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Characterization of Fusion Determinants Points to the Involvement of Three Discrete Regions of Both E1 and E2 Glycoproteins in the Membrane Fusion Process of Hepatitis C Virus[▽]

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Infection of eukaryotic cells by enveloped viruses requires the merging of viral and cellular membranes. Highly specific viral surface glycoproteins, named fusion proteins, catalyze this reaction by overcoming inherent energy barriers. Hepatitis C virus (HCV) is an enveloped virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. Little is known about the molecular events that mediate cell entry and membrane fusion for HCV, although significant progress has been made due to recent developments in infection assays. Here, using infectious HCV pseudoparticles (HCVpp), we investigated the molecular basis of HCV membrane fusion. By searching for classical features of fusion peptides through the alignment of sequences from various HCV genotypes, we identified six regions of HCV E1 and E2 glycoproteins that present such characteristics. We introduced conserved and nonconserved amino acid substitutions in these regions and analyzed the phenotype of HCVpp generated with mutant E1E2 glycoproteins. This was achieved by (i) quantifying the infectivity of the pseudoparticles, (ii) studying the incorporation of E1E2 and their capacity to mediate receptor binding, and (iii) determining their fusion capacity in cell-cell and liposome/HCVpp fusion assays. We propose that at least three of these regions (i.e., at positions 270 to 284, 416 to 430, and 600 to 620) play a role in the membrane fusion process. These regions may contribute to the merging of viral and cellular membranes either by interacting directly with lipid membranes or by assisting the fusion process through their involvement in the conformational changes of the E1E2 complex at low pH.

Enveloped viruses penetrate their host cells through a complex series of interactions between the viral surface and the cell membrane. This requires the attachment of the viral envelope glycoproteins to specific cell surface receptors and subsequent membrane fusion. Highly specific viral surface glycoproteins, named fusion proteins, catalyze the latter reaction by overcoming inherent energy barriers (10, 33). To date, two classes of virus fusion proteins have been defined (33): class I fusion proteins, the most well characterized of which is influenza virus hemagglutinin (HA) (62), and class II viral fusion proteins, exemplified by the E glycoprotein of tick-borne encephalitis virus (51), a flavivirus from the family *Flaviviridae*, and the E1 glycoprotein of Semliki Forest virus (26), an alphavirus from the *Togaviridae* family. Whereas their structural characteristics are markedly different, evidence suggests that class I and class II fusion proteins share an overall similar mechanism of membrane fusion (reviewed in reference 33). At an essential stage during fusion, the fusion protein bridges the gap between the viral and cell membranes by simultaneously interacting with them. The exposure and membrane insertion of a hydrophobic stretch of about 15 residues, called the "fusion peptides" or "fusion loops," mediate this crucial step (20, 54). For influenza virus HA and several other class I fusion proteins, the fusion peptide is located at the amino terminus of the transmembrane subunit. In contrast, the fusion peptide for other class I and class II fusion proteins is located internally and is thought to insert into the membrane as loops (33).

Recent evidence indicates that the simple picture of a viral fusion protein interacting with cell and viral membranes by means of only two localized segments, i.e., the fusion peptide and the transmembrane domain, is oversimplified. Instead, a more complex concerted action of different membranotropic segments of the fusion proteins seems to be required (33, 46). Further conformational changes are necessary to achieve the complete merging of the two lipid bilayers. Both class I and class II fusion proteins share common conformational rearrangements in order to drive the formation of different fusion intermediates (33, 54). Several regions of the fusion protein complex indirectly assist the fusion process, for example, the "stem" regions (24, 33, 46, 54). In contrast to the relatively simple organization of fusion peptides in influenza virus HA or in flavivirus E proteins, the two recently resolved crystal structures of herpes simplex virus type 1 glycoprotein gB (29) and vesicular stomatitis virus protein G (52, 53) revealed a bipartite structural fusion peptide composed of two relatively apolar hydrophobic loops.

Hepatitis C virus (HCV) is an important public health concern worldwide, as it is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. HCV is an enveloped virus that belongs to the hepacivirus genus of the *Flaviviridae* family (39).

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The two surface proteins, E1 (residues 192 to 383) and E2 (residues 384 to 746), are processed by signal peptidases of the endoplasmic reticulum from a 3,000-amino-acid-long polyprotein encoded by the HCV genome (reviewed in reference 48). The E1 (~31 kDa) and E2 (~70 kDa) proteins are glycosylated in their large amino-terminal ectodomains and are anchored into the membrane by their carboxy-terminal transmembrane domains. E1 and E2 form a heterodimer stabilized by noncovalent interactions. This oligomer is thought to be the prebudding form of the functional complex (41), which is present at the surface of HCV particles (42) and is involved in viral entry. HCV E2 is responsible for virion attachment to target cells and can bind different receptors that include several capture molecules, the CD81 tetraspanin, and the scavenger receptor BI (SR-BI) (reviewed in references 4 and 12). The role of E1 in HCV infection remains unclear; however, several antibodies directed against E1 are able to neutralize cell entry, presumably at a stage distinct from receptor binding (14, 32, 50).

Little is known about the molecular events that mediate cell entry and membrane fusion for HCV. Significant progress has been made with the development of HCV pseudoparticles (HCVpp), consisting of unmodified HCV E1E2 glycoproteins that are assembled with retroviral core particles (5, 17, 31), and of cell culture-grown infectious HCV (38, 61, 66). Extensive characterization of HCVpp showed that they mimic the early steps of the HCV life cycle (4, 12). Both this infection assay and a novel in vitro liposome fusion assay (36) have established that the fusion process for HCV is pH dependent (7, 31, 36), suggesting that cell entry of HCV occurs upon endocytosis (8) and that the low endosomal pH promotes the rearrangement of the fusion protein to its active form. This was confirmed by cell-cell fusion assays (34) and with cell culture-grown infectious HCV (8, 60), which behaves like HCVpp for cell entry steps (2, 4, 12).

In this report, we focused on identifying the determinants of HCV membrane fusion. By searching for classical features of fusion regions through the alignment of sequences from various HCV genotypes and subtypes, we identified six regions of HCV E1 and E2 glycoproteins. We introduced amino acid substitutions in these regions and analyzed the phenotypes of HCVpp generated with mutant E1E2 glycoproteins. We propose that at least three regions play a necessary role in membrane fusion. Altogether, our data are consistent with the notion that both E1 and E2 proteins harbor membrane fusion determinants, suggesting a complex figure in which several segments of HCV E1E2 are directly or indirectly involved in membrane fusion.

MATERIALS AND METHODS

Sequence analysis and structure predictions. Most analyses were performed using the IBCP euHCVdb database website facilities (http://euhcvdb.ibcp.fr) (13). Multiple sequence alignments and amino acid conservation were carried out with the CLUSTAL W program (58). The secondary structure of proteins was predicted using a combination of various methods available at the Network Protein Sequence Analysis website (58), including DPM, DSC, HNNC, MLRC, PHD, Predator, and SOPM (see http://npsa-pbil.ibcp.fr/NPSA and references therein). Sequences with a propensity to partition into the lipid bilayer were identified with Membrane Protein Explorer (MPEx; S. White laboratory [http://blanco.biomol.uci.edu/mpex/]) by using the interfacial setting based on experimentally determined hydrophobicity scales (63).

Cell lines. Huh-7 (40) and 293T (ATCC CRL-1573) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Perbio). Huh-7-Tat indicator cells were generated by the transduction of Huh-7 cells with a retroviral vector transducing the Tat plasmid (LXSN-tat, a kind gift from Olivier Schwartz, Institut Pasteur, Paris, France) and selected for G418 resistance. CHO cells (ATCC CRL-1582) and CHO-derived cells that express CD81 and SR-BI, CHO-CD81 and CHO-SR-BI, respectively, were maintained in RPMI (Invitrogen) with 10% fetal bovine serum.

Production of HCVpp. Mutations were introduced into E1E2 from genotype 1a strain H77 (58) (EMBL accession number AF009606) by site-directed mutagenesis (primer sequences are available upon request). All mutants were sequenced to ensure that the clones possessed only the expected mutation. For infection assays and Western blots, HCVpp were produced, as previously described (5), from 293T cells cotransfected with a murine leukemia virus (MLV) Gag-Pol packaging construct, an MLV-based transfer vector encoding a neomycin resistance gene, and each of the E1E2 expression constructs. For fusion assays (36), 293T cells were cotransfected with a human immunodeficiency virus type 1 (HIV-1) Gag-Pol packaging construct, an HIV-based transfer vector encoding the green fluorescent protein, the E1E2-expressing constructs, and, in order to specifically incorporate beta-lactamase (BlaM) into HIV-based particles, a chimeric protein encoding BlaM linked to the N terminus of HIV-1 viral protein R (Vpr) (9) (BlaM-Vpr was kindly provided by W. C. Green, Gladstone Institutes, San Francisco, CA). For Western blotting, immunoprecipitation assays, binding assays, and fusion assays, the pseudoparticles were purified and concentrated from the cell culture medium by ultracentrifugation at 82,000 × *g* for 1 h 45 min at 4°C through 5 ml of a 20% sucrose cushion in an SW28 rotor (Beckman Coulter). Viral pellets were suspended in phosphate-buffered saline (PBS) to concentrate the viral particles 100-fold. As a control for infection assays, immunoprecipitation assays, binding assays, and fusion assays, pseudoparticles devoid of viral glycoproteins were produced in parallel.

Incorporation of E1E2 glycoproteins onto viral particles. Viral pellets were subjected to immunoblot analysis using a mouse anti-HCV E1 antibody (A4) (19), a mouse anti-HCV E2 antibody (H52) (22), and a goat anti-MLV-CA antibody (anti-p30; Viromed). Viral pellet samples were mixed with 6× buffer (375 mM Tris-HCl [pH 6.8], 3% sodium dodecyl sulfate [SDS], 10% glycerol, and 0.06% bromophenol blue), and the samples were analyzed by electrophoresis in 10% polyacrylamide gels in the presence of 0.1% SDS. After protein transfer onto nitrocellulose filters, the blots were blocked in Tris-buffered saline (1 M, pH 7.4) with 5% milk powder and 0.1% Tween 20 (Sigma). The blots were probed with the different primary and secondary antibodies (1:10,000-diluted horseradish peroxidase-conjugated anti-mouse or anti-goat; Dako) in Tris-buffered saline–5% milk–0.1% Tween 20. Bound enzyme-labeled antibody was visualized using an enhanced chemiluminescence kit (SuperSignal West Pico chemiluminescent substrate; Pierce).

To perform immunoprecipitation assays, 293T cells were transfected with HCVpp expression plasmids, and the viral pellets were generated as described above for the production of HCVpp. The E1E2-transfected cells or pelleted virions were lysed in an immunoprecipitation buffer (0.05 M Tris-HCl, 0.15 M NaCl, 0.5% NP-40, 0.1% sodium deoxycholate, 0.1% SDS), and the cell lysates and medium containing HCVpp were precleared by overnight incubation with a 1:1 mixture of protein A- and protein G-Sepharose beads (Amersham Biosciences) at 4°C. After a centrifugation at 13,000 × *g* at 4°C for 5 min, the supernatants were incubated with the conformation-dependent anti-E2 monoclonal antibody H53 for 2 h at 4°C, and the immune complexes were precipitated using a 1:1 mixture of protein A- and protein G-Sepharose beads for 1 h at 4°C. The complexes were washed three times with the immunoprecipitation buffer and were then analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting using anti-E1 (A4) and anti-E2 (H52) antibodies.

Infection assay. Supernatants containing HCVpp were harvested 36 h after transfection and filtered through 0.45-μm-pore-size membranes. Huh-7 target cells (8×10^4 cells/well in a 12-well plates) were incubated with different dilutions of HCVpp harboring the mutant glycoproteins for 4 h at 37°C. Supernatants were removed, and cells were incubated in regular medium for 72 h at 37°C. Cells were detached and seeded in 6-well plates and selected with neomycin for 2 weeks at a concentration of 0.35 mg/ml. The infectious titers were expressed as infectious units (IU) per ml, as deduced from the number of resistant clones counted.

Binding assays. Fifty microliters of concentrated virus was mixed with 10^6 CHO, CHO-CD81, or CHO-SR-BI cells in the presence of 0.1% sodium azide for 1 h at 37°C. Cells were then washed twice with PBFA (PBS, 2% fetal bovine serum, and 0.1% sodium azide) and incubated in 100 μl at 40 μg/ml of the mouse anti-HCV E1 (A4) or mouse anti-HCV E2 (H53) (22) antibody for 1 h at 4°C. After two washes, cells were incubated with 100 μl of allophycocyanin goat anti-mouse immunoglobulin G (Jackson Immunoresearch) diluted in PBFA (1:200) for 45 min at 4°C. Five minutes before the two final washes in PBFA, cells

were counterstained with 20 μg/ml of propidium iodide (Sigma). Fluorescence of 10,000 living cells was analyzed with a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson). As a negative control for binding assays with CHO-CD81 and CHO-SR-BI cells, the binding of HCVpp on parental CHO cells that do not express either HCV receptor was determined. Each experiment was repeated at least three times.

Fusion assays. Both HCVpp/liposome lipid mixing and content mixing assays were performed as previously described (36). Liposomes were large unilamellar vesicles (100 nm) consisting of egg yolk phosphatidylcholine-cholesterol (Sigma). Briefly, for lipid mixing, R_{18}-labeled liposomes were obtained by mixing octadecyl rhodamine B chloride (R_{18}; Molecular Probes) and lipids in ethanol and chloroform solutions, respectively. Liposomes were prepared by extrusion over polycarbonate filters in PBS (pH 7.4). Lipid mixing between HCVpp and liposomes was monitored by the quenching of R_{18}. R_{18}-labeled liposomes (final lipid concentration, 15 mM) were added to a 37°C thermostable cuvette containing pseudoparticles in PBS (pH 7.4). After temperature equilibration and a pH decrease to 5, fusion kinetics were recorded on an SLM Aminco 8000 spectrofluorimeter over a 30-min time period, with an excitation wavelength (λ_{exc}) at 560 nm and an emission wavelength (λ_{em}) at 590 nm. Maximal R_{18} quenching was measured after the disruption of liposomes by the addition of 0.1% Triton X-100 (final concentration, vol/vol).

For HCVpp/liposome content mixing experiments, CCF2-loaded liposomes were obtained by resuspending the egg yolk phosphatidylcholine-cholesterol lipid film into a 100 μM coumarin cephalosporin fluorescein (CCF2)-FA (free-acid form; Invitrogen Life Technologies) solution in 25 mM HEPES–150 mM NaCl (pH 7.4). Unencapsulated CCF2 was removed by gel filtration over a PD-10 column (Amersham Biosciences). Pseudoparticles and liposomes were mixed in a cuvette, and the pH was decreased to 5. The final concentration of CCF2 used in the assay was 25 μM, and the total lipid concentration was 150 μM. CCF2 is a cephalosporin-derived molecule bearing coumarin-derived and fluorescein moieties in close proximity due to their association with the beta-lactam ring. In the intact substrate, excitation of the coumarin donor at 409 nm leads to internal fluorescence resonance energy transfer to the fluorescein acceptor and emission of green light (520 nm). When liposome and pseudoparticle contents coalesce as a result of fusion, beta-lactamase-catalyzed hydrolysis of CCF2 separates the donor and acceptor. The coumarin donor then emits blue fluorescence (maximum, 447 to 450 nm), whereas the fluorescein acceptor is quenched. Content mixing was visualized by monitoring the increase of coumarin fluorescence kinetically at λ_{exc} at 409 nm and λ_{em} at 450 nm.

For cell-cell fusion assays, 293T "donor" cells (2.5×10^5 cells/well seeded in six-well tissue culture dishes 24 h prior to transfection) were cotransfected using calcium phosphate reagent with 2 μg of phCMV-H77-wt or mutated E1E2 glycoproteins and 20 ng of an HIV-1 long terminal repeat (LTR)-luciferase reporter plasmid (a kind gift of Françoise Bex, Institut de Recherches Microbiologiques Jean-Marie Wiame, Belgium) (35). For a positive control, cells were cotransfected with 1 μg of phCMV-HA and 1 μg of phCMV-NA, encoding the fowl plaque virus hemagglutinin and neuraminidase, respectively (57), and 20 ng of the HIV-1–LTR–luciferase reporter plasmid. For a negative control, cells were cotransfected with 2 μg of empty phCMV plasmid and 20 ng of the HIV-1–LTR–luciferase reporter plasmid. Twelve hours later, transfected cells were detached with Versene (0.53 mM EDTA; Invitrogen), counted, and reseeded at the same concentration (10^5 cells/well) in six-well plates. Huh-7-Tat indicator cells (4×10^5 cells per well), detached with EDTA and washed, were then added to the transfected cells. After 24 h of cocultivation, the cells were washed with PBS, incubated for 5 min in a pH 5 fusion buffer (130 mM NaCl, 15 mM sodium citrate, 10 mM MES [morpholineethanesulfonic acid], 5 mM HEPES), and then washed three times with medium. The luciferase activity was measured 24 h later using a luciferase assay kit according to the manufacturer's instructions (Promega).

RESULTS

Predictions for localization of fusion sequences. Assuming that HCV glycoproteins belong to class II fusion proteins, one may expect that its putative fusion peptide or loop should exhibit structural features similar to those of other phylogenetically related flavivirus E and alphavirus E1 glycoproteins. First, the class II fusion loops harbor highly conserved sequence motifs (e.g., DRGWGNGCGLFGKG for most flaviviruses [51] and GVYPFMWGGAYCFCDSEN for most alphaviruses [26]). Second, the fusion sequences contain particular residues that are crucial for the capacity of the peptide to interact and destabilize target lipid membranes. These sequences are generally rich in glycine residues. They also include hydrophobic residues (20), especially aromatic residues such as tryptophan and, to a lesser extent, tyrosine. The aromatic residues are known to preferentially interact at the membrane interface (28, 65). Typically, the fusion region from alphaviruses and flaviviruses includes at least three glycines, a tryptophan, two phenylalanines, and some other large hydrophobic residues (Y, L, and M in the one-letter amino acid code). Meanwhile only a few charged residues have been found (D, E, R, and K). In addition, these fusion sequences may include cysteine residues that are involved in specific disulfide bridges essential for the folding stability of the fusion motif (26, 51). Thirdly, fusion peptides exhibit an inherent hydrophobicity, allowing their preferential interaction with lipid membranes. Typically, the internal fusion peptides of class II viral fusion proteins display a high interfacial hydrophobicity when analyzed with the Wimley and White interfacial hydrophobicity scale (63). Finally, class II fusion peptides form a loop which does not exhibit a periodic secondary structure and which is located at the end of a rod-like domain composed of antiparallel beta-sheets (26, 51).

Using these criteria to analyze the HCV E1 and E2 amino acid sequences, we selected one region in E1 and five regions in E2 that could potentially exhibit fusogenic-region features (Fig. 1A). To identify the most conserved residues in these regions, we analyzed the variability of amino acids at each sequence position after aligning 26 reference sequences from confirmed HCV genotypes/subtypes (55). The interfacial hydrophobicity was calculated using the Wimley and White interfacial hydrophobicity scale, and all selected regions were predicted to have a propensity to interact with membranes (Fig. 1B). The secondary structure of E1 and E2 was predicted using a large set of prediction methods (Fig. 1C). Sequence segments that were predicted to be an alpha-helix or that did not contain conserved glycine, tryptophan, or hydrophobic residues as well as any neighboring cysteine residues were ignored. Regions I and IV matched previously predicted fusion peptide candidates (25, 64). Two other regions (regions II and III) were located between hypervariable region 1 (HVR1) and HVR2 of E2. Region II was the most conserved but included three potential glycosylation sites, while region III contained a GWG motif observed in flavivirus fusion peptides. Regions V and VI were located between HVR2 and the stem region of E2 that seems to be critical for E1E2 dimerization and cell entry (18).

The functional role of these six regions in cell entry and membrane fusion was assessed by in vitro site-directed mutagenesis of conserved amino acids, i.e., glycine, tryptophan, and tyrosine residues (1). The aim was to introduce a loss of activity, as reported previously for other fusion peptides. The effect of conservative (e.g., W to F or G to A) and nonconservative (e.g., W to A and G to D) substitutions (Table 1) was investigated by incorporating mutated E1E2 into HCVpp. Each substitution was characterized in cell infection and cell binding assays and by determining in vitro membrane fusion properties for the most relevant mutants. We also introduced mutations into a set of conserved amino acids in HVR1 (6, 47) or into residues involved in CD81 binding (15, 44) that were

FIG. 1. Definition and localization of fusion peptide candidates in HCV E1 and E2 glycoproteins. (A) The E1 and E2 glycoproteins are drawn to scale as boxes. Amino acid positions relate to the HCV H77 sequence (genotype 1a) (EMBL accession number AF009606). The transmembrane domains (TMD) of E1 and E2 are represented as black boxes, HVR1 and HVR2 are shown as vertical shaded boxes, and the stem region is shown as an oblique shaded box. The locations of the six regions defined as fusion peptide candidates in this study are indicated by roman numerals I to VI. The sequence features of each region are reported for the HCV strain H77 sequence used in this study in the middle and the bottom of the figure. (B) Interfacial hydrophobicity plots corresponding to E1 and E2 were generated with MPEx Web facilities (http://blanco.biomol.uci.edu/mpex/) by using the Wimley and White interfacial hydrophobicity scale (63). The segments exhibiting a propensity to partition into the lipid bilayer are indicated by black bars on the top of the graph. (C) Amino acid similarity at each position between the various HCV genotypes was deduced from an alignment of 26 reference sequences of confirmed HCV genotypes/subtypes (55). Identical, strongly conservative, and weakly conservative amino acids are indicated by stars, colons, and dots, respectively, according to ClustalW conventions (58). Sec.struct.cons, secondary structure consensus deduced from seven prediction methods (DPM, DSC, HNNC, MLRC, PHD, Predator, and SOPM). Predictions were done according to three folding states. h, alpha-helix, e, extended (beta-sheet), c, coil (aperiodic structure). The mutated amino acids are indicated in boldface type in the HCV H77 sequence. The various point mutations and the corresponding amino acid substitutions performed in this study are indicated below the HCV sequence (boldface type).

not localized in regions predicted to be fusion peptide candidates (Table 1). Since these residues were not likely to affect cell entry at a membrane fusion stage, the corresponding mutants were used as controls and were referred to as "control mutants" in Fig. 2 through 6 and as belonging to "control regions."

Cell entry properties of HCVpp harboring mutant E1 and E2 glycoproteins. To analyze the phenotype of each mutation, we derived 29 E1E2 glycoproteins harboring point substitutions (listed in Table 1) spanning all six regions selected by sequence analysis (Fig. 1). HCVpp containing these mutant E1E2 glycoproteins or containing the control mutant E1E2 glycoproteins

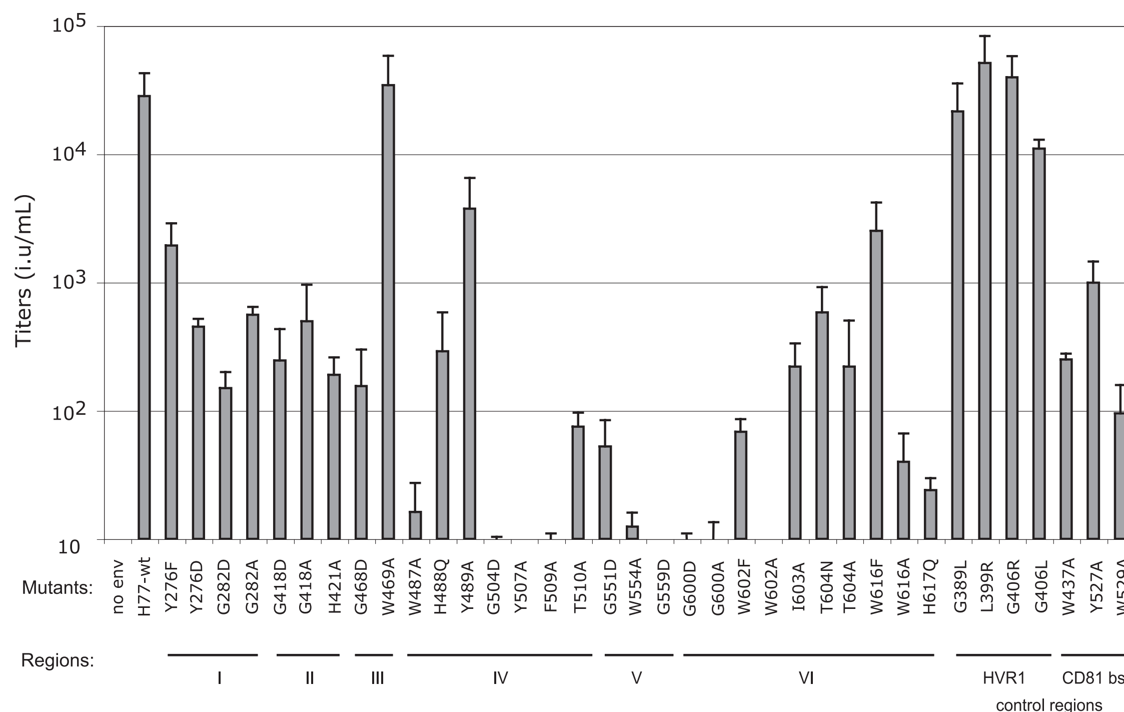

FIG. 2. Cell entry properties of HCVpp harboring E1E2 mutants. Shown are results of cell entry assays using Huh-7 cells of the different HCVpp generated with the different E1E2 point mutants in regions I to VI as well as in a "control" regions, i.e., HVR1 and CD81 binding sites. Huh-7 target cells were seeded in 12-well plates at a density of 8×10^4 cells per well and incubated with HCVpp for 4 h at 37°C. The infectious titers, expressed as IU per ml, were determined as the number of resistant clones after G418 selection. The data are the means of three independent experiments. H77-wt, wild-type H77 E1E2 envelope glycoproteins.

were prepared with a recombinant genome encoding the neomycin resistance (Neo^r) marker, which allows a precise determination of infectious titers within a range of 4 logs (ca. 10 to 100,000 IU/ml). HCVpp harboring mutations in conserved residues of HVR1 (G389L, L399R, G406R, and G406L) had wild-type titers, whereas mutations in residues involved in CD81 binding (W437A, Y527A, and W529A) reduced HCVpp infectivity by 20- to 200-fold (Fig. 2). As for mutations in the selected fusion region candidates, only a few of the E1E2 mutants turned out to be functional in cell entry assays using Huh-7 target cells (Fig. 2 and Table 1). Two mutants were nearly as infectious as the wild type, one in region III (W469A) and one in region IV (Y489A). Eight out of the 29 mutants were completely noninfectious and were localized in regions IV (W487A, G504D, Y507A, and F509A), V (G559D), and VI (G600D, G600A, and W602A). Thus, a large number of mutant HCVpp had a reduced cell entry capacity, with a 10- to 1,000-fold reduction in infectious titers compared to HCVpp harboring wild-type E1E2 glycoproteins. These relative differences in infectious titers were consistent even when PLC/PRF/5 or Hep3B hepatocarcinoma cells were used as target cells, rather than the Huh-7 cells (data not shown).

Viral incorporation of E1E2 mutant glycoproteins on HCVpp. In order to dissect at which stage of infection the defective mutants were affected, we analyzed the expression and incorporation of the different E1E2 glycoproteins on HCVpp. We analyzed the expression of E1E2 loaded on HCVpp by Western blotting using the A4 anti-E1 and H52 anti-E2 antibodies that recognize linear epitopes outside the fusion peptide regions. We verified that all the mutant glycoproteins were correctly synthesized. The Western blot analysis of HCVpp producer cell lysates indicated that all E1 and E2 mutants had expression levels and patterns similar to those of the wild-type glycoproteins (Fig. 3A). After virus purification, we compared the levels of incorporation of E1 and E2 on HCVpp. Wild-type E2 and E1 glycoproteins, which contain complex carbohydrates, migrate diffusely at 80 kDa and 30 kDa, respectively. HCVpp generated with mutants in control regions, i.e., HVR1 and the CD81 binding site, incorporated wild-type levels of E1E2 glycoproteins (Fig. 3B), as previously reported (6, 15, 44). In contrast, a number of mutants in the putative fusion regions exhibited altered E1 and/or E2 incorporation levels, i.e., Y276D, G282D, H421A, G468D, W487A, H488Q, G504D, Y507A, F509A, T510A, G551D, W554A, G559D, G600D, G600A, W602A, W602F, I603A, T604N, T604A, and H617Q. The results indicated that these conserved residues are crucial for the structure and/or assembly of the E1E2 complex. With the exception of mutants in region V, some E1E2 mutant glycoproteins were incorporated at densities similar to that of wild-type E1E2. Some discrepancies in the size of incorporated E2 were also observed for some mutants, i.e., G282D, H421A, G504D, Y507A, F509A, and G559D, and correlated with reduced E1 incorporation and a loss of infectivity. Treatment of these viral particles with PNGase indicated that the size differences were due to differences in their glycosylation patterns (data not shown). The comparison of E1E2 incorporation to the infectious titer allowed us to distinguish three phenotypes. For 21 out of 29 mutants (Table 1), a defect in the incorporation of E1 and/or

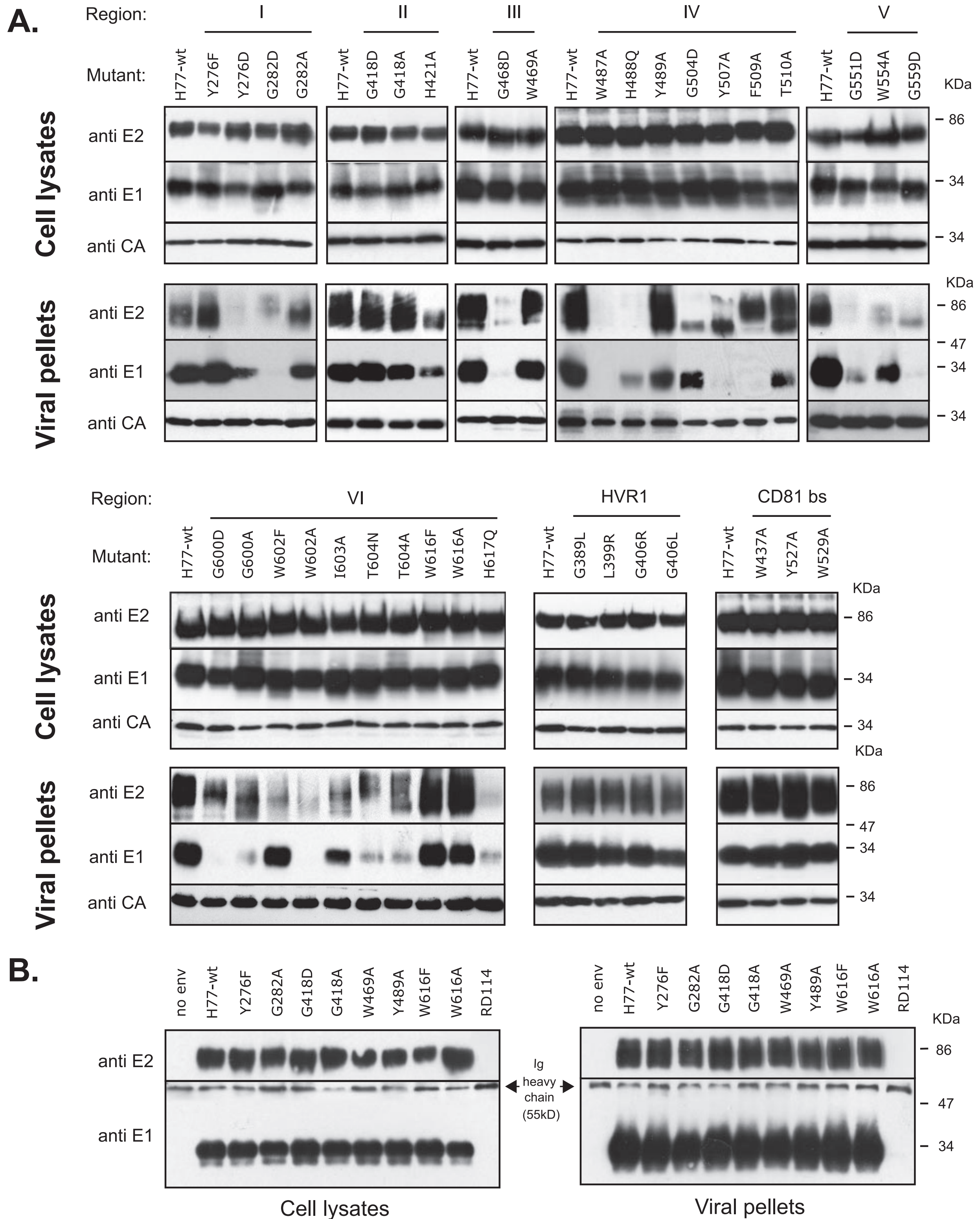

FIG. 3. Expression and incorporation of E1E2 glycoproteins onto HCVpp. (A) The expression of the mutant E1E2 glycoprotein was verified by immunoblots of lysates of HCVpp producer cells using an anti-E1 antibody (A4) and an anti-E2 antibody (H52). The incorporation of the E1E2 envelope was analyzed by immunoblots of viral particles pelleted and passed through 20% sucrose cushions. Viral pellets were analyzed by Western blotting using theA4 and H52 antibodies and an anticapsid (anti-p30, MLV-CA) antiserum (ViroMed Biosafety Laboratories). (C) The folding and heterodimerization of E1 and E2 glycoproteins that were incorporated on the HCVpp, i.e., mutants Y276F, G282A, G418A, G418D, Y489A, W616F, W616A, and W469A, were analyzed by coimmunoprecipitation of purified viral particles with the H53 antibody, which recognizes a conformational epitope on E2, followed by immunoblots of pellets using E1 (A4) and E2 (H52) antibodies. H77-wt, wild-type H77 E1E2 envelope glycoproteins.

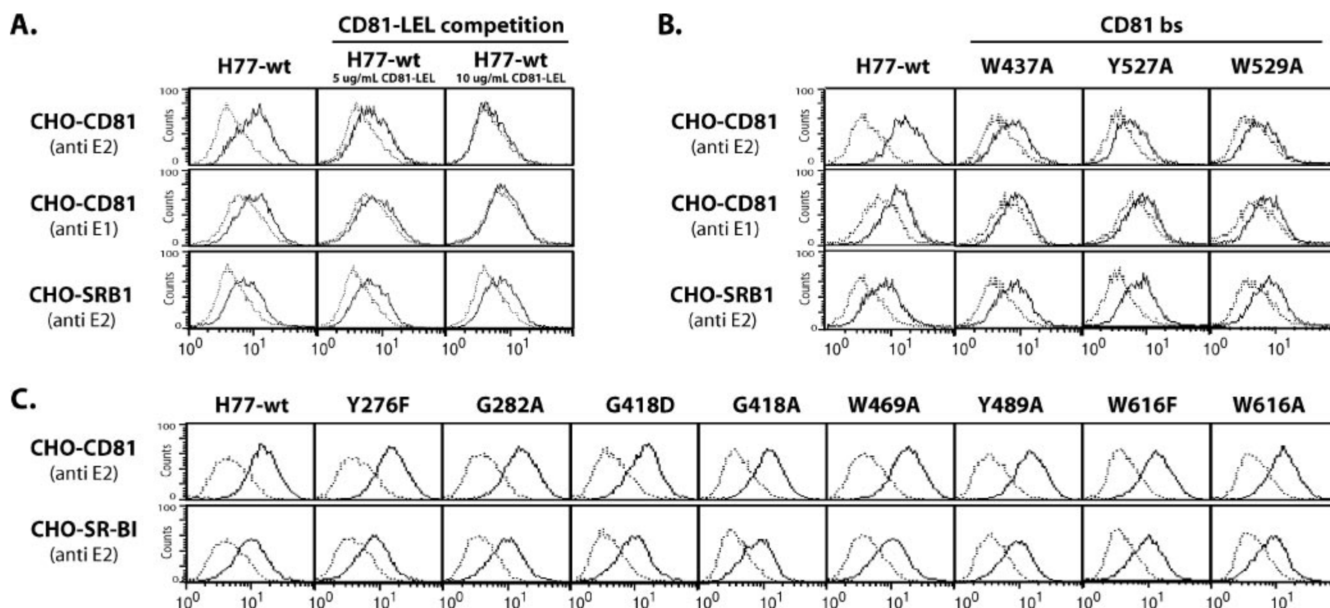

FIG. 4. Cell surface binding of HCVpp harboring E1E2 mutants. CHO, CHO-CD81, or CHO-SR-BI cells were incubated with 50 μl of the concentrated viral particles for 1 h at 37°C in the presence of sodium azide. The HCVpp bound on native CHO (dotted lines) or on either HCV receptor-expressing CHO cell line (plain lines) were detected with anti-E2 (H53) or with anti-E1 (A4) mouse antibodies and using anti-mouse allophycocyanin-conjugated antibodies. The fluorescence was analyzed with a fluorescence-activated cell sorter (FACSCalibur; Becton Dickinson). The data are representative of three independent experiments. (A and B) Control binding assays were performed using CD81 LEL as a competitor for the binding of wild-type HCVpp (H77-wt) or using HCVpp harboring E1E2 glycoproteins mutated in CD81 binding sites. (C) Binding assays of HCVpp harboring selected mutations in regions I, II, III, IV, and VI of E1E2.

E2 glycoproteins correlated with a defect of the infectious titer. Since these mutations had a dramatic effect on protein folding and/or assembly, they were not further investigated. For two other mutants, i.e., W469A and Y489A, no or little difference in either E1E2 incorporation or infectious titers was observed compared to wild-type E1E2. The fact that these nonconservative mutations had no effect on their phenotype suggests the absence of a fusion peptide in regions III and IV. Interestingly, six mutants, i.e., Y276F, G282A, G418A, G418D, W616F, and W616A, exhibited a profile of E1 and E2 incorporation similar to that of the wild-type glycoproteins. However, their infectious titers were reduced by 50-fold (Y276F, G282A, and W616F) or greater (G418A, G418D, and W616A). To further analyze the biochemical properties of these mutants, we immunoprecipitated the corresponding viral particles with the H53 conformation-dependent antibody (42). The pellets of precipitates were then analyzed by immunoblotting using E1 or E2 antibodies (Fig. 3B). Reflecting proper E1E2 folding and heterodimerization, we could detect both HCV glycoproteins from the HCVpp harboring these selected mutants, and no significant difference could be observed compared to wild-type HCVpp. Taken together, these data indicated that while the folding and incorporation of these E1E2 mutants were not altered, they could not fulfill all the cell entry steps. We conclude that regions I, II, and VI, which include these six mutants (Y276F, G282A, G418A, G418D, W616F, and W616A), may harbor fusion region candidates.

Receptor binding properties of cell entry-defective E1E2 mutant glycoproteins. The cell entry process starts with the binding of the viral particles to the surface of the target cells via interactions between viral glycoproteins and specific cell surface receptors. In principle, mutations in the fusion regions should not affect such interactions. Therefore, to further characterize our mutants, i.e., Y276F, G282A, G418A, G418D, W469A, Y489A, W616F, and W616A, we studied the ability of HCVpp harboring the corresponding E1E2 glycoproteins to bind the surface of Huh-7 cells. The binding of purified HCVpp was assessed by flow cytometry using either anti-E2 or anti-E1 antibodies, as described previously for determining receptor binding levels of alternative pseudoparticles (37). No difference was observed between HCVpp harboring E1E2 mutant glycoproteins and wild-type E1E2 in binding to Huh-7 cells (data not shown). Of note, the binding of HCVpp to Huh-7 cells reflects the contribution of all HCV receptors, CD81, SR-BI, and, particularly, capture molecules such as heparan sulfates (3). Thus, to measure the ability of our selected mutants to interact with the CD81 and SR-BI HCV cell entry receptors, we next compared the binding of purified HCVpp on native CHO cells and CHO cells expressing either CD81 or SR-BI.

The specificity of HCVpp binding to CD81 was demonstrated by competition assays or by using CD81 binding-deficient E2 glycoproteins incorporated on the HCV particles (Fig. 4A and B). The addition of the purified CD81 large extracellular loop (LEL) during binding assays abrogated the binding of HCVpp harboring wild-type E1E2 glycoproteins on CHO-CD81 cells (Fig. 4A). Furthermore, HCVpp with mutations in E2 that impair binding to CD81, e.g., the W437A, Y527A, and W529A point mutants (15, 44), had strongly reduced binding to the CHO-CD81 target cells, as detected using E1 or E2 antibodies (Fig. 4B). As expected, no alteration of binding to CHO-SR-BI cells was detected for these CD81-binding mu-

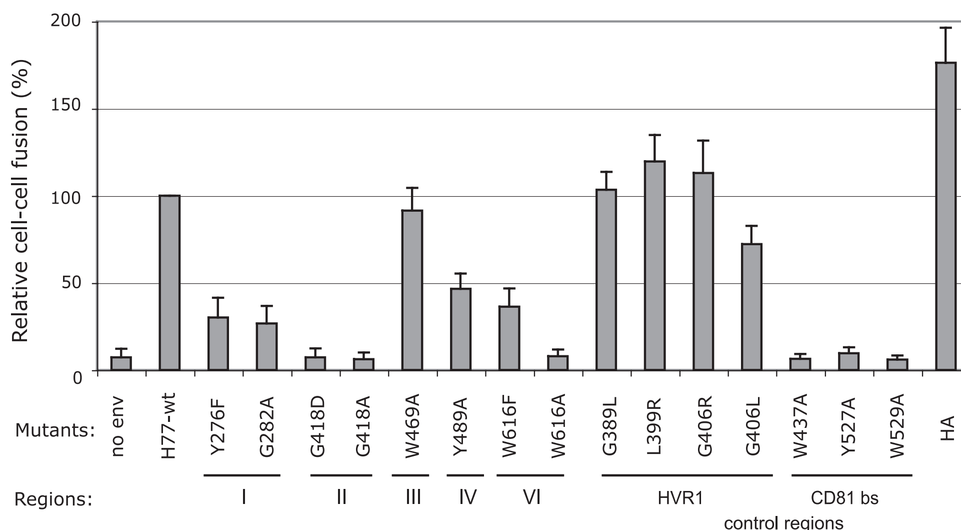

FIG. 5. Cell-cell properties of E1E2 mutants. 293T "donor" cells coexpressing the HCV E1E2 or HA envelope glycoproteins and a luciferase marker gene under the control of the HIV-1 promoter were cocultured with Huh-7-Tat "indicator" cells expressing the HIV-1 Tat protein. After 24 h, the cells were treated at pH 5 for 5 min, and the luciferase activity induced by the fusion between donor and indicator cells was measured 24 h later. Fusion mediated by wild-type H77 E1E2 envelope glycoproteins (H77-wt) with Huh-7-Tat indicator cells at pH 5 was taken as 100%. The graphs represent the averages of three independent experiments.

tants or when using CD81 LEL in binding assays with wild-type HCVpp (Fig. 4A and B). Altogether, these results validated the HCVpp binding assays.

HCVpp with the selected panel of mutations in the putative fusion regions had binding levels similar to those of HCVpp harboring wild-type E1E2 glycoproteins on either CHO-CD81 or CHO-SR-BI cells as determined using anti-E2 (Fig. 4C) and anti-E1 (data not shown) antibodies. The low infectious titers of the Y276F, G282A, G418A, G418D, W616F, and W616A mutants thus could not be explained by a defect in their cell binding capacity.

Mutations in region I of E1 and regions II and VI of E2 inhibit HCVpp membrane fusion. Since our results indicated a normal capacity of the selected mutant proteins to assemble on viral particles (Fig. 3) and to mediate cell binding (Fig. 4) compared to wild-type E1E2 proteins, the loss in infectivity of the mutants could be due to a defect in the membrane fusion process. To address this point, we performed cell-cell fusion (syncytium) assays (34), whereby 293T "donor" cells, expressing a luciferase marker gene under the control of the HIV-1 promoter, were cocultured with Huh-7-Tat "indicator" cells, expressing the HIV-1 transactivator of transcription (Tat) protein. Since the HIV-1 promoter requires Tat for efficient expression, only fused cells should express detectable levels of luciferase. To test the presence of putative fusion determinants in the selected regions, donor cells were transfected with expression plasmids encoding wild-type or mutant E1E2 glycoproteins. The transfected cells were then cocultivated with the Huh-7-Tat cells for 1 day. The medium of the coculture was briefly acidified at pH 5 to trigger E1E2 fusogenicity, and the next day, the luciferase activity of the coculture cell lysates was determined as a measurement of fusion extent. Although cell-cell fusion events could be detected at a neutral pH, as reported elsewhere previously (21), lowering the pH of the culture enhanced syncytium formation between E1E2-expressing cells and target cells (data not shown). Overall, the results were in agreement with the results of infection assays. Mutants in the CD81 binding sites had reduced cell-cell fusion activity (Fig. 5), concomitant to decreased infectious titers (Fig. 2), which was expected, given their inability to bind CD81 (Fig. 4). Reduced cell-cell fusion events were detected with the Y276F, G282A, G418A, G418D, Y489A, W616F, and W616A mutants compared to wild-type E1E2 (Fig. 5). Thus, since the mutants in the putative fusion regions were fully competent for CD81 binding, the results of cell-cell fusion assays indicated that they were impaired in their capacity to mediate membrane fusion.

To further address the membrane fusion properties of our set of E1E2 mutants, we used an in vitro liposome/HCVpp fusion assays that we recently developed (36). In the first step of the fusion process, the fusion peptide interacts with the target membrane and destabilizes it without generating a fusion pore (lipid mixing). This leads to the formation of a hemifusion intermediate characterized by the formation of a stalk between the two membranes. In the second step, the conformational rearrangements of the fusion proteins provide the driving force to open a fusion pore (33) to allow the mixing of the contents of viral particles and cells (content mixing). In order to investigate the HCV-mediated membrane fusion process, we recently developed a variety of virus-liposome fusion assays to study lipid mixing or content mixing (36). The lipid mixing assay is based upon the direct measurement of mixing between HCVpp and liposome lipids. Briefly, R_{18} fluorescent lipid dye was incorporated into liposomes. Lipid dilution upon fusion between liposome and viral membranes at low pH leads to fluorescence dequenching of this probe for pH-dependent virions (30), but no dequenching was observed for pH-independent viruses such as MLV (36). As reported previously for wild-type HCVpp, R_{18} dequenching was observed only when the pH was decreased to 5.0, and no significant dequenching could be detected at a neutral pH even after long incubation

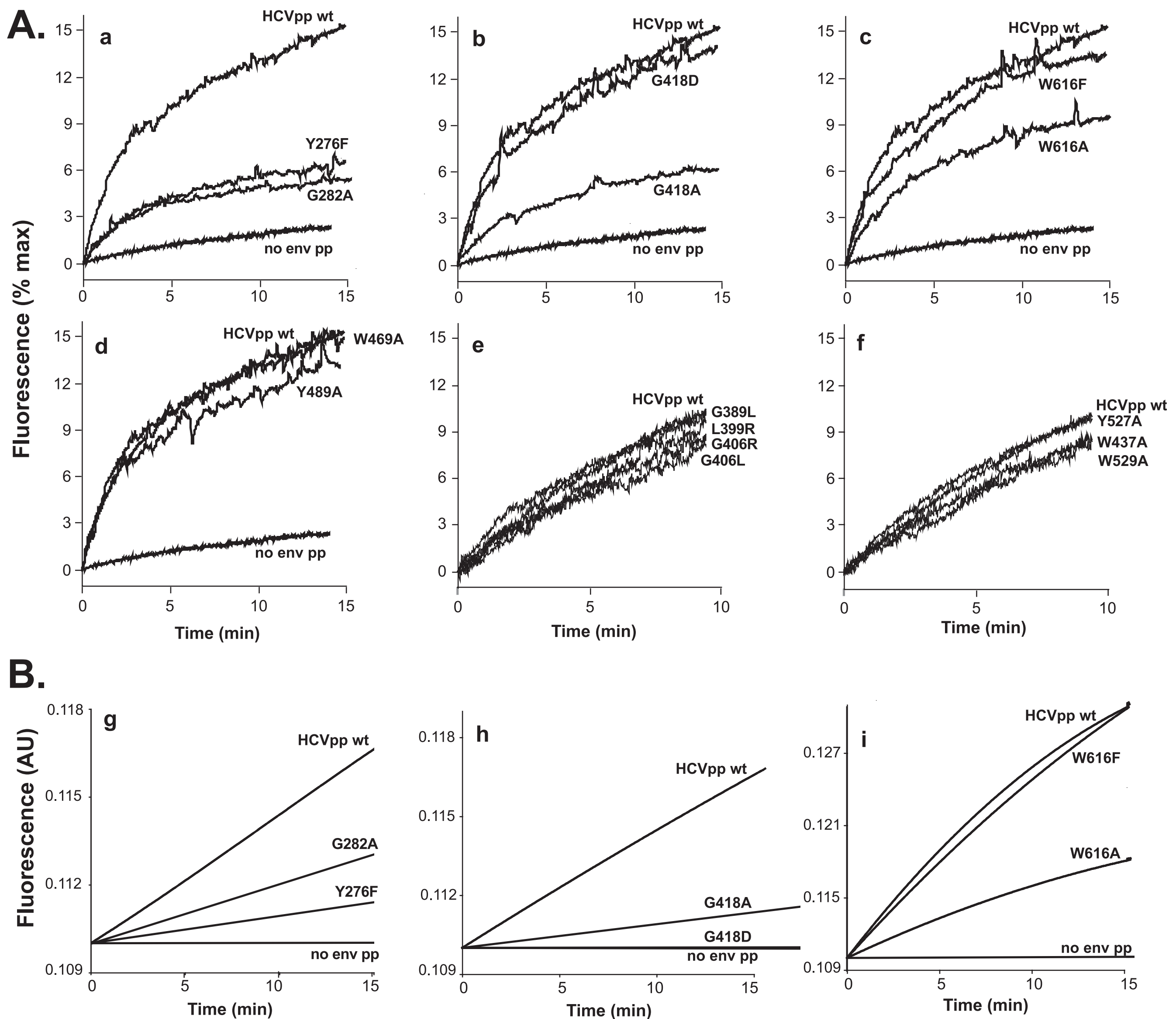

FIG. 6. Membrane fusion properties of HCVpp harboring E1E2 mutants. The fusion capacities of HCVpp harboring wild-type (HCVpp wt) or mutant E1E2 proteins, as indicated, were tested using either lipid mixing or content mixing assays. (A) For lipid mixing assays, 40 μl of purified pseudoparticles was added to R_{18}-labeled liposomes (final lipid concentration, 15 mM) in PBS (pH 7.4). After a 2-min equilibration at 37°C, fusion was initiated by decreasing the pH to 5 in the cuvette (time zero of the fusion kinetics). The results are expressed as percentages of maximal fluorescence obtained by the addition of Triton X-100 (final, 0.1% [vol/vol]) to the pseudoparticle/liposome suspensions. (B) For content mixing, 80 μl pseudoparticles was added to CCF2-containing liposomes in PBS (pH 7.4). After a 2-min equilibration at 37°C, fusion was initiated by decreasing the pH to 5 in the cuvette (time zero of the fusion kinetics). The results are presented as arbitrary units (AU) of fluorescence, and noisy curves were subjected to mathematical smoothing. For all panels, experiments were repeated four times, and the most representative curves are presented here. For the sake of comparison, only the first 15 min of the reaction are shown. (a and g) Mutants in region I. (b and h) Mutants in region II. (c and i) Mutants in region VI. (d) Mutants in regions III and IV. (e) Mutants in HVR1. (f) Mutants in CD81 binding sites.

times (36). The content mixing assay is based on the ability to measure enzymatic cleavage of the CCF2 aqueous fluorescent probe (encapsulated in the liposomes) by beta-lactamase (BlaM, contained in HCVpp). There is no maximal value measurable in this assay; therefore, the results are expressed as absolute values of fluorescence. As a consequence, a direct comparison of numerical values of lipid mixing and content mixing results is not possible. Instead, the comparison was performed in terms of tendency of the fusion behavior.

We used these two assays to investigate the fusion properties of selected E1E2 mutants containing point mutations in regions I (positions 276 and 282), II (position 418), III and IV (positions 469 and 489), and VI (position 616). Wild-type HCVpp and pseudoparticles devoid of viral surface protein were used as references for "fusion-responding" and "fusion-defective" viral particles, respectively (Fig. 6). We also used control pseudoparticles harboring mutations in HVR1 or in CD81 binding sites to validate this fusion assay. Compared to

wild-type E1E2, mutations of residues in HVR1 or in the CD81 binding site did not significantly alter the capacity of the corresponding HCVpp to mediate liposome fusion (Fig. 6A, panels e and f), which reflects the fact that membrane fusion occurs in the absence of HCV receptors in this experimental system. In contrast, the fusogenicity of mutants in region I (Y276F and G282A) was impaired compared to wild-type HCVpp in both lipid and content mixing assays (Fig. 6A, panel a, and B, panel g). Therefore, E1 most probably contains a membrane fusion determinant. Concerning region II, the G418A mutant was strongly impaired in both lipid and content mixing assays. Surprisingly, the G418D mutant displayed a normal lipid mixing pattern but a defect in content mixing (Fig. 6A, panel b, and B, panel h). This suggests a block at the stage of hemifusion, for which the outer leaflets of the viral and liposome membranes are mixed, while the inner leaflets are still distinct. Such a situation would indeed lead to R_{18} dequenching (lipid mixing) but not to the cleavage of CCF2 by BlaM (content mixing). Together, these findings revealed a role for region II of E2 in the membrane fusion process. In region VI, whereas the W616F change led to no significant alteration of the fusion phenotype compared to wild-type E1E2 (Fig. 6A, panel c, and B, panel i), the W616A substitution induced a substantial decrease in the fusion capacities of the corresponding mutant HCVpp in lipid- and content-mixing assays. This observation points to the importance of an aromatic side chain at position 616 and supports the possible involvement of region VI in the HCV fusion process. Finally, HCVpp harboring E2 glycoproteins mutated in regions III (W469A) and IV (Y489A) exhibited no significant alteration in their fusion pattern compared to that of wild-type HCVpp (Fig. 6A, panel d). Together with the data for infectivity (Table 1), this strongly suggests that the two latter regions do not contain a fusion determinant.

In summary, these results revealed that one region of E1 and two regions of E2 could contain a fusion determinant involved in the membrane fusion process of HCV: regions I (residues 273 to 290), II (residues 417 to 433), and VI (residues 597 to 620).

DISCUSSION

Based on the similarity to other flaviviruses, it is likely that HCV harbors a class II fusion protein; however, the HCV cell entry and membrane fusion steps differ from other flaviviruses in many respects (4). First, the identity of the HCV fusion protein remains controversial because opposing studies have found that the localization of fusion peptides and loops could be in either E1 or E2 (11, 16, 23, 25, 45, 49, 64). The typical class II fusion protein from flaviviruses and alphaviruses is expressed as a noncovalent heterodimer with a second membrane glycoprotein, known as the “companion” protein, that is located N terminal to the former protein (prM for the flaviviruses and pE2 for alphaviruses). Shortly before release from the cell, activation of the fusogenic potential occurs by cleavage of the companion protein (33). In contrast to alphavirus and flavivirus companion proteins, the HCV E1 and E2 glycoproteins are highly glycosylated and are not matured by a cellular endoprotease (42). Second, different from the glycoproteins of influenza viruses, flaviviruses, and alphaviruses, the HCV glycoproteins are resistant to inactivation by low pH (60), suggesting that HCV pH sensitivity occurs during cell entry. Finally, cell entry of HCV requires E1E2 interactions with at least three cell surface molecules (4, 12), which could trigger conformational rearrangements and/or promote acid pH sensitivity of the E1E2 glycoproteins.

In this study, we evaluated the role of six possible fusion regions that we and others (11, 16, 23, 25, 45, 49, 64) identified in conserved regions within E1 and E2 HCV glycoproteins of different genotypes and subtypes. We introduced conserved changes at amino acids predicted to be critical for the properties of fusion and analyzed the phenotypes of the mutants in assays based on infectious HCVpp. Altogether, the results gathered from cell entry, receptor binding, and fusion assays allowed us to distinguish between three mutant phenotypes.

A first group of mutants had a primary defect in the incorporation of E1 and E2 into HCVpp, likely due to glycoprotein misfolding, which most likely explained their low infectious titers. These mutants could therefore not be further investigated with additional techniques used in this study.

A second group of mutants had a wild-type phenotype, suggesting that corresponding regions III and IV may not harbor a fusion peptide or loop. Indeed, the nonconservative W469A mutation in region III, containing a GWG motif known to be critical in the fusion peptide of flaviviruses (51), had no effect on infectivity and fusion. Similarly, the Y489A mutation in the WHY motif in region IV led to a near-wild-type phenotype. Based on computational analyses to generate models of HCV E2, this region, region IV, has been proposed by others to harbor a fusion peptide (64); however, our results provided no experimental data to support this hypothesis.

In the last group of mutants, six E1E2 mutants (Y276F, G282A, G418A, G418D, W616A, and W616F) had no defect in incorporation onto HCVpp and cell surface binding; however, the infectious titers of these viral particles were reduced by at least 20-fold compared to the wild type. These mutations disrupted the membrane fusion step as judged from cell-cell and liposome/HCVpp fusion assays (Fig. 5 and 6). The behavior of these mutants suggests a role for region I of E1 and regions II and VI of E2 in the HCV fusion process.

Of the four mutants generated in region I of E1 (positions 265 to 296) at positions 276 and 282, the two conservative mutations (Y276F and G282A) did not affect the incorporation of the E1E2 complex but significantly reduced the infectious titers of HCVpp (Table 1). Interestingly, this region had been previously proposed to be a fusion peptide on the basis of secondary structure predictions (25). Moreover, the inhibition of cell entry in the presence of these mutants was clearly established using the in vitro liposome/HCVpp fusion assay (Fig. 6). Our results suggest that region I of E1 is a convincing fusion region candidate for virus-cell fusion. These results are also supported by a recent study (16) that indicated that mutations in this region abolished viral entry without affecting E1E2 heterodimerization. Moreover, another report using peptide libraries derived from E1E2 that had analyzed their effect on membrane integrity indicated an active role for the E1 segment at positions 265 to 296 (49). While the latter study did not provide conclusive evidence because the effect of peptides on membranes does not constitute very specific criteria,

TABLE 1. Phenotypes of different E1E2 mutants[a]

Region	Mutant	Phenotype					
		Titer	E1E2 content	Binding	Cell-cell fusion	Lipid mixing	Content mixing
WT	H77-wt	++++	++	++	++	++	++
I	Y276F	+++	++	++	+	+	+
	Y276D	++	−	ND	ND	ND	ND
	G282D	+	−	ND	ND	ND	ND
	G282A	++	++	++	+	+	+
II	G418D	++	++	++	−	++	−
	G418A	++	++	++	−	+	−
	H421A	++	+	ND	ND	ND	ND
III	G468D	+	−	ND	ND	ND	ND
	W469A	++++	++	++	++	++	++
IV	W487A	−	−	ND	ND	ND	ND
	H488Q	++	−	ND	ND	ND	ND
	Y489A	+++	++	++	+	++	++
	G504D	−	−	ND	ND	ND	ND
	Y507A	−	−	ND	ND	ND	ND
	F509A	−	−	ND	ND	ND	ND
	T510A	+	+	ND	ND	ND	ND
V	G551D	+	−	ND	ND	ND	ND
	W554A	+	+	ND	ND	ND	ND
	G559D	−	−	ND	ND	ND	ND
VI	G600D	−	+	ND	ND	ND	ND
	G600A	−	+	ND	ND	ND	ND
	W602F	+	+	ND	ND	ND	ND
	W602A	−	−	ND	ND	ND	ND
	I603A	++	+	ND	ND	ND	ND
	T604N	++	+	ND	ND	ND	ND
	T604A	++	+	ND	ND	ND	ND
	W616F	+++	++	++	+	++	++
	W616A	+	++	++	−	+	+
	H617Q	+	−	ND	ND	ND	ND
HVR1	G389L	++++	++	ND	++	++	ND
	L399R	++++	++	ND	++	++	ND
	G406R	++++	++	ND	++	++	ND
	G406L	++++	++	ND	++	++	ND
CD81 bs	W437A	++	++	−	−	++	ND
	Y527A	++	++	−	−	++	ND
	W529A	+	++	−	−	++	ND

[a] The infectivity of HCVpp (titers) harboring the different E1E2 glycoproteins was detected using Huh-7 cells (Fig. 2). ++++, infectious titers higher than 10^4 IU/ml; +++, titers between 10^3 and 10^4 IU/ml; ++, titers between 10^2 and 10^3 IU/ml; +, titers between 10 and 10^2 IU/ml; −, titers lower than 10 IU/ml, which corresponds to the threshold of detection of infected cells with the neomycin resistance gene, as determined using pseudoparticles generated in the absence of viral envelope glycoproteins. The incorporation of the E1E2 glycoproteins (E1E2 content) was analyzed by immunoblots of viral particles pelleted through 20% sucrose cushions (Fig. 3). ++, E1 or E2 incorporation similar to that of the wild type; +, 5- to 10-fold reduced levels of E1E2 incorporation; −, absence of E1E2 incorporation. For the binding assay, cells were incubated with HCVpp purified on a 20% sucrose cushion for 1 h (Fig. 4). The binding of the particles was detected with anti-E2 or anti-E1 antibodies and analyzed by flow cytometry. The results of cell binding assays of HCVpp that incorporated wild-type levels of E1E2 were similar for all mutants and are reported as ++. Cell-cell fusion was measured with the luciferase expression induced by the mixing of cytoplasms of "donor" 293T viral envelope-expressing cells with the Huh-7-Tat "indicator" cells. Fusion mediated by wild-type H77 E1E2 envelope glycoproteins (H77-wt) with Huh-7 target cells at pH 5 was taken as 100%. ++, percentage of fusion higher than 75%; +, percentage of fusion between 10% and 75%; −, fusion lower than 10%. Lipid mixing was measured as the fusion-induced quenching of the R_{18} fluorescent probe incorporated inside HCVpp as a fusion protein with Vpr (BlaM-Vpr). Upon full fusion, the BlaM protein is released into the liposome, and content mixing is revealed by monitoring the cleavage of the CCF2 molecule encapsulated into the liposome (Fig. 5). For both lipid mixing and content mixing, + and ++ indicate a fusion reduced or similar compared to that of wild-type HCVpp, respectively. − indicates the absence of membrane fusion. ND, not determined.

concomitant with our findings, the data argue for an active role of E1 in the membrane fusion process.

Interestingly, other membrane-disturbing activities have been detected in HCV E2, as recently shown with the peptide libraries (45, 49). In addition, structural homologies with other fusion proteins suggested that E2 itself could carry a fusion peptide (23, 64). Our results support the notion that the E2 glycoprotein harbors fusion determinants in regions II and VI. Indeed, mutations in either region II (positions 416 to 430) or region VI (positions 600 to 620) abolished infectivity and membrane fusion. These mutations did not disrupt E2 folding or incorporation with E1 into HCVpp as well as subsequent HCVpp cell binding. In region II, the conservative G418A substitution strongly impaired both lipid and content mixing,

whereas the nonconservative G418D substitution displayed a block at the stage of hemifusion in content mixing assays (Fig. 6, panels b and h). The discrepancy between the behavior of G418A and G418D mutants remains elusive in the absence of E2 structural data. One possibility is that the hydrophobic nature of alanine might locally hinder the protein dynamics, while the hydrophilic aspartic acid would not have such a deleterious effect. Although these results argue for the presence of a fusion determinant in region II, the possibility that this region is a classical fusion peptide is unlikely for several reasons. First, region II harbors three N-linked glycans at positions 417, 423, and 430 (27), a feature which is a priori not expected to be close or within fusion peptides. Second, as shown in a previous report (43), antibodies targeting the E2 segment at positions 412 to 423 were able to block the interaction of E2 with CD81. Third, the W420A mutation reduced the CD81 binding of intracellular E1E2 complexes (44). Taken together, these data suggest that region II may not include a bona fide fusion peptide but rather that it may play an indirect role in the fusion process, possibly through a structural rearrangement of E2 during the fusion process.

In region VI, while the nonconservative replacement of W616 by an alanine led to a strong decrease in the fusion capacities of the mutant glycoproteins, its semiconservative replacement by a phenylalanine did not alter membrane fusion (Fig. 6, panels c and i). It may be that the latter change retains the physical and chemical properties of the W616 residue required for membrane fusion. These findings thus point to a role for region VI in the membrane fusion process.

In summary, our data are consistent with the notion that both E1 and E2 proteins contain membrane fusion determinants, which suggests a complex structural feature in which several segments of HCV E1E2 (i.e., at positions 270 to 284, 416 to 430, and 600 to 620) contribute to membrane fusion. The mechanism by which viral fusion proteins facilitate the formation of fusion intermediates is a complex process and requires the concerted action of different membranotropic segments (20, 33, 46, 54). Indeed, for membrane fusion to occur, fusion proteins must pull viral and cellular membranes together via major structural conformation changes to create membrane alterations that induce hemifusion. This results in complete membrane fusion and subsequent pore formation, stabilization, and enlargement (33, 54). While the fusion peptides are responsible mainly for the first steps of membrane fusion, other HCV E1 and E2 segments may contribute to membrane fusion at later stages. The different segments thus found in E1 and/or E2 could be involved in promoting membrane destabilization, pore formation, and/or enlargement, which probably occur in combination with other E1 and/or E2 regions. Such an involvement of different segments of the fusion protein during the membrane fusion process has indeed been proposed for other enveloped viruses harboring additional membrane fusion motifs (46). The recently reported crystal structure of glycoprotein G from vesicular stomatitis virus and glycoprotein gB from herpes simplex virus revealed that unlike flavivirus fusion proteins, the fusogenic motif is made of two loops (29, 52). Furthermore, it is not unprecedented to have more than one surface protein of enveloped viruses contribute directly to membrane fusion. For example, membrane fusion of herpesviruses and poxviruses involves several viral membrane proteins that interact to induce fusion at low pH (56, 59). Our results lend additional evidence to having multiple surface proteins contributing to membrane fusion and suggest that distinct regions in both HCV E1 and E2 may cooperate to drive the fusion process to completion. Similar to other enveloped viruses, the definitive characterization of fusion peptides in HCV will require resolving the crystal structure of the HCV E1E2 glycoprotein complex, which remains highly challenging. Nonetheless, the segments at positions 270 to 284, 416 to 430, and 600 to 620 constitute important membrane fusion determinants of HCV that are attractive targets for the further development of new antiviral compounds.

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