Hepatitis C Virus E2 Has Three Immunogenic Domains Containing Conformational Epitopes with Distinct Properties and Biological Functions

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Mechanisms of virion attachment, interaction with its receptor, and cell entry are poorly understood for hepatitis C virus (HCV) because of a lack of an efficient and reliable in vitro system for virus propagation. Infectious HCV retroviral pseudotype particles (HCVpp) were recently shown to express native E1E2 glycoproteins, as defined in part by HCV human monoclonal antibodies (HMAbs) to conformational epitopes on E2, and some of these antibodies block HCVpp infection (A. Op De Beeck, C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Y. Keck, S. Fong, F. L. Cosset, and J. Dubuisson, *J. Virol.* 78:2994–3002, 2004). Why some HMAbs are neutralizing and others are nonneutralizing is looked at in this report by a series of studies to determine the expression of their epitopes on E2 associated with HCVpp and the role of antibody binding affinity. Antibody cross-competition defined three E2 immunogenic domains with neutralizing HMAbs restricted to two domains that were also able to block E2 interaction with CD81, a putative receptor for HCV. HCVpp immunoprecipitation showed that neutralizing and nonneutralizing domains are expressed on E2 associated with HCVpp, and affinity studies found moderate-to-high-affinity antibodies in all domains. These findings support the perspective that HCV-specific epitopes are responsible for functional steps in virus infection, with specific antibodies blocking distinct steps of virus attachment and entry, rather than the perspective that virus neutralization correlates with increased antibody binding to any virion surface site, independent of the epitope recognized by the antibody. Segregation of virus neutralization and sensitivity to low pH to specific regions supports a model of HCV E2 immunogenic domains similar to the antigenic structural and functional domains of other flavivirus envelope E glycoproteins.

Hepatitis C virus (HCV) infects over 170 million individuals worldwide. Although acute infection is usually silent, most HCV infections progress to chronicity that is not cleared by an apparently robust immune response (3, 24). The virus is a member of the family *Flaviviridae* (37), with a 9.5-kb positive-strand RNA genome that encodes three structural proteins, the capsid and viral envelope proteins E1 and E2, and at least six nonstructural proteins, NS2 to NS5b (29). The envelope proteins are thought to be the primary mediators of virion attachment and cell entry (13). HCV E2 is a ~70-kDa glycoprotein that shows large variations among HCV genotypes and contains a 27-amino-acid (aa) sequence at its amino terminus that is highly variable and is designated the hypervariable region 1, or HVR1 (reviewed in references 3 and 6). This linear region on E2 is likely to be involved in virus infection, since neutralizing antisera to HVR1 have been reported in in vitro and in vivo models, although other studies showed that HCV with HVR1 deleted remains infectious (15, 19, 34, 41, 47). Unfortunately, a leading contributor to disease progression is the emergence of new viral mutants or “quasispecies” in HVR1 induced by immune selection. Increased diversity or mutations in HVR1 correlate with progressive disease, and

decreased diversity correlates with resolving disease (14). HCV E2 is thought to mediate attachment to target cells and binds to human CD81, a member of the tetraspannin family of proteins (28). Interaction of E2 with CD81 on B or T cells has been reported to result in B-cell aggregation and a lowering of the threshold for T- and B-cell activation (17, 43). Other alternative receptors that have been proposed include the low-density lipoprotein receptor (1, 44), two receptors on HepG2 cells, the scavenger receptor type B class I (5, 40), and two closely related membrane-associated C-type mannose-binding lectins, DC-SIGN and L-SIGN (20, 30, 33).

Mechanisms of virion attachment, entry, and virus replication have been difficult to study because of difficulties in having an efficient and reliable in vitro system for virus propagation. The development of infectious HCV retroviral pseudotype particles expressing E1E2 (HCVpp) has permitted a more detailed characterization of functional envelope glycoproteins involved in virion attachment and entry (4, 25). HCVpp preferentially infect human hepatocytes and hepatocellular cell lines and express noncovalent E1E2 heterodimers as defined in part by HCV human monoclonal antibodies (HMAbs) to conformational epitopes on E2 (32). Production of HMAbs provides information on the immune response to native E1 and E2 proteins, as they are recognized during natural infection and should be useful in determining the function and structure of specific immunogenic domains of E1 or E2. HMAbs and re-

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combinant antibodies to E2 have been isolated to conformational epitopes that are conserved between subtypes 1a and 1b (2, 7, 9, 22) and genotypes 1 and 2 (23). These HMAbs include antibodies that are effective and ineffective in inhibiting the binding of E2 to CD81 and HCVpp entry into target cells (2, 7, 22, 23, 32). Our investigators developed a panel of HMAbs to HCV E2, of which the majority were to conformational epitopes (23). Each of the HCV HMAbs was secreted from a human hybridoma expressing a unique immunoglobulin G1 (IgG1) gene that had undergone affinity maturation in vivo (10). Some of the epitopes recognized by the HMAbs were broadly conserved across different HCV genotypes and were able to inhibit the binding of E2 to human CD81, but only three blocked HCVpp entry into Huh-7 cells (32). Why only a subset of the HCV HMAbs to E2 was able to block HCVpp entry remains unclear. One explanation is the availability of the different antibody binding epitopes on the surface of HCVpp. A second possibility is the binding affinity of the antibodies, and a third possibility is that virus entry is mediated by only specific epitopes on the virus surface. For some viruses, the prevailing view is that inhibition of virus entry or virus neutralization correlates with increased antibody binding to any virion surface site, independent of the epitope 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 (8). Higher-affinity antibodies will have higher neutralizing activities. Nonneutralizing antibodies either do not bind to the virion surface or are poor binders with low affinity. In contrast, the role of specific epitopes responsible for functional steps in virus entry has been documented for other viruses, with specific antibodies blocking distinct steps of virus attachment, interaction with receptor and coreceptor, and initiation of viral envelope fusion with the cellular membrane (26).

In this report, antibody competition studies showed three immunogenic domains on HCV E2 that contained conserved conformational epitopes. The lack of HVR1 involvement with these domains was determined by binding studies to HVR1 deletion mutants. Expression of these epitopes on native proteins was analyzed by the ability of HCV HMAbs to immunoprecipitate HCVpp. Affinity studies of HCV HMAbs were performed to correlate antibody binding affinity and blocking of HCVpp entry to target cells. Also, a collective analysis of previous and present findings suggests that the three immunogenic domains are associated with distinct properties similar to the antigenic structure and function of other flavivirus envelope E glycoproteins.

MATERIALS AND METHODS

Cell culture and viruses. HeLa and HEK293T cells were from the American Type Culture Collection (Manassas, Va.). Cells were grown in minimal essential medium (MEM; Invitrogen, Carlsbad, Calif.) and Dulbecco's MEM (Invitrogen), respectively, supplemented with 10% fetal calf serum (Gemini Bioproducts Inc., Calabasas, Calif.). A cell line constitutively expressing sf1b-E2 on the cell surface was derived by transfecting Chinese hamster ovary (CHO) cells with plasmid expressing sf1b-E2. After selection with Geneticin (Invitrogen), CHO cells expressing E2 were identified via fixed-cell immunofluorescence using CBH-5 and hemagglutinin (HA) MAbs, essentially as described elsewhere (23). Cells expressing high levels of E2 were subjected to single-cell cloning. Recombinant vaccinia virus expressing HCV envelope proteins was constructed and grown as described previously (23). Vaccinia virus 1488 expressing the structural

proteins of HCV 1a strain H (21) was generously provided by Charles Rice (Rockefeller University).

MAbs. The production, purification, and biotinylation of the HCV HMAbs were performed as described previously (23). Rat MAb 3/11 to HCV E2 was cultured as described elsewhere (17) and generously provided by Jane McKeating (Rockefeller University). Rat MAb to the influenza virus HA epitope was from Roche Applied Sciences (Indianapolis, Ind.). Murine MAb to the *c-myc* epitope was from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Competition assays. Monolayers of HeLa cells were grown to 80% confluence and infected at 5 PFU/cell with both wild-type virus and recombinant vaccinia virus or wild-type virus only. Cells were harvested after 1 day of infection. Extracts were prepared by washing the cells with phosphate-buffered saline (PBS) and then resuspending $\sim 25 \times 10^6$ cells in 1 ml of lysis buffer. Extracts prepared in this manner contained approximately 25 μ g of E2 protein/ml. Nuclei were pelleted by centrifugation at $18,000 \times g$ at 4°C for 10 min, and resulting cytoplasmic extracts were stored at 4°C and used for enzyme-linked immunosorbent assays within 24 h of preparation. Microtiter plates were prepared by coating wells with 500 ng of purified *Galanthus nivalis* lectin (GNA; Sigma, St. Louis, Mo.) in 100 μ l of PBS for 1 h at 37°C. Wells were washed with Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris-HCl; pH 7.5) and then blocked with BLOTTO (TBS, 0.1% Tween 20, 2.5% normal goat serum, and 2.5% nonfat dry milk) by incubation for 1 h at room temperature (RT). Plates were washed twice with TBS, followed by the addition to each well of 15 μ l of cytoplasmic extract containing E2 diluted with 85 μ l of BLOTTO. After 1.5 h at RT, plates were washed three times with TBS followed by the addition to each well of 50 μ l of BLOTTO containing competing antibodies at various concentrations. After 30 min, 50 μ l of a 2- μ g/ml solution of the biotinylated test antibody was added. After incubation for 1.5 h at RT, the plates were washed three times with TBS, and 100 μ l of 1/1,000-diluted alkaline phosphatase-conjugated streptavidin (Amersham-Pharmacia Biotech, Piscataway, N.J.) was added. After 1 h at RT, the plates were washed four times with TBS followed by a 30-min incubation with a 1-mg/ml solution of *p*-nitrophenyl phosphate. Absorbance was measured at 405 nm with a multiwell plate reader (BioTek Instruments, Winooski, Vt.). Each test HMAb at 2 μ g/ml with competing HMAb ranging from 0.2 to 50 μ g/ml was tested in duplicate in at least two different experiments. To develop a cross-competition matrix for percentage of test antibody bound to E2, the mean signal with biotinylated test antibody to E2 with competing antibody at 20 μ g/ml was divided by the signal in the absence of the competing HMAb, followed by multiplying by 100.

Phylogenetic grouping of HCV HMAbs. Without knowing the exact conformational epitopes targeted by these antibodies, we attempted to determine their spatial relationship based on competition study results. When two antibodies cross-competed, the extent of bidirectional inhibition was interpreted as the extent of epitope overlap by the competing antibodies. For unidirectional inhibition or enhancement, effects were interpreted as proximal, but not overlapping epitopes (31). Using the principles of UPGMA (unweighted pair-group method using arithmetic averages) to perform a sequential cluster analysis, spatial relationships were developed as a phylogenetic tree to correlate the relatedness of the epitopes as identified by this panel of antibodies (16, 42). In this analysis, antibodies with the highest bidirectional inhibition were placed next to each other (see Fig. 1C). The paired antibodies were averaged and used to compare other antibodies according to the degree of their cross-competition with the paired antibodies. The (third) identified antibody with the strongest bidirectional inhibition was placed next to the first pair of antibodies, and a new average was obtained between the third antibody and the average of the first pair. The new average was then used for another cycle of comparison with the other antibodies in this panel until the matrix was completely reduced.

Isolation and cloning of HCV E2 HVR1 deletion constructs. The vaccinia virus recombinant Q1b (GenBank accession no. AF348705) (23), sf1b-E2, and the HCV 1b deletion constructs were derived from the same HCV genotype 1b-positive serum. DNA encoding the E2 protein was prepared by reverse transcription-PCR using *Pfu* *Taq* polymerase (Stratagene, La Jolla, Calif.) with HCV-specific oligonucleotide primers (forward_{1b}, 5'-AGATCTACCACCTACACGACGGGGGGGGC-3'; forward_{1b411}, 5'-AGATCTATCCAGCTCATAAACACCAACGGC-3'; reverse_{1b}, 5'-CTGCAGCTCTGATCTGTCCCTATCCTCC AAG-3'). HCV 1a constructs were amplified by PCR from viral stocks of vaccinia virus construct vv1488 (21) with the HCV-specific oligonucleotide primers forward_{1a} (5'-AGATCTGAAACCCACGTACCGGGGG-3'), forward_{1a411} (5'-AGATCTATCCAACCTGATCAACACCAAC-3'), and reverse_{1a} (5'-CTGCAGCTCGACCTGTCCCTGTCTTC-3'). Flanking *Bgl*II or *Pst*I restriction sites in the primer sequences are underlined. Amplified DNA fragments were ligated into the pDisplay vector (Invitrogen) in frame with HA and *c-myc* as tags,

FIG. 1. Competition analysis of HCV HMAs. (A) Binding of biotinylated test antibody (as indicated on top of each panel) to HCV Q1b E2 protein captured onto GNA lectin-coated microtiter plates was competed by various concentrations of competing antibodies, as described in Materials and Methods. The bound biotinylated test antibody was detected by using alkaline phosphatase-conjugated streptavidin and *p*-nitrophenyl phosphate as the substrate. The y axis value (mean $\pm$ standard deviation; *n* = 2) is the absorbance reading obtained in the presence of competing antibody as a percentage of the reading in the absence of competing antibody. Competing antibodies were CBH-2 ($\blacktriangle$), CBH-5 ($\blacksquare$), CBH-8C ($\blacklozenge$), CBH-8E ($\blacktriangledown$), CBH-11 ($\bullet$), CBH-7 ($\square$), CBH-4G (∇), CBH-4B ($\triangle$), and R04 (*). R04 is an isotype-matched control antibody to a cytomegalovirus protein. (B) Cross-competition matrix. (C) Phylogenetic grouping of HCV HMAs based on the competitive binding assay. Solid lines with numbers indicate the relatedness of the two adjoining antibodies. Circles are clusters of antibodies in a specific domain. Competition results used for calculation are the mean values obtained from two to five separate experiments.

which were used for purification and detection of expressed proteins. In-frame HCV inserts and deletion sites were confirmed by DNA sequencing (PE-Applied Biosystems, Foster City, Calif.).

Analyses of HCV E2 deletion constructs. HEK293T cells were seeded to obtain 60 to 70% confluence by the following day. For transfection of a T-75 flask, a mixture of 40 µg of the plasmid DNA and 240 µg of PerFect Lipid Pfx-2 (Invitrogen) were combined in 1 ml of serum-free Dulbecco's MEM. After 4 h of incubation at 37°C, the transfection solution was replaced with 20 ml of complete medium and cells were grown for 24 h. Cell extracts were prepared by washing cells with PBS and resuspending them in 1 ml of lysis buffer. Nuclei were pelleted by centrifugation at 18,000 × g at 4°C for 10 min. For microtiter plate assays, the plates were prepared by coating wells with 500 ng of purified GNA lectin in 100 µl of PBS for 1 h at 37°C. Wells were washed with TBS and then blocked with 150 µl of BLOTTO by incubation for 1 h at RT. Wells were washed twice with TBS, followed by the addition of 25 µl of extract from HEK293 cells transfected with E2 deletion constructs diluted in 75 µl of BLOTTO. After 1.5 h at RT, plates were washed three times with TBS followed by the addition of 100 µl of BLOTTO containing various MABs at 10 µg/ml. Plates were incubated for 1.5 h and washed three times with TBS, and then 100 µl of alkaline phosphatase-conjugated secondary antibody, diluted in BLOTTO as recommended by the manufacturer, was added (for anti-human and anti-mouse antibodies [Promega, Madison, Wis.] and for anti-rat antibody [Kirkegard & Perry, South San Francisco, Calif.]). Bound secondary antibody was detected and quantified as described above.

For Western blot analysis, 293T cells were transfected with either full-length E2 or constructs with HVR1 deleted overnight using a calcium phosphate transfection kit (Clontech, Palo Alto, Calif.). After washing once with PBS, the cells were lysed in lysis buffer. Ten micrograms of the proteins was denatured in Laemmli sodium dodecyl sulfate (SDS) sample buffer and loaded onto SDS-polyacrylamide gels. Proteins were separated by electrophoresis and transferred to nitrocellulose membranes. Blots were blocked for 1 h in 5% (wt/vol) nonfat dry milk dissolved in TBS–0.1% Tween 20 (TBST). Blots were then probed with rat anti-HA antibody overnight at 4°C in blocking buffer. After washing with TBST three times, the blots were probed with a secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG from Santa Cruz Biotech) for 1 h at RT. Blots were washed three times in TBST and then developed with enhanced chemiluminescence. Western blot images were captured using ChemiDoc imager system (Bio-Rad, Richmond, Calif.).

Immunoprecipitation. Production of HCVpp was carried out as described previously (4). Briefly, 293T cells were transfected with expression vectors encoding the viral components, i.e., E1E2 glycoproteins, retroviral core proteins, and packaging-competent green fluorescent protein-containing retroviral transfer vectors, by using a calcium-phosphate transfection protocol. 293T cells were metabolically labeled from 16 to 40 h posttransfection with 50 µCi of ³⁵S-labeled protein labeling mix (Amersham Biosciences)/ml. Metabolically labeled 293T cells and supernatant containing HCVpp were lysed with 0.5% Igepal CA-630 in TBS (50 mM Tris-HCl [pH 7.5], 150 mM NaCl). Approximately 10⁵ infectious pseudotype particles were used per immunoprecipitation reaction mixture. Immunoprecipitations were carried out as described elsewhere (12, 13). Briefly, 7 µg of MAB was incubated with protein A-Sepharose (Sigma) for 1 h at 4°C in TBS containing 0.2% Igepal CA-630. Beads were then incubated with the antigen for 1 h at 4°C. Between each step, beads were washed once with TBS-Igepal. After the last step, they were washed three times with this buffer and once with distilled water. The precipitates were then heated at 70°C for 5 min in SDS-polyacrylamide gel electrophoresis sample buffer and run on a polyacrylamide gel. After electrophoresis, gels were treated with sodium salicylate, dried, and exposed at –80°C to an autoradiograph (Amersham).

Antibody affinity measurements. A range of 0.001 to 100 µg of each HCV HMAb/ml was incubated with either genotype 1b E2 constitutively expressed in CHO cells or transiently expressed 1a E2 in 293T cells for 45 min and washed twice, followed by incubation with fluorescein isothiocyanate-labeled goat anti-human IgG (4 µg/ml; Jackson ImmunoResearch, West Grove, Pa.) for 45 min on ice. Cells were then washed in PBS containing 1% fetal calf serum at 4°C and resuspended in fixative solution. Fluorescence of HMAb-bound cells was analyzed by flow cytometry using a FACSCalibur (Becton-Dickinson, San Jose, Calif.), and the mean fluorescence intensity (MFI) values of cell populations were obtained. The MFI value of nonspecific fluorescence was measured by using an isotype-matched control HMAb (R04), the fluorescence of which was subtracted from the MFI values of the specific HMABs. The MFI values of the cell populations incubated with different amounts of antibodies were analyzed using Prism software, and the saturation binding curves were fit by nonlinear regression.

TABLE 1. Selected characteristics of HCV HMABs

HMAb ^a	Genotypes ^b	IgG1 isotype	E2 binding to CD81 ^c
CBH-2	1a 1b 2a 2b	κ	+
CBH-4B	1a 1b	κ	–
CBH-4D	1a 1b	λ	–
CBH-4G	1a 1b 2a 2b	κ	–
CBH-5	1a 1b 2a 2b	κ	+
CBH-7	1a 1b 2a 2b	κ	+
CBH-8C	1a 1b 2a 2b	κ	+
CBH-8E	1a 1b 2a 2b	κ	+
CBH-11	1b 2a 2b	κ	+
CBH-17	1a 1b	λ	+

^a Designation of HMABs generated previously.

^b Reactivity with separate isolates of the indicated genotypes.

^c Ability of antibody to block HCV E2 binding to CD81-LEL. +, inhibitory; –, not inhibitory.

RESULTS

Competition analyses of HCV HMABs. Competition studies were performed to determine the spatial proximity of each conformational epitope to other epitopes on HCV E2 as defined by a panel of HCV HMABs (Table 1) (23). These antibodies were derived from peripheral B cells of an individual who had an asymptomatic HCV genotype 1b infection. Sequence analysis of the IgG1 genes of the HMABs confirmed that they were derived from independent B cells (10, 23). As described previously, the antibodies varied in the breadth of their reactivities with different genotypes of HCV E2, with some broadly reactive with genotypes 1a, 1b, 2a, and 2b and in their ability to inhibit the interaction of HCV E2 with human CD81 (Table 1). Genotype-specific plasmids used to produce HCV E2 in the vaccinia virus expression system in this analysis were derived from sera of other patients infected with different HCV genotypes. Complete inhibition of antibody reactivity to denatured E2 protein confirmed that all HMABs were to conformational epitopes except for CBH-17, which retained reactivity. Competition studies were performed with genotype 1b E2, since the original B cells were from an individual infected with HCV 1b. Each HMAb was purified and biotinylated, and the binding of the antibodies in increasing concentrations of competing antibody was determined. Representative binding curves are presented in Fig. 1A. The cross-competition matrix (Fig. 1B) shows the mean signals of biotinylated test HMAb with competing HMAb divided by signals without competing HMAb from multiple experiments. The antibodies are approximately ordered on both axes to reflect bidirectional inhibition along the diagonal axis of the matrix. Strong inhibition is indicated by values of ≤50%, and enhancement is indicated by values of ≥125%. HMABs CBH-2, -5, -8C, -8E, and -11 (as shown for CBH-2 and -5 in Fig. 1A) form one cluster, where each antibody showed strong bidirectional inhibition with all other antibodies in the cluster (Fig. 1B). No significant inhibition was observed with a control HMAb, R04, or CBH-4B -4D, -4G, and -7. HMABs CBH-4B, -4D, and -4G form another cluster with strong bidirectional inhibition to each other (as shown for CBH-4B in Fig. 1A). CBH-7 is to itself and is not significantly inhibited by CBH-2, -5, -8C, -8E, -11, or the control antibody. The relationship between CBH-7 and the CBH-4B, -4D, and -4G cluster is unusual, with either strongly inhib-

itory (CBH-4B) or enhancing (CBH-4G) effects on binding. While uneven bidirectional inhibition was observed within each cluster, a predominately unidirectional effect was observed between CBH-7 and the CBH-4B, -4D, and -4G cluster (Fig. 1B).

Many techniques have been developed for determining phylogenetic relationships in comparative biology. The UPGMA algorithm is a straightforward approach for cluster analysis, weighting each data point in the cluster equally (16, 42). The competition data were analyzed and spatially placed by pairing antibodies with the highest bidirectional inhibition as most related (shown next to each other in Fig. 1C). For example, the binding of CBH-5 to E2 was reduced to 25% by CBH-11, and the binding of CBH-11 was reduced to 9% by CBH-5. The average of these two values is 17%, or 0.17 (as shown above the line in the figure). A relationship tree was generated with the closest two antibodies (e.g., CBH-5 and -11) 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 tree. This approach identified three distinct immunogenic domains. HMABs CBH-4G, -4B and -4D constitute domain A. A second domain, B, includes CBH-2, -5, -8C, -8E, and -11; CBH-7 is in a separate domain, C. Within each domain, strong bidirectional inhibition was observed, with values generally less than 0.5. Virtually no cross-competition was observed between domains A and B, with a relational value of 0.84. While domain C had a theoretical relational value with the other two domains of 0.97, domain C has a more complex relationship with domain A, as CBH-7 cross-competes with CBH-4B but enhances CBH-4G binding. Because CBH-7 affects these antibodies in opposite directions, it is possible that CBH-7 is either in proximity to domain A or induces structural changes that affect domain A. CBH-17 was the only antibody to a linear epitope and did not influence the binding of the other antibodies (data not shown). These studies suggest that eight HCV HMABs define three immunogenic domains containing conformational epitopes on the HCV E2 glycoprotein.

HVR1 is not involved in conformational epitopes on E2. To determine whether HVR1 is involved in the conformational epitopes as defined by domain A to C antibodies, constructs of 1b (pDN-411) and 1a strain H (pDNH-411) E2 without their HVR (from aa 384 to 410) (Fig. 2A) were produced with HA and *c-myc* tags at the N and C termini, respectively. To test whether tag proteins have an effect on antibody binding, intracellularly expressed 1a and 1b E2s without tags were tested and shown to be without detectable differences by immunofluorescence assay (data not shown). DNA sequencing confirmed the junction of deletion, and the expected sequences resulted in no frameshifts or premature terminations. The expression of the E2 deletion constructs was verified by Western blot analysis of cytoplasmic extracts of transiently transfected HEK293 cells by using a MAb to the tag-HA epitope (Fig. 2B). Wild-type and HCV E2 deletion constructs were then transfected into HEK293T cells, and intracellular forms of E2 and E2 deletions were captured onto GNA lectin-coated microtiter plates. The reactivities of the HCV HMABs with E2 and the E2 deletion were then determined. Anti-tag antibodies (HA and *c-myc*) were used as positive controls for protein expression and for GNA capture (Fig. 2C and D). All HCV HMABs reacted with wild-type genotype 1b sf1b E2 protein, and none reacted with

proteins captured from extracts of mock-transfected HEK293 cells (Fig. 2C). All HCV HMABs retained reactivity with E2 produced by the pDN-411 deletion construct, indicating that the epitopes recognized by the antibodies did not include HVR1, although CBH-2 and -8E were reduced. No reactivity was observed with a control antibody (R04) to either wild-type E2 or the protein with E2 deleted. Antibody 3/11 was used as a positive control in these studies, since the epitope of this antibody has been defined as being outside of HVR1 (aa 412 to 423) (17). Next, the same panel of antibodies was tested against analogous genotype 1a E2 derived from strain H with (sfH1a-E2) or without (pDNH-411) HVR1 (Fig. 2D). HMABs CBH-8C and CBH-11 did not recognize either sfH1a-E2 or pDNH-411; CBH-2 had a significant reduction. For CBH-11 this was expected, since this antibody does not react to 1a E2. However, the reduced or lack of reactivity for CBH-2 and -8C was more isolate specific, as previous studies showed these antibodies binding equally well to other 1a E2 proteins (Table 1) (23). The other HCV HMABs and control antibodies had equivalent reactivities with the strain H-derived E2 proteins and the genotype 1b E2 proteins. All HMABs reactive with sfH1a-E2 retained reactivity with the HVR1-deficient construct pDNH-411, confirming that the epitopes recognized by these HMABs were outside HVR1. The reduction in binding of CBH-2 and -8E to genotype 1b and not to 1a HVR1 proteins with E2 deleted may reflect structural differences between genotypes and the involvement of HVR1 in these two epitopes in 1b E2 but not with the other E2 HMABs.

Conformational epitopes on E2 associated with HCVpp. Only eight of nine HCV HMABs to conformational epitopes were tested with HCVpp, because of an inadequate amount of CBH-8E antibodies. The hybridoma producing this HMAB was unstable and will require alternative production for further studies. Of the remaining eight antibodies, only CBH-5 and -7 had strong activity, and CBH-2 had weak activity, to block HCVpp entry to target cells as shown previously (32). The other antibodies had no neutralizing activity. To assess whether virus neutralization is caused by the expression or lack of expression of their respective epitopes on E2 associated with HCVpp, immunoprecipitation studies were performed with HCVpp and cell lysate-associated E1E2 glycoproteins (Fig. 3). It is worth noting that HCVpp-associated E2 had a slower and more diffuse migration pattern than the cell-associated form. This is due to modifications of the glycans by Golgi enzymes, as previously shown (32). With HCVpp (Fig. 3A), CBH-4B, -4D, and -7 showed strong binding, CBH-5 showed moderate binding, CBH-2 and -4G showed weak binding, and no detectable binding was seen with CBH-8C or -11. The lack of reactivity with CBH-8C and -11 was expected, since the HCVpp were derived from the 1a H strain, which is not recognized by these antibodies (Fig. 2D). For CBH-4G, a lower antibody affinity could be a contributing factor, as discussed below. Another explanation is the masking of this epitope on the surface of HCVpp. From these studies, it is reasonable to conclude that conformational epitopes as identified by antibodies to domains A, B, and C, CBH-2, -4B, -4D, -5, and -7, are present to some degree on the virion surface, although virus neutralization is restricted to domains B and C, CBH-2, -5, and -7. The strong binding of domain A antibodies CBH-4D and -4B, while being nonneutralizing, supports the view that virus neutralization for

FIG. 2. Epitopes recognized by HCV HMAs are located outside of HVR1. (A) Construct designation (on the left) showing the first amino acid sequence included in the two E2 deletion constructs employed in this study. All constructs were expressed in the pDisplay vector, which includes a heterologous signal sequence and TM domain (solid black lines) as well as epitopes recognized by MAb to influenza virus HA (vertical bars) and *c-myc* (horizontal bars). HCV 1b E2 sequences are indicated in white, and strain H E2 sequences are shaded. (B) Western blot analysis showing the expression of HCV E2 proteins. Wild-type 1b and 1a (strain H) E2 proteins are shown as sf1b and sfH1a, respectively. The HVR1 1b and 1a deletions (aa 384 to 410 were deleted) are shown as pDN-411 and pDNH-411, respectively. A protein size marker is indicated in kilodaltons. (C) Reactivity of HCV HMAs with E2 deletion constructs. HEK293 cells were mock transfected (white bars) or transfected with the HCV E2 constructs sf1b (stippled bars) or pDN-411 (black bars). Twenty-four hours posttransfection, cytoplasmic extracts were prepared and equivalent aliquots were captured onto GNA lectin-coated microtiter plates as described in Materials and Methods. The captured E2 proteins were then incubated with 10 μ g of the indicated HCV HMAb/ml (x axis), and the amount of bound antibody was determined. Bars represent the mean absorbance values obtained from duplicate wells. Error bars indicate one standard deviation from the mean. (D) Same experiment as in panel C, except that HEK293 cells were transfected with sfH1a E2 (stippled bars) or pDNH-411 (black bars).

HCV is mediated in part by restricted virion surface E2 epitopes in specific domains.

Immunoprecipitation of intracellular E1E2 showed binding with all conformation-sensitive antibodies except for CBH-8C and -11. Interestingly, differences in reactivity were observed between HCVpp-associated envelope proteins and their intracellular forms. Domain B antibodies, CBH-2 and -5, had stronger reactivities against cell-associated E1E2. Indeed, when compared to CBH-7, these antibodies precipitated 4 to 5 times less HCVpp-associated E2 protein. Since HCVpp-associated envelope proteins are modified by Golgi enzymes (32), the glycans added to these proteins in this compartment might

potentially reduce the accessibility of the epitopes recognized by CBH-2 and -5. Alternatively, we cannot exclude some local structural changes induced by modified glycans in domain B.

Antibody affinity and virus neutralization. To estimate affinity, saturation of HCV HMAb binding to cell surface-expressed genotype 1a and 1b E2 was measured by flow cytometry, and the data were analyzed using Prism software (GraphPad). Saturation profiles for domain A-, B-, and C-specific HMAs to 1a E2 are shown in Fig. 4. As summarized in Table 2, the antibodies displayed a wide range of dissociation constants (K_d). In general, affinity tended to be higher to 1b E2 than to 1a E2, except with CBH-4G and -7. Moderate-to-high

FIG. 3. Epitopes exposed on HCV glycoproteins associated with HCVpp. HEK293T cells transfected to produce HCVpp were labeled for 24 h. Supernatants (HCVpp) (A) and cell lysates (B) were immunoprecipitated with HCV HMAs as noted.

affinity antibodies of $<5 \times 10^{-8} K_d$ were observed in all three groups to 1b E2 and in domains A and C to 1a E2. In domain B, CBH-5 had a similar affinity with CBH-2 to 1a E2 but higher neutralizing activity for HCVpp derived from strain H77. The difference suggests that the number of CBH-5 epitopes is greater than the number of CBH-2 epitopes on the surface of HCVpp. This is supported by the observation that CBH-5 has a higher total binding than CBH-2 to 1a E2, as shown in Fig. 2D. CBH-4G weakly precipitated E1E2 associated with HCVpp and weakly bound to intracellular E1E2 compared to the other two antibodies in domain A. A possible explanation is that this epitope is partly masked on the surface of HCVpp.

Collectively, there are distinct biological activities between the antibody clusters (Table 2). Domain A antibodies, CBH-4B, -4D, and -4G, had no neutralization activity but showed greater recognition of HCVpp than intracellular E1E2, except for CBH-4G. One of the antibodies, CBH-4D, in previous studies was low-pH sensitive, with a 40% reduction in binding (32). Domain B antibodies, CBH-5 and CBH-2, have neutralizing activities but show reduced recognition of HCVpp compared to intracellular E1E2. The domain C antibody, CBH-7, has neutralization activity and equal accessibility of its epitope on E2 in HCVpp and intracellular E1E2.

DISCUSSION

Precise information on the mechanisms of virion attachment and entry will be critical in the successful development of newer therapeutics and an effective vaccine for HCV. Studies with MAbs on some viruses tend to support two different views on virus neutralization. One perspective is that virus neutralization correlates with antibody binding affinity to any virion surface site and is irrespective of the epitopes recognized by these antibodies. Antibody binding of a critical number of sites on the virion surface prevents virus entry through steric hindrance (8). The other perspective is that specific antibodies blocking distinct steps of virus attachment, interaction with receptor and coreceptor, and initiation of viral envelope fusion lead to virus neutralization (26). The development of HCVpp provides a useful tool to study the functional roles of specific immunogenic domains on envelope glycoproteins in virion binding and entry. We and other colleagues recently showed that HCVpp-associated envelope proteins are noncovalent E1E2 heterodimers, recognized by a panel of MAbs to conformational epitopes and CD81, and some epitopes are sensitive to low-pH treatment. Consequently, HCVpp are likely to contain envelope proteins in a similar structure as native virions (32).

Of eight HCV HMAs to conformational epitopes on E2, only three antibodies, CBH-2, -5, and -7, were able to inhibit HCVpp attachment and entry to Huh-7 cells (Table 2) (32). Competition analyses of these HMAs showed that conformational epitopes on E2 were clustered into three distinct domains. Domain A consisted of CBH-4B, -4D, and -4G; domain B contained CBH-2, -5, -8C, -8E, and -11; and domain C contained HMAb CBH-7. In domain B, CBH-2 and -5 were neutralizing. The lack of neutralizing activity of the other two tested antibodies, CBH-8C and -11, was explained by their lack of recognition of the genotype 1a H strain, which was used to construct the HCVpp. The neutralizing antibodies in domains B and C (CBH-2, -5, and -7) are to conformational epitopes on proteins associated with the virion surface and do not involve HVR1, as shown by HVR1 deletion studies. All antibodies in domains B and C inhibited the E2-CD81 interaction (Table 1), supporting the involvement of CD81 in virus entry (46). Domain A anti-

FIG. 4. Saturation binding of HMAs to HCV E2. Transiently transfected 293T cells with 1a E2 were incubated with the HCV HMAs in increasing concentrations as indicated. Staining and flow cytometry analysis were performed as described in Materials and Methods. The data points are means of two determinations and are representative of three independent experiments. Binding affinity data shown in Table 2 were analyzed using Prism software.

TABLE 2. Comparison of biological activities of HCV HMABs

Group ^a	HMAb ^b	K_d ^c		IP-E1E2 associated with:		Low pH sensitivity ^e (%)	Neutralization activity ^f
		1b	1a	Lysate ^d	Particles ^d		
A	CBH-4B	1.62×10^{-8}	4.50×10^{-8}	34	55	3	—
	CBH-4D	4.58×10^{-8}	1.67×10^{-7}	64	77	40	—
	CBH-4G	1.22×10^{-7}	9.03×10^{-8}	Weak	—	NT	—
B	CBH-2	1.27×10^{-8}	1.10×10^{-7}	77	16	17	+
	CBH-5	9.11×10^{-8}	2.22×10^{-7}	82	26	10	+
	CBH-8C	2.72×10^{-8}	No binding	—	—	NT	—
	CBH-11	6.60×10^{-8}	No binding	—	—	NT	—
C	CBH-7	1.88×10^{-8}	1.11×10^{-8}	87	93	3	+

^a Grouping of HCV HMABs based on results of competition and phylogenetic analyses.

^b HMABs analyzed.

^c Affinity disassociation of antibodies with genotypes 1b (GenBank AF348705) and 1a (AF009606).

^d Reported in arbitrary quantitative units. "Weak", low reactivity; —, no reactivity.

^e HMAb binding ability to HCVpp after low-pH treatment. Percentage denotes reduction in binding (32). NT, not tested.

^f Ability of HMABs to neutralize HCVpp infectivity of Huh-7 cells. "+" or "—" denotes neutralization or no neutralization (32).

bodies were nonneutralizing and did not block the E2-CD81 interaction. Their ability to precipitate E1E2 associated with HCVpp suggests that their epitopes are also on the surface of virions, supporting the perspective that HCV virion attachment and entry are restricted to specific virion surface domains.

This perspective is further supported by antibody affinity studies. CBH-4B has a moderate affinity to 1a E2 that is higher than that of CBH-2 or -5 (Table 2). But CBH-4B in domain A, whose epitope is on E2 associated with the virion surface, is nonneutralizing. Nonetheless, higher-affinity antibodies will tend to have higher neutralizing activities, with CBH-7 having higher activity than CBH-2 or -5 (32). More studies of antibodies in a specific domain, such as domain B, using HCVpp constructed with genotype 1b are required to further support this relationship of antibody affinity and virus neutralization activity.

The clustering of these epitopes on HCV E2 into three antigenic domains is in agreement with topological mapping by similar MAb cross-competition studies with other flavivirus E glycoproteins and the crystal structure of tick-borne encephalitis virus E glycoprotein (35, 39). The flavivirus E glycoprotein is the dominant antigen inducing neutralizing antibodies and is the protein responsible for virus attachment to cell receptors and initiation of viral envelope fusion leading to cell entry (36, 38). Epitopes on flavivirus E protein are clustered into three structural domains. A central domain I containing nonneutralizing epitopes is felt to be a hinge region involved in low-pH-induced conformational changes (39). Our HCV E2 domain A has similar properties for nonneutralizing epitopes, and at least one of the epitopes, CBH-4D, was shown to be pH sensitive, with 40% binding reduction under low pH (Table 2). The varied effects of CBH-7 on CBH-4G (increase) and CBH-4B (decrease) in binding to E2 suggest that a conformational change is possibly induced with CBH-7 binding, consistent with domain A as a possible hinge region. Flavivirus E protein domain II is involved in dimerization and membrane fusion and is able to elicit neutralizing and nonneutralizing antibodies (35, 39). For HCV, the identity of the fusion protein is unclear. At first, a putative fusion peptide in E1 led to the proposal that this protein is the fusion protein (18). But, other homology studies suggested that E2 is responsible for virus-induced fusion (27, 45). Flavivirus E protein domain III containing distal projecting loops from the virion surface elicits the strongest

neutralizing antibodies, is minimally affected by low pH, and is felt to be the receptor binding motif (35, 38, 39). Currently, it is not possible to correlate our HCV domain B and C antibodies in a similar manner. Both domains contain neutralizing antibodies and are able to inhibit the E2-CD81 interaction. One possible clue is that previous studies showed that CBH-2 is uniquely able to recognize noncovalent E1E2 heterodimers and not high-molecular-weight E1E2 aggregates that are misfolded. Furthermore, CBH-2 will only recognize 1a H strain E2 when complexed with E1 (11). These findings suggest that the HCV domain B antibodies are potentially correlated with the flavivirus E protein domain II involved in envelope protein dimerization. Further studies are required to substantiate this model for HCV. Differences are to be expected in the structural organization of HCV E2 and flavivirus E glycoproteins, since a major determinant is the number of disulfate bonds, which are different between these glycoproteins. Epitope mapping and more detailed investigation on structure-function properties of this panel of HCV HMABs will be useful to advance our knowledge on E2 immunogenic structures, which in turn should facilitate effective vaccine design.

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