. Fung, D. R. Burton, S. Beddows, and J. P. Moore.** 2003. Nonneutralizing antibodies to the CD4-binding site on the gp120 subunit of human immunodeficiency virus type 1 do not interfere with the activity of a neutralizing antibody against the same site. J. Virol. **77:**1084–1091.
9. **Hofmann-Lehmann, R., J. Vlasak, R. A. Rasmussen, B. A. Smith, T. W. Baba, V. Liska, F. Ferrantelli, D. C. Montefiori, H. M. McClure, D. C. Anderson, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, H. Katinger, G. Stiegler, L. A. Cavacini, M. R. Posner, T.-C. Chou, J. Andersen, and R. M. Ruprecht.** 2001. Postnatal passive immunization of neonatal macaques with a triple combination of human monoclonal antibodies against oral simian-human immunodeficiency virus challenge. J. Virol. **75:**7470–7480.
10. **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.
11. **Keck, Z. Y., A. Op De Beeck, K. G. Hadlock, J. Xia, T.-K. Li, J. Dubuisson, and S. K. H. Foung.** 2004. Hepatitis C virus E2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. J. Virol. **78:**9224–9232.
12. **Keck, Z. Y., V. M. H. Sung, P. Perkins, J. Rowe, P. Sudhir, T. J. Liang, M. M. C. Lai, and S. K. H. Foung.** 2004. Human monoclonal antibody to hepatitis C virus E1 glycoprotein that blocks virus attachment and viral infectivity. J Virol. **78:**7257–7263.
13. **Lescar, J., A. Roussel, M. W. Wien, J. Navaza, S. D. Fuller, G. Wengler, and F. A. Rey.** 2001. The fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH. Cell **105:**137–148.
14. **Levy, S., S. C. Todd, and H. T. Maecker.** 1998. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. Annu. Rev. Immunol. **16:**89–109.
15. **Lindenbach, B. D., and C. M. Rice.** 2001. *Flaviviridae*: the viruses and their replication, p. 991–1042. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
16. **Lozach, P. Y., H. Lortat-Jacob, A. De Lacroix De Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J. L. Virelizier, F. Arenzana-Seisdedos, and R. Altmeyer.** 2003. DC-SIGN and L-SIGN are high-affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. **278:**20358–20366.
17. **Major, M. E., B. Rebermann, and S. M. Feinstone.** 2001. Hepatitis C viruses, p. 1127–1162. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Field virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
18. **McLauchlan, J., M. K. Lemberg, R. G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. **21:**3980–3988.
19. **McMinn, P. C.** 1997. The molecular basis of virulence of the encephalitogenic flaviviruses. J. Gen. Virol. **78:**2711–2722.
20. **Monazahian, M., I. Bohme, S. Bonk, A. Koch, C. Scholz, S. Grethe, and R. Thomssen.** 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J. Med. Virol. **57:**223–229.
21. **Op De Beeck, A., C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Y. Keck, S. Foung, F. L. Cosset, and J. Dubuisson.** 2004. Characterization of functional hepatitis C virus envelope glycoproteins. J. Virol. **78:**2994–3002.
22. **Pohlmann, S., J. Zhang, F. Baribaud, Z. Chen, G. J. Leslie, G. Lin, A. Granelli Piperno, R. W. Doms, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. **77:**4070–4080.
23. **Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. G. Harrison.** 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature (London). **375:**291–298.
24. **Robertson, B., G. Myers, C. Howard, T. Brettin, J. Bukh, B. Gaschen, T. Gojobori, G. Maertens, M. Mizokami, O. Nainan, S. Netesov, K. Nishioka, T. Shini, P. Simmonds, D. Smith, L. Stuyver, A. Weiner, et al.** 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. Arch. Virol. **143:**2493–2503.
25. **Roehrig, J. T., A. R. Hunt, A. J. Johnson, and R. A. Hawkes.** 1989. Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray valley encephalitis virus elicit antiviral antibody. Virology **171:**49–60.
26. **Rosa, D., S. Campagnoli, C. Moretto, E. Guenzi, L. Cousens, M. Chin, C. Dong, A. J. Weiner, J. Y. N. Lau, Q.-L. Choo, D. Chien, P. Pileri, M. Houghton, and S. Abrignani.** 1996. A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. Proc. Natl. Acad. Sci. USA **93:**1759–1763.
27. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21:**5017–5025.
28. **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.