The Hypervariable Region 1 of the E2 Glycoprotein of Hepatitis C Virus Binds to Glycosaminoglycans, but This Binding Does Not Lead to Infection in a Pseudotype System

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The hypervariable region 1 (HVR1) of hepatitis C virus (HCV) E2 envelope glycoprotein is a 27-amino-acid sequence located at its N terminus. In this study, we investigated the functional role of HVR1 for interaction with the mammalian cell surface. The C-terminal truncated E2 glycoprotein was appended to a transmembrane domain and cytoplasmic tail of vesicular stomatitis virus (VSV) G protein for generation of the chimeric E2-G gene construct. A deletion of the HVR1 sequence from E2 was created for the construction of E2ΔHVR1-G. Pseudotype virus, generated separately by infection of a stable cell line expressing E2-G or E2ΔHVR1-G with a temperature-sensitive mutant of VSV (VSVts045), displayed unique functional properties compared to VSVts045 as a negative control. Virus generated from E2ΔHVR1-G had a reduced plaquing efficiency (~50%) in HepG2 cells compared to that for the E2-G virus. Cells prior treated with pronase (0.5 U/ml) displayed a complete inhibition of infectivity of the E2ΔHVR1-G or E2-G pseudotypes, whereas heparinase I treatment (8 U/ml) reduced 40% E2-G pseudotype virus titre only. E2ΔHVR1-G pseudotypes were not sensitive to heparin (6 to 50 μg/ml) as an inhibitor of plaque formation compared to the E2-G pseudotype virus. Although the HVR1 sequence itself does not match with the known heparin-binding domain, a synthetic peptide representing 27 amino acids of the E2 HVR1 displayed a strong affinity for heparin in an enzyme-linked immunosorbent assay. This binding was competitively inhibited by a peptide from the V3 loop of a human immunodeficiency virus glycoprotein subunit (gp120) known to bind with cell surface heparin. Taken together, our results suggest that the HVR1 of E2 glycoprotein binds to the cell surface proteoglycans and may facilitate virus-host interaction for replication cycle of HCV.

Hepatitis C virus (HCV) is a major causative agent of parentally transmitted hepatitis (9) and is associated with liver cirrhosis and hepatocellular carcinoma (1). Approximately 25% of infected individuals appear to clear viremia without therapeutic intervention (2, 31), and the mechanism leading to this natural resolution of HCV infection is not completely understood. However, the majority of HCV-infected individuals do not resolve infection, leading to the development of chronic hepatitis. The HCV genome is a linear, positive sense, single-stranded RNA molecule of ~9,500 nucleotides. It encodes a polyprotein precursor of ~3,000 amino acids (10). This polyprotein is cleaved by both host and viral proteases to generate several distinct polypeptides (24, 29, 36). The structural proteins, core, E1 and E2, of HCV physically interact and may have a role in virus assembly (4, 11, 36). The glycosylated polypeptides (E1 and E2) are most likely anchored onto the envelope lipid bilayer of the virus and facilitate virus entry by interaction with the cell surface. In vitro expression studies have suggested that the glycoproteins of HCV associate to form two types of complexes: (i) a heterodimer stabilized by noncovalent interaction and (ii) a high-molecular-weight disulfide-linked aggregate. Both types of complexes accumulate in the endoplasmic reticulum, a proposed site for HCV assembly and budding (47). The noncovalent interactions between E1 and E2 may represent the functional subunit of HCV envelope glycoproteins (47). If HCV entry occurs through a heteromeric complex of the envelope glycoproteins, the specific role for E1 and/or E2 in this active complex remains to be understood.

E2 exhibits the highest degree of genetic heterogeneity, especially in hypervariable region 1 (HVR1), which is located at the N terminus (61). A binding site for neutralizing antibodies has been identified in the HVR1 region (52). Interestingly, despite strong amino acid sequence variability, the chemico-physical properties and conformation of HVR1 are highly conserved (50). Prospective studies of serological responses to HVR1 sequence of patients with acute and chronic HCV infection displayed extensive cross-reactivity with an unrelated HVR1 peptide (59). A significant correlation has been found between HVR1 sequence variation and the intensity and cross-reactivity of humoral immune responses. This provided strong evidence in support of the contention that HCV variant selection is driven by immune pressure. Monoclonal antibodies (MAbs) generated after immunization of mice with peptides derived from a natural HVR1 sequence were observed to generate a neutralization response for several HVR1 sequences, attesting to the existence of conserved amino acid motifs among different variants (52). These findings suggest the possibility for an induction of a broadly cross-reactive immune response to HVR1 and that this mechanism can be used to generate protective immunity. Antibodies to HVR1 can inhibit the binding of recombinant E2 to the cell surface in vitro, and...
rabbit hyperimmune serum generated against the HVR1 can neutralize the ability of HCV to infect chimpanzees (19, 41, 47, 55). An HCV clone lacking HVR1 is infectious but attenuated in the chimpanzee model (22). However, HVR1 is always present in strains infecting humans, suggesting that virus containing intact HVR1 may have a significant survival advantage (50).

Using vesicular stomatitis virus (VSV) particles pseudotyped with HCV envelope proteins we have previously shown that both the E1 and E2 envelope glycoproteins may take part in the process of entry into mammalian cells (33, 40). In contrast, the single E glycoproteins of tick-borne encephalitis virus, dengue virus, and other members of the Flavivirus genus mediate both receptor binding and membrane fusion functions. Proteoglycans are a group of proteins that carry sulfated polysaccharide side chains, called glycosaminoglycans (GAGs), consisting of repeating disaccharide units (5). A variety of microorganisms bind the GAG chains of proteoglycans, and many appear to use this association as a way to attach to target cells. Many viruses, such as herpesvirus (58), human immunodeficiency virus (49), human T-cell leukemia virus type 1 (51), flavivirus (8), coxsackievirus B3 variant PD (63), and alphavirus (7), utilize highly sulfated GAGs or heparan sulfate (HS) to mediate attachment to target cells through basic residues of a virus protein. It seems possible that HS could help this virus adhere to a cell before more specific, high-affinity receptors induce adhesion and mediate virus entry (56). Interestingly, the structural requirement of HS for attachment seems to vary among microorganisms and for different virus types of the same family (28, 35, 60). HVR1 is a stretch of amino acids with basic residues at conserved positions (50). Although HVR1 sequences vary between HCV strains, the overall physiochemical (charge) properties remain conserved. This conservation of positively charged residues indicates that HVR1 might interact with negatively charged molecules such as lipids, proteins, or GAGs. Treatment of HCV with heparin led to ~70% inhibition of attachment with cells (26). Polyanionic compounds similar to heparin, such as dextran sulfate and suramin, can inhibit HCV binding to peripheral blood mononuclear cells and human hepatoma cells (13, 25). As with many other viruses, it seems possible that the interaction of HVR1 with GAGs could play a role in attachment to host cells.

Generation of pseudotypes from a number of virus envelope glycoproteins by using VSV or its temperature-sensitive mutant (VSVts045) has been described earlier (15, 17, 48, 57). We have previously reported some of the functional properties of HCV E1 and E2 glycoproteins by using pseudotype virus generated from chimeric gene constructs expressing the ectodomain of E1 or E2 glycoprotein of HCV genotype 1a (33, 40). One such pseudovirus, carrying an HVR1 segment, has been shown to infect both cell lines and human hepatoma cells (13, 25).

Perturbation of the C-terminal region of E2 have no detectable effect on pseudotype virus generation.

Incorporation of HCV chimeric envelope glycoproteins into a temperature-sensitive mutant of VSV (VSVts045) by phenotypic mixing was used to understand the functional properties of the HCV glycoproteins (33, 40, 41). VSVts045 has a G protein with a single amino acid change in the ectodomain and a thermoreversible folding phenotype (reviewed in reference 17). At nonpermissive temperature (40.5°C), the G protein of VSVts045 is synthesized and core glycosylated normally but does not fold correctly. As a result, G is not transported on cell surface for incorporation on virus particles. However, this temperature-sensitive mutant virus does not tightly regulate this process, and the possibility for leakage of G cannot be ruled out. To safeguard this, we have used a carefully selected stock of VSVts045. This virus stock was generated after four rounds of plaque purification, and the temperature-sensitive phenotype of the stock virus was verified by lack of growth at the nonpermissive temperature (40.5°C) in a plaque assay. This virus stock displayed much lower leakage of G in repeated experiments. Plaque-purified VSVts045 was used for generation of VSV/HCV pseudotype virus by infecting BHK stable transfectants expressing E2-G or E2ΔHVR1-G chimeric gene and grown at 40.5°C, as described previously (40). Mock-transfected cells were similarly treated with VSVts045 as a negative control. The culture fluid was flash frozen in aliquots and each aliquot was thawed only once for use in each plaque assay at a permissive temperature (32°C) for VSVts045. The pseudovirus showed titers in the order of ca. 10³ to 10⁶ PFU/ml, whereas mock-transfected negative control displayed <2% of pseudotype titer. The pseudotype virus from E2 or E2ΔHVR1 was neutralized (61 to 85% at 1:20 dilution and 29 to 73% at 1:50 dilution) by six different HCV-infected patient sera (34), and none of these sera at similar dilutions exhibited a detectable neutralization titer (<5%) for VSVts045 used as a negative control. Treatment of pseudotype virus with an antiserum to VSV-G did not significantly alter virus titer, suggesting the absence of revertant VSV-G in the virus preparation. Two different E2-G pseudotypes were generated by using two different C-terminally truncated E2 genomic regions, one ending at amino acid (aa) 711 and the other ending at aa 742; and both produced a similar virus titer. This indicated that the additional 31 aa in the C-terminal region of E2 have no detectable effect on pseudotype virus generation or infectivity.
Enzymatic modification of cell surface for virus plaque assay. BHK and HepG2 cells were treated separately with pronase and heparinase I (Sigma, St. Louis, Mo.) for 60 min at 37°C to destroy a possible receptor-binding property of cells with the pseudotype virus. After incubation, cells were extensively washed with Dulbecco modified Eagle medium to remove residual enzyme and a predetermined titer of virus inoculum was added on to the cell monolayer for plaque assay (40).

Synthetic peptides. The E2 HVR1 peptide (ETHVTGGSAGHTVSGFVSLL APGAKQNY), encompassing amino acid residues 384 to 411, was generated from the sequence of HCV 1a strain (accession number M 62321 in GenBank). The peptide was synthesized by Fmoc (9-fluorenylmethoxy carbonyl) chemistry under continuous flow conditions with Rapp-Polymeric PEG-Poly styrene resins (Sigma-Genosys) and purified by reversed-phase chromatography. A peptide derived from V3 loop (aa 308 to 331) of human immunodeficiency virus (HIV) gp120 (NNTRKSIQRQPGRFAVTIGKIG), a different antigenic peptide (aa 474 to 497) from HIV gp120 (CGKIEPLGVAPTKAKRRVVQREKR) and a peptide from a different region (aa 554 to 569) of HCV 1a E2 (WMNSTGFTKVCGA PPC) were procured (BACHEM) as controls. The peptide from the V3 loop of gp120 was used as a positive control for binding with heparin. Two other peptides, unrelated to E2 HVR1 or gp120 V3 loop, were used as negative controls in a similar analysis.

ELISA. HVR1 peptide was examined for affinity to heparin by enzyme-linked immunosorbent assay (ELISA). Briefly, ELISA plates (Maxisorp; Nunc Corp.) were coated with 100 µl of peptide (10 µg/well) in phosphate-buffered saline (PBS; pH 7.4) or 2-N-morpholinoethanesulfonic acid (MES) buffer (50 mM MES, 50 mM NaCl [pH 6.0]) at 4°C overnight. Analysis of peptide concentration from colorimetric assay (Bio-Rad Protein Assay Kit) before and after adsorption on ELISA wells suggested −1 µg of peptides was adsorbed on each well of ELISA plate. Plates were washed and blocked with 2% bovine serum albumin in PBS for 1 h at 37°C. Plates were washed either with PBS or MES buffer, and heparin-biotin conjugate (Calbiochem, La Jolla, Calif.) was added at serial dilutions in the respective buffer to triplicate wells. After incubation for 1 h at 37°C, plates were washed, and bound heparin was detected by adding 100 µl of streptavidin-horse radish peroxidase (HRP) conjugate (0.1 µg/ml). After incubation for 1 h at 37°C, plates were washed, and the color reaction was developed using the biotin analogue EZ-Link-sulfo-NHS-LC-Biotin (Pierce, Rockford, Ill.).

RESULTS

Deletion of HVR1 does not alter cell surface expression of the chimeric E2 glycoprotein. First, we sought to determine whether a deletion of the HVR1 region from the E2-G chimeric gene construct would retain E2 glycoprotein expression on the cell surface. MAbs H52 (recognizing a linear epitope of E2) and H53 (directed to a conformational epitope) were used for the detection of chimeric protein expression on transfected cell surface (unfixed) by immunofluorescence. Both the antibodies recognized cell surface expression of E2-G or E2AHVR1-G protein from the chimeric gene constructs in nonpermeabilized BHK transfectants (Fig. 1A to D). On the other hand, mock-transfected BHK cells, used as a negative control, did not exhibit a detectable fluorescence with either of the MAbs (Figs. 1E and F). Thus, the presence or absence of HVR1 in the chimeric E2-G protein did not affect cell surface expression. Translocation of chimeric E2-G onto the cell surface may modify glycosylation and conformation of the native E2. However, the binding of a conformation-sensitive MAb (H53) was not altered after expression of the chimeric E2-G or E2AHVR1-G. This result suggests that the conformational epitope of E2 recognized by H53 has undergone proper folding.

FIG. 1. Expression of HCV E2-G chimeric glycoprotein on BHK cell surface. Expression from E2AHVR1-G (A and C) and E2-G (B and D) in BHK cells (nonpermeabilized) was observed by indirect immunofluorescence with a conformation-independent MAb H52 (A and B) and a conformation-dependent MAb H53 (C and D). (E and F) Mask-transfected BHK cells were similarly used as negative controls.
heparinase I (which digests highly sulfated heparin-like structures on HS) were used to investigate the role of proteoglycans in virus cell interaction. Plaque formation by pseudotype virus from E2-G or E2ΔHVR1-G significantly decreased in cells prior treated with increasing pronase concentration (0.25 and 0.5 U/ml). On the other hand, control VSVts045 displayed a 35 and 65% reduction of virus titer when HepG2 cells were treated with 0.25 and 0.5 U of pronase/ml, respectively (Fig. 3A). A further increase in the pronase concentration to 1 U/ml altered the morphology of the cells. Although several different receptors can be utilized by VSV based on the cell type used, the implications of this partial reduction of VSVts045 titer for pronase treatment remain to be understood. The treatment of cells with heparinase I (8 or 16 U/ml) resulted in a maximum reduction of E2-G pseudotype virus plaque number of ~40%, but not for E2ΔHVR1-G, in both HepG2 (Fig. 3B, open bars) and BHK cells (data not shown). The differences between plaque numbers of E2-G in untreated and heparinase I-treated cells were highly significant (P < 0.0001). On the other hand, when Sindbis virus (Sin Toto 1101) was used as a control, heparinase I dose-dependent virus plaque reduction was observed. The Sindbis virus titer decreased by >80% after the treatment of BHK cells with 16 U of heparinase I/ml (data not shown). These results suggest a possible

in the chimeric glycoproteins E2-G and E2ΔHVR1-G, as observed earlier with other chimeric E2 expression (22).

**E2ΔHVR1-G displays pseudotype virus plaque formation.** We examined whether the deletion of HVR1 interferes with pseudotype virus plaque formation of the E2-G glycoprotein in epithelial cells. For this, pseudotype viruses were generated by expressing E2-G or E2ΔHVR1-G, and the plaquing efficiency was compared in three different cell lines at 32°C. To allow direct comparison, a known titer of pseudotype virus (~60 PFU), previously determined by plaquing in BHK cells, was concurrently examined for plaque formation in HepG2 and MCF7 cells. Plaque numbers with pseudotypes bearing E2-G were ~2-fold higher in HepG2 and MCF7 cells compared to BHK (Fig. 2). In contrast, plaque numbers of E2ΔHVR1-G pseudotype were similar in HepG2 and MCF7 cells but were ~2-fold higher in MCF7 cells. Thus, MCF7 cells appeared to support higher plaque numbers, irrespective of the presence or absence of HVR1 in E2-G. The difference in plaque numbers of the E2-G and E2ΔHVR1-G pseudotypes in HepG2 cells may be related to the HVR1 region of E2-G protein. HVR1 may facilitate the recognition of distinct receptors or coreceptors by the pseudotype virus for fixation or internalization; however, its exact role is not clear from these experimental findings. On the other hand, VSVts045 (grown at 32°C) when used as an unrelated control displayed negligible plaque number in HepG2 cells, while remaining relatively unchanged on MCF7 cells. The VSVts045 used here were the same as those used to generate the pseudotype. These results suggest that the parental temperature-sensitive mutant of VSV interacts in a manner that is distinct from the E2 pseudotypes.

**Enzymatic modification of the cell surface alters E2ΔHVR1-G pseudotype virus infectivity.** The enzymatic digestion of cell surface proteins by pronase and removal of attached GAGs by

FIG. 2. Plaquing efficiency of the pseudotype virus in mammalian cells. The E2-G or E2ΔHVR1-G pseudotype virus was generated from stable transfectants of BHK cells expressing chimeric glycoprotein by infecting with VSVts045. Pseudotype virus titers were determined by plaque assay on BHK cells, and a known titer of the virus (~60 PFU) was used for determining the plaquing efficiency in two other epithelial cell lines, HepG2 and MCF7. VSVts045 was also included in this assay to distinguish the plaquing efficiency of the pseudotype virus on specific cell types. Plaque assay was done at 32°C, and the results from three independent assays are presented with the standard deviations indicated.

![Graph](image.png)

**FIG. 3. Infectivity of pseudotype virus following pronase (A) and heparinase (B) treatment of HepG2 cells.** The indicated doses of enzymes were used to treat HepG2 cells for 1 h at 37°C. The cells were washed extensively and incubated with a known titer (ca. 80 to 120 PFU) of E2-G, E2ΔHVR1-G, or VSVts045 for plaque assay. The results presented are the means from three independent experiments, together with the standard deviations.
interaction between E2-G and cell surface proteoglycans during virus attachment and/or entry. The interaction may occur through the HVR1 region of the E2 glycoprotein since treatment of cells with a similar dose of heparinase I had a marginal effect (<10%) in plaquing efficiency of E2ΔHVR1-G virus (Fig. 3). On the other hand, when cells were treated similarly with heparinase I, VSVts045 did not exhibit a reduction in plaque number in HepG2 cells (Fig. 3) and BHK cells (data not shown). The results from heparinase I treatment suggest that E2-G interaction is partially inhibited. The interaction of E2-G with cell surface may also depend on other susceptible structures or additional cell surface molecule(s) are involved in E2-G pseudotype virus attachment and/or entry into a susceptible host.

The absence of HVR1 significantly alters heparin- or suramin-mediated inhibition of E2 pseudotype virus infectivity. To further verify our results observed with heparinase treatment, we examined whether biologically important polyanionic ligands can inhibit E2 glycoprotein interaction with cell surface. For this purpose, E2-G or E2ΔHVR1-G pseudotype virus was prior incubated with various concentrations of heparin or suramin at 32°C and subsequently added onto the cell surface for adsorption. An inhibition of virus infectivity (~67%) at ~6 μg of heparin/ml for E2-G was observed, whereas there was no significant effect on E2ΔHVR1-G plaque number even at 100 μg of heparin/ml in HepG2 (Fig. 4A) and BHK cells. However, the E2-G titer did not change significantly with further increases in doses of heparin of up to 100 μg/ml. When Sindbis virus (Sin Toto 1101) was used as a positive control, a gradual plaque reduction up to ~90% was apparent over a range of 6 to 100 μg of heparin/ml in BHK cells (data not shown). On the other hand, VSVts045 control did not display a significant reduction in plaque number at similar doses of heparin. Inhibition of E2-G pseudotype virus bearing the HVR1 domain but not with E2ΔHVR1-G, by heparin further suggested that HVR1 interacts with cell surface GAGs. Preincubation of virus with suramin reduced (>50%) plaque formation from E2-G pseudotypes at 50 μg/ml. On the other hand, a much higher dose (250 μg/ml) was required for ~50% plaque reduction by E2ΔHVR1-G pseudotype virus (Fig. 4B), further suggesting the involvement of HVR1 in interaction with cell surface proteoglycans. The VSVts045 control did not display a significant change in plaque number over the same range of suramin concentration. The higher quantity of suramin required for virus plaque reduction could be due to the different backbone structure and the degree of sulfonation of suramin. The binding of the E2ΔHVR1-G pseudotype virus with increasing doses of suramin could also be due to nonspecific interactions or interaction with a different domain predicted for E2 (62). The results from these experiments suggested that pseudotype virus generated from E2ΔHVR1-G is less sensitive to heparin or suramin as inhibitors of plaque formation compared to E2-G pseudotype virus.

HVR1 peptide binds with heparin. Our earlier results indicated that E2 pseudotype virus might interact through HVR1 with heparin to facilitate binding with the cell surface. In the present study we investigated by ELISA the ability of HVR1 peptide to bind to heparin. Similarly, a peptide from the V3 loop of HIV GP120 (aa 308 to 331), known to bind with cell surface heparin, was used as a positive control. Two other peptides, E2 (aa 554 to 569) and HIV GP120 (aa 474 to 497), were used as unrelated controls. Peptide coated ELISA plates were incubated with serial dilutions of biotinylated heparin at mildly acidic (pH 6.0) or neutral (pH 7.4) conditions. Mean adsorption at 405 nm was read against a blank without heparin to calculate the concentration of biotinylated heparin bound to HVR1-coated ELISA wells. Absorbance values were found to be linearly related to the concentration of biotinylated heparin at ranges of 0.3 and 3 μg/ml under mildly acidic conditions in the ELISA (Fig. 5A) and 5 to 50 μg/ml under neutral conditions (data not shown). The binding of protein to HS relies on the release of cations, most notably sodium ions, from polyanionic HS chains by positively charged components of the protein (38). Thus, differences in the heparin-binding capacities of the HVR1 molecule vary with the pH of its incubation medium. The association constant between HVR1 and biotinylated heparin was estimated to be ~6 × 10⁶ M⁻¹ at pH 6.0 and ~2 × 10⁷ M⁻¹ at pH 7.4. This behavior may be due, at least in part, to a general electrostatic effect, since the overall positive charge of HVR1 decreases with increasing pH, which potentially weakens the interactions with the negatively charged polysaccharide (45).

The specificity of HVR1 binding with biotinylated heparin

![FIG. 4. Effect of heparin and suramin on the infectivity of the pseudotype virus. E2-G, E2ΔHVR1-G, or VSVts045 pseudotype virus of known titer were pretreated with various concentrations of heparin (A) or suramin (B) at 32°C for 60 min and examined for plaque formation. The results are presented as mean from three independent assays, together with standard deviations.](http://jvi.asm.org/)
was also verified by competition ELISA with unbiotinylated heparin, the peptide from V3 loop of GP120 (aa 308 to 331), E2 peptide (aa 554 to 569), a recombinant E2 glycoprotein of HCV (Biogenesis, Poole, United Kingdom), and unlabeled HVR1. The results suggested that the inhibition of binding by biotinylated heparin with HVR1 occurs in a concentration-dependent manner with the unbiotinylated heparin, peptide from V3 loop and the recombinant E2 glycoprotein of HCV (Fig. 5B). The quantity of peptide from V3 loop required for 50% inhibition of HVR1-heparin binding was determined to be ~3 μg/ml at pH 6.0, whereas >3-fold higher peptide concentration (10 μg/ml) was required for similar inhibition at pH 7.4 (data not shown). On the other hand, the unrelated (negative control) peptides did not display competition in binding of HVR1 with heparin. Biotinylated HVR1 peptide also exhibited binding with MCF7, BHK and HepG2 cell surface molecules by ELISA. The binding was competitively inhibited by unbiotinylated HVR1, heparin, or GP 120 (aa 308 to 331), whereas an unrelated peptide from E2 (aa 554 to 569) displayed absence of inhibitory activity in competition ELISA (Fig. 5C). The results from these studies suggested that HVR1 specifically binds with heparin.

Treatment of cells with HVR1 peptide does not inhibit E2 pseudotype virus plaque formation. To investigate whether HVR1 peptide binding with cell surface inhibits pseudotype virus infectivity by blocking the interaction between E2 and heparin, an inhibition assay was carried out. Cells were incubated with different doses of HVR1 peptide (10, 20, 40, and 80 μg/ml) at ~4°C for 30 min, prior to addition of E2-G or E2ΔHVR1-G pseudotype. Cells and virus inoculum were incubated at ~4°C for 1 h. A predetermined virus titer of ~100 PFU was used for virus infectivity in the presence or absence of HVR1 peptide. Virus plaque number altered <10% at any one of these doses of HVR1 peptide. Similar results were also obtained when cells were treated with HVR1 and infected with E2-G or E2ΔHVR1-G pseudotype virus at 32°C. These experimental data suggested that prior incubation of cells with HVR1 does not inhibit virus entry. The peptide from V3 loop of HIV gp120 (aa 308 to 331), when tested similarly, did not inhibit E2 pseudotype titer at 4 or 32°C at the same concentrations. Our earlier results have shown that HVR1 or peptide from the V3 loop of HIV binds to HS present on cell surface proteoglycans. Taken together, these results indicate that HVR1 of E2 may provide an additional means of cell association but does not have a direct effect to trigger virus entry into cells.

DISCUSSION

Our results highlight a number of interesting features of the HVR1 from HCV E2 glycoprotein, which include the following: (i) the presence or absence of HVR1 did not alter cell surface expression of the chimeric E2-G as recognized by a conformation-dependent MAb; (ii) E2ΔHVR1-G pseudotype

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**FIG. 5.** (A) Concentration-dependent binding of biotinylated heparin to HVR1. HVR1 peptide (10 μg/well in MES [pH 6.0]) was used to coat ELISA plates. The binding of biotinylated heparin was measured at the indicated concentration. A peptide from HIV GP 120 (aa 308 to 331) known to bind with heparin was used similarly as a positive control. Two other peptides from different regions of E2 (aa 554 to 569) and HIV GP120 (aa 474 to 497) were included as negative controls in this assay. (B) Competitive inhibition of HVR1-heparin binding in ELISA. Serially diluted unlabeled recombinant E2, GP 120 (aa 308 to 331), heparin, or HVR1 was added as a competitor to HVR1-coated wells of a microtiter plate, and biotin-conjugated heparin at a fixed concentration was added immediately into wells. The binding of biotinylated heparin was measured after incubation with a streptavidin-HRP conjugate. The results from similar analyses with negative control peptides, E2 (aa 554 to 569) and GP120 (aa 474 to 497), are also shown. (C) Competitive inhibition of HVR1-HepG2 cell binding in ELISA. Serially diluted unlabeled HVR1, HIV GP120 (aa 308 to 331), E2 (aa 554 to 569) peptide, and heparin as competitors were added to cells fixed on a microtiter plate. Biotin-conjugated HVR1 at a fixed concentration was immediately added into the wells. The binding of biotinylated HVR1 was measured after incubation with streptavidin-HRP conjugate. The results are presented as the means, together with the standard deviations, from three independent assays.
virus displayed a decreased plaque number in HepG2 cells; (iii) heparinase I treatment of HepG2 cells reduced E2-G pseudotype virus infectivity, and incubation of pseudotype with heparin significantly inhibited infection of E2-G virus but not E2ΔHVR1 virus; and (iv) the binding of HVR1 with mammalian cells did not have a direct role on E2 pseudotype virus entry. Proteoglycans are found abundantly in the extracellular matrices or cell surfaces and mediate many fundamental cellular processes (27). A proteoglycan is formed by the linkage of GAGs such as HS or chondroitin sulfate to a protein core. HS is the initial binding target of many viruses. Heparin-binding proteins are known to interact with heparin via electrostatic charge interactions generated between the negatively charged sulfate groups on heparin and the positively charged amino acids within the protein’s heparin-binding domain. This event is followed by an initial virus-cell contact that concludes an interaction between the viral attachment protein(s) and the receptor molecule(s). Such a cascade of events is of great importance in the stable cell adherence of complex pathogens such as enveloped viruses in cell culture. Pseudotype virus generated from E2ΔHVR1-G was able to interact with mammalian cell surface (BHK, HepG2, and MCF7) for virus plaque initiation, suggesting that the binding of HVR1 is not solely responsible for E2-G pseudotype virus entry. Modification of the cell surface by treatment with pronase inhibited both E2-G and E2ΔHVR1-G pseudotype virus infectivity. Pseudotype virus generated from E2ΔHVR1-G was not heparinase I sensitive or sensitive to the use of heparin as an inhibitor of plaque formation, a finding which contrasted with results obtained with the E2-G pseudotype virus. When a portion of E2 is expressed alone or together with E1 for the generation of HCV-like particles, the reactivity of HVR1 was accessible to a specific MAb and recognized with comparable affinity (11). Thus, the HVR1 domain of E2 may be accessible to the cell surface even in the presence of the E1 glycoprotein to bind with HS, which potentially provides an additional means to facilitate virus attachment to host cells.

We have reported that both E1 and E2 glycoproteins of HCV interact independently with a number of mammalian cell lines to initiate VSV pseudotype entry (33). Matsuura et al. (39) subsequently reported that both the chimeric proteins are required for "maximal" infectivity. A viral transmembrane domain may play a role in the fusion process. For VSV-G, the fusion peptide appears to reside at an internal location of the protein and also requires some form of membrane anchoring in order to promote membrane fusion (12, 18, 21, 46, 64). However, replacement of glycine residues by alanine at amino acid positions 16 and 18 of the G transmembrane domain of chimeric E2-G did not alter pseudotype virus infectivity (unpublished data). To determine whether coexpression of E1 and E2 was able to enhance pseudotype titer, we recently generated stable transfecants of BHK cells expressing both the chimeric glycoproteins on the cell surface. BHK stable transfecants were generated first by transfection with E1-G under the control of a cytomegalovirus promoter. G418 resistant BHK cells were selected and transfected with E2-G under the control of a murine leukemia virus (MuLV) promoter. Stable transfecants expressing E2-G were selected by treatment of cells with puromycin. Selected cells expressing both E1-G and E2-G were used for generating the pseudotype by infection with VSVts045. We observed a four- to eightfold-higher virus titer in pseudotype generated from cell lines expressing both E1-G and E2-G in comparison to E1-G alone (unpublished results). However, we do not know at this time whether the increase in pseudotype titer is due to an association between the E1 and E2 ectodomains, and this will require careful examination. Interestingly, pseudotypes generated from the expression of both HCV E1 and E2 when treated with heparin exhibited a similar reduction of virus infectivity, as was observed with E2-G pseudotype alone. However, E1/E2ΔHVR1 required at least a sixfold-higher level of heparin for a similar reduction of E1/E2 pseudotype infectivity.

Buonocore et al. (6) have expressed both HCV glycoproteins from a recombinant VSV vector that also expressed VSV-G and found that all of the infectivity could be neutralized with antiserum to G. These authors demonstrated that VSV with either E1 or E2 separately or with E1 and E2 together without any G protein are not infectious for a number of cells, including BHK cells, HepG2 cells, or even primary hepatocytes. We do not know at this time why the results with recombinant viruses are different from those of others by using pseudotyped VSV (33, 39), and this would require additional study. Recently, Bartosch et al. (3) presented data suggesting that the MuLV/E1 or E2 pseudotype is able to independently infect cells, but to a much lower extent than E1 and E2 together. These authors suggested that "high" infectivity of the pseudotype-particles required both E1 and E2 HCV glycoproteins. However, individual expression or coexpression of E1 and E2 from distinct expression units in rats led to normal levels of recombinant protein synthesis compared to higher expression of both the glycoproteins in cis from a single E1E2 polyprotein. Therefore, we cannot rule out the possibility that a weak infectivity of MuLV pseudotype bearing individual HCV glycoprotein is due to a lower level of E1 or E2 expression on the cell surface. Pseudotypes with lentivirus bearing E1 and E2 have also been shown by a different group of investigators (30) to infect cells. Surprisingly, pseudotypes produced from MuLV and lentivirus systems were derived from the surface expression of HCV envelope sequences without genetic manipulation. These glycoproteins have been previously observed to be retained in the endoplasmic reticulum of a number of mammalian cells. Interestingly, Bartosch et al. (3) also observed partial inhibition of MuLV/E1E2 infectivity with human CD81 large extracellular loop polypeptide or anti-CD81 antibody and a weak inhibition by inhibitors of LDL-R. Although there are apparent differences, the overall observations made by Bartosch et al. (3) have some similarities to those reported earlier (33, 39, 40). Whether the cytomegalovirus promoter driving the green fluorescent protein reporter gene provides a reliable expression level across different cell types (37) and a reliable quantification of infectious pseudotype titer remain to be examined to understand the reported discrepancies in susceptibility of cell types to MuLV or lentivirus E1/E2 pseudotype (3, 30) and VSV pseudotype infectivity (33, 39, 40). Although pseudotypes are a necessary tool, they may not actually reflect the specificity of the wild-type virus until an efficient cell culture for HCV infection is developed.

We and others have previously suggested that HCV E2 glycoprotein interacts with GAGs located on the mammalian cell surface (40, 50, 62). Several viruses appear to use HS proteo-
glycans (HSPG) at a step prior to interaction with their specific receptors. HIV type 1 (HIV-1) binds cell surface HSPG, and the type and quantity of HSPG on a given cell type can modulate virus infectivity in vitro and may influence virus tropism in vivo. HIV-1 interacts with soluble polyanions and cell surface HSPG via the V3 loop of gp120 (43, 44, 53). The level of positive charge present on the gp120 surface may relate directly to coreceptor utilization. Similarly, the positive charge level on HCV E2 and the type and quantity of HSPG on a given cell type may modulate pseudotype virus infectivity. No specific function has thus far been attributed to HVR1 of HCV E2 glycoprotein. However, our results suggest that an early interaction between HCV and cell surface GAGs may facilitate virus attachment to target cells. A recent study with tick-borne encephalitis virus also suggested the existence of a host-cell receptor, other than HS, that can mediate virus entry (32). Basic amino acid residues are most frequently observed at positions 1, 3, 11, 14, 15, 25, and 27 of E2 HVR1 sequences of HCV (50). Both basic residues and basic patterns are conserved within the HVR1 sequence, arguing for involvement of positively charged residues of HVR1 in a biological function. However, the HVR1 sequence does not match with the known heparin-binding domain. Thus, the basic stretch of amino acids in HVR1 is likely to be involved in interactions with negatively charged molecules such as GAGs. HVR1 could thus be involved in the initial step of virus attachment and may possibly influence cell tropism.

An earlier study has shown that an infectious HCV cDNA clone lacking E2 HVR1 sequences is infectious but attenuated in the chimpanzee model (23). During the first few weeks of infection, ΔHVR1 virus replicated at unusually low levels; however, the virus titer increased with time of incubation (10 to 20 weeks). Some of the observed mutations in the HCV E2 region were implicated for the increase in virus titers. This ΔHVR1 virus stock from infected chimpanzee plasma, when transmitted to a naïve chimpanzee, displayed an increased virus titer to a peak level within a short time of incubation (3 to 9 weeks). These observations indicated that the HVR1 is not essential for the life cycle of HCV. Instead, the compensating mutations observed in E2 with ΔHVR1 virus were suggested to play a role in adaptation of virus fitness in chimpanzee hepatocytes. Interestingly, a different report (55) implicated human scavenger receptor class B type 1 (SR-BI) as a candidate receptor for HCV. The importance of HVR1 for binding of E2 with SR-BI on HepG2 cell surface suggested such hypothesis. However, our results from the present study suggest that deletion of HVR1 does not inhibit infection in a pseudotype system. This indicates additional regions of E2 ΔHVR1 may recognize alternate cell surface receptors for virus entry, although less efficiently in HepG2 cells. HSPG may provide advantages to E2 for attachment to cells over ΔHVR1. Cell types that carry large amounts of HSPG are endothelial and epithelial cells (41), and certain tissues, such as the liver, are very rich in HSPG expression (54). Thus, cell surface proteoglycans appear to be important for E2-G pseudotype virus attachment, and the presence of HVR1 is beneficial for HCV, especially with HepG2 cells, in early virus-host interaction. A further understanding of the strategies appropriate for mimic virus-host cell interaction may help in elucidating the mechanism of specific receptor recognition for HCV entry.

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ADDITIONAL PROOF


REFERENCES

19. Farci, P., A. Shimoda, D. Wong, T. Cabezon, D. DeGioannis, A. Strazzera, Y.


