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Functional Analysis of Hepatitis C Virus Envelope Proteins, Using a Cell-Cell Fusion Assay

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Hepatitis C virus (HCV) envelope proteins mediate the entry of virus into cells by binding to cellular receptors, resulting in fusion of the viral membrane with the host cell membrane and permitting the viral genome to enter the cytoplasm. We report the development of a robust and reproducible cell-cell fusion assay using envelope proteins from commonly occurring genotypes of HCV. The assay scored HCV envelope protein-mediated fusion by the production of fluorescent green syncytia and allowed us to elucidate many aspects of HCV fusion, including the pH of fusion, cell types that permit viral entry, and the conformation of envelope proteins essential for fusion. We found that fusion could be specifically inhibited by anti-HCV antibodies and by at least one peptide. We also generated a number of insertional mutations in the envelope proteins and tested nine of these using the fusion assay. We demonstrate that this fusion assay is a powerful tool for understanding the mechanism of HCV-mediated fusion, elucidating mutant function, and testing antiviral agents.

Hepatitis C virus (HCV) infects an estimated 2 to 4% of the world's population. Chronic hepatitis C develops in 60 to 70% of infected individuals, and many subsequently develop cirrhosis and hepatocellular carcinoma (4). Current antiviral therapies cure only about 40% of patients, are expensive, and are associated with severe toxicity (34). HCV is an enveloped, positive-sense, single-stranded RNA virus from the *Flaviviridae* family. Its envelope proteins, E1 and E2, are believed to be class II fusion proteins. While class I fusion proteins, such as influenza virus hemagglutinin or the human immunodeficiency virus (HIV) *env* protein, are mostly α-helical, exist as trimers, and are oriented perpendicular to the cell membrane, class II fusion proteins consist mostly of β-sheets, form dimers, and lie parallel to the membrane (39, 43, 48). Since the discovery of the virus over 15 years ago, functional methods to study HCV entry have remained elusive. The recent generation of pseudotyped particles bearing HCV envelope proteins with a retrovirus core allowed the first opportunity to study viral entry mediated by HCV envelope proteins (6, 25). More recently three groups were able to replicate HCV in vitro, using a single, rare, and unusually virulent isolate from HCV genotype 2a (29, 47, 50). Here we present a new, robust system for studying HCV infection, utilizing envelope proteins from HCV genotypes 1a and 1b, which together account for over 70% of the disease in the United States. The assay provides a reproducible and quantitative measure of fusion mediated by HCV envelope proteins. It has allowed us to define many characteristics of HCV fusion and offers a rapid and convenient method to perform a screen for antiviral agents or to search for viral receptor(s).

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HCV envelope proteins are anchored to the membrane via a single C-terminal transmembrane (TM) domain and contain an N-terminal ectodomain (36) (38). The TM domains of E1 and E2 also encode the signal sequence of the downstream proteins (E2 and p7, respectively), and the signal sequence of E1 is encoded within the C-terminal region of the core protein. The TM domains are essential for correct heterodimerization and also contain signals for retention of the proteins in the endoplasmic reticulum (ER) (37; reviewed in reference 17). It is believed that the viral cores bud into the ER, with the virus being transported outside the cell via the secretory pathway. More recently it has been shown that a small fraction of E1 and E2 escapes ER retention and is expressed on the cell surface (6, 16, 20). The envelope proteins on the cell surface play an important role in the generation of retroviral pseudotypes of HCV and in the cell-cell fusion process that we describe here.

High-resolution structures of several flavivirus envelope proteins have been determined. Electron cryomicroscopic analysis of the West Nile virus E protein (27) and X-ray crystallographic studies of tick-borne encephalitis virus and Dengue virus E proteins (32, 39) have revealed that each monomer of the E protein consists of three beta-barrel domains. Domain I is located in the center and contains the N terminus. It is flanked on one side by domain II, which is important for dimerization, and on the other by domain III, which is immunoglobulin like and possibly contains receptor-binding sites. Not much is known about the structure of prM, the second flavivirus envelope protein. Flavivirus envelope proteins undergo major conformational changes as they mature and then fuse (26, 33). They begin as prM-E heterodimers in the ER and envelop the viral core, forming particles that bud into the ER and follow the secretory pathway. The prM protein is proteolytically cleaved to form M. The M-E heterodimers now reorganize to E-E homodimers, which lie flat on the surface of the viral membrane. A second major rearrangement occurs upon exposure to low pH in the endocytic pathway. The antiparallel E homodimers dissociate into monomers, and a hinge motion between domains I and II possibly results in the formation of

homotrimers. The hydrophobic fusion peptide/loop that was buried between domains I and III of adjacent monomers within each dimer now gets exposed and inserts into the target membrane. The structure of this postfusion trimer resembles that of the class I protein trimers.

HCV has been placed in a genus separate from the other flaviviruses and it not clear to what extent HCV envelope proteins behave like those of other members of the family *Flaviviridae*. There is controversy regarding which of the two HCV envelope proteins, E1 or E2, mediates membrane fusion. Sequence comparisons with other envelope proteins have identified a putative fusion peptide in E1 (21), and a model for HCV E1 based on the structure of the tick-borne encephalitis virus fusion protein has been proposed (22). Another model suggests that HCV E2 is the fusion protein (48). However, like other flavivirus proteins, HCV envelope proteins also appear to need a low pH to fuse, since HIV pseudotypes bearing HCV envelope proteins cannot transduce cells in the presence of 10 mM ammonium chloride or 25 nM concanamycin A (25). The cell-cell fusion assay that we describe here defines a pH of 5.4 or lower as optimal for HCV fusion.

Cell-cell fusion assays have been very useful for studying entry of many viruses, including retroviruses, rhabdoviruses, and herpesviruses (1, 14, 40, 41). The assays typically consist of expressing viral glycoproteins on one cell type and viral receptors on a second cell type. Cocultivating the two types of cells and, in case of viruses that fuse in endosomes, also transiently lowering the pH of the culture result in cell-cell fusion and syncytium formation. A reporter system for fusion usually consists of a plasmid containing the genes for beta-galactosidase or luciferase downstream of a T7 promoter in one cell type, with the other cell type expressing T7 polymerase. Fusion of the two cell types allows for expression of the reporter, which is measured enzymatically upon cell lysis (35). Cell-cell fusion assays have revealed extensive information about viral entry. Even in the case of HIV, where efficient systems for viral replication and genetic manipulation exist, such assays have been used to identify viral coreceptors (19) and inhibitors of fusion used as antivirals (reviewed in reference 13). Our HCV assay differs from the typical cell-cell fusion assay in that it uses green fluorescent protein (GFP) as a reporter, permitting us to measure fusion even when not all cells in the culture form syncytia. The assay allowed us to elucidate many characteristics of HCV fusion, and we demonstrate how it can be a powerful tool for understanding the mechanism of HCV fusion.

MATERIALS AND METHODS

Cells, expression constructs, and virus. 293T cells were used as effector cells for the fusion assay. Target cells were Huh-7.5, Huh-7, Hep3B, HepG2/hCD81 (gifts of Charles Rice, Rockefeller University, New York), HepG2 (gift of Chris Schindler, Columbia University, New York), and FLC4 cells (gift of Yoshiharu Matsuura, Osaka University) (5, 8, 49). HepG2 and HepG2/hCD81 were propagated on plates coated with collagen type 1 (~56 μg/ml in 0.02 M acetic acid; no. 354236; BD Biosciences). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2.2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a mixture of 95% air and 5% CO_2. All cells were regularly tested for mycoplasma (no. 302008; Stratagene) and kept mycoplasma free.

Constructs expressing envelope proteins were as follows. pcDNA3.1-HE1E2 expressed 22 amino acids from the C terminus of the C protein, all sequences from the E1 and E2 proteins of HCV genotype 1a(H) (gift of Jane McKeating, University of Birmingham, Birmingham, England) (21). Chimeric constructs pCAV340V and pCAV711V, consisting of ectodomains of the E1 and E2 sequences from HCV genotype 1b and signal sequence, transmembrane domain, and cytoplasmic tail from the vesicular stomatitis virus G glycoprotein (VSV-G) and pCAGVSV to express the VSV-G protein were as described previously (44) (kind gifts of Yoshiharu Matsuura, Osaka University). pcDNA3.1 (Invitrogen Corp., Carlsbad, CA) was used as an empty vector control. pT7IRES-GFP was constructed by cloning encephalomyocarditis virus internal ribosome entry site from pNCA-IRES (from Jeremy Luban, Columbia University) into the XbaI/NheI site of pQBI T7-GFP (Quantum Biotechnologies).

Replication-defective lentivirus vector pCMVΔR8.91 (51) was used to express Gag and Pol proteins, and the packaging vector pCSRW (15) encoded the fluorescent reporter DsRed (both gifts of Jeremy Luban, Columbia University, New York). To generate pseudotyped particles, the above lentivirus vectors were introduced into cells with expression constructs for either wild-type or mutant E1 and E2 proteins as described below.

Vaccinia virus VTF1.1 (2) (gift of Bernard Moss, NIAID, NIH) was used to express T7 polymerase via the viral late promoter in target cells used for fusion.

Fusion assay. 293T cells, 8×10^5 cells/per well seeded in six-well tissue culture dishes 24 h prior to transfection, were transfected with pcDNA3.1-HE1E2, an expression construct for the HCV E1 and E2 glycoproteins. For the negative control, cells were transfected with pcDNA3.1, which lacked sequences for the E1 and E2 proteins. For the positive control, cells were transfected with pCAGVSV, an expression construct for the VSV-G protein. All cells were cotransfected with pT7IRES-GFP, encoding GFP under control of the T7 promoter. Lipofectamine and Plus reagent in Opti-MEM (from Invitrogen Corp., Carlsbad, CA) were used for transfection, following the manufacturer's directions. Transfections were deemed successful when a control well transfected with a plasmid expressing a fluorescent reporter indicated an ~80% rate of transfection. We experimented with growing cells at different temperatures between 28 to 37°C to test if lower temperatures might result in better folding of the E1 and E2 proteins and more efficient transport to the cell surface. All temperatures tested resulted in similar efficiencies of fusion. Cells were incubated for 18 to 28 h posttransfection before they were dissociated from wells using nonenzymatic cell dissociation solution following the manufacturer's directions (catalog no. C5789; Sigma), resuspended in DMEM containing 10% FBS, 100 μg/ml rifampin, and 10 μM AraC, and mixed with target cells.

Huh-7.5 target cells were plated on fibronectin-coated (14 μg/ml, following manufacturer's protocol; no. F1141; Sigma) coverslips in a 24-well plate at ~1.5×10^5 cells/well and grown for ~24 h at 37°C. Cells were infected with vaccinia virus vTF1.1 (2) containing the T7 polymerase gene downstream of the viral late promoter. Viral inoculum contained in ~200 μl serum-free DMEM was added to cells for ~3 h at 37°C. One well containing cells transfected with a T7IRES-GFP plasmid and a red fluorescent protein expression plasmid was also infected with the same vaccinia virus to determine the efficiencies of transfection and infection. When ~50% of cells in this well turned green, the transfection and infection processes were judged to be at acceptable levels for the assay to work efficiently. Viral inoculum was removed by washing cells with DMEM containing 10% FBS, 100 μg/ml rifampin, and 10 μM AraC. Effector cells were placed on top of target cells, ~5×10^5 effector cells/well in 24-well plates, and cocultured for 4 to 6 h at 37°C. To initiate fusion, medium was removed and replaced with fusion buffer (135 mM NaCl, 15 mM sodium citrate, 10 mM morpholineethanesulfonic acid, 5 mM HEPES, 1 mM EDTA [made fresh from 10× stock and adjusted to the desired pH between 4.8 and 7.0 using HCl]) for ≤1 min (9). The low-pH buffer was replaced with DMEM containing 10% FBS, 100 μg/ml rifampin, and 10 μM AraC. After incubating the cells for 12 (range, 5 to 18) h at 37°C, cells were examined by fluorescence microscopy using filters with emission spectra of 510 to 560 nm. Green multinucleated cells were counted in each well.

Modifications to the assay involved testing different constructs for expression of the E1 and E2 proteins and various cell types as target cells (described in "Cells, expression constructs, and virus"). HCV antisera were pooled from over 25 patients with high titers of anti-HCV antibodies. Peptides SAYQ (amino acid sequence: SAYQVRNSSGLYHVTNDC) and SSGLY (SSGLYHVTNDCPNSSIVY), corresponding to amino acids 190 to 207 and 197 to 214, respectively, in E1 (23), were synthesized and purified by high-performance liquid chromatography (NeoMPS Inc., San Diego, CA). Antisera and peptides were added to the coculture in serum-free DMEM at different concentrations and incubated at 4°C for ~45 to 90 min immediately before the addition of low-pH buffer for fusion. All subsequent incubations before scoring for fusion, including incubation with fusion buffer, were without antisera or peptides. U.S. Patent no. 60/669,643 is pending for the fusion assays described here.

Generation of library of mutations in HCV E1 and E2 sequences. We created 15-nucleotide insertional mutations in a plasmid derived from pcDNA3.1-HE1E2, using TnsABC transposase (New England Biolabs, Beverly, MA) (11). To facilitate the mutagenesis, we first altered two PmeI restriction endonuclease

sites located in noncoding regions of the plasmid using site-directed mutagenesis (Quikchange; Stratagene). This new construct, pHE1E2Δ2*Pme*, behaved like the original construct in fusion and pseudotyping assays. Mutations are denoted by the position of the amino acid just N-terminal to the insertion, with 1 being the first methionine of the C protein. Mutations reported in this study, along with their insertional sequences, are as follows: 384-TLFKQ, 388-TGVST, 394-HTVST, 545-LCLNT, 548-WCLNN, 722-FLFKQ, and 735-WCLNM.

Immunofluorescence. Indirect immunofluorescence was performed mostly as described in reference 24. To determine whether mutations in E2 resulted in envelope protein expression, 293T cells growing in six-well dishes were transfected with constructs expressing mutant proteins. Constructs expressing wild-type proteins served as controls. Twenty-four hours after transfection, cells were plated onto fibronectin-coated coverslips, and another 24 h later fixed with paraformaldehyde (4%). Half the set was permeabilized using 0.1% Triton X-100 in phosphate-buffered saline, while the other half was left untreated to visualize surface expression of the E1 and E2 proteins. Nonspecific binding was blocked by incubating cells with 10% goat serum. Human sera pooled from ~25 patients with high titers of antibodies to HCV were used as primary antibody. Alexa Fluor 594-conjugated goat antihuman immunoglobulin G (IgG) was the secondary antibody (Invitrogen). Staining was visualized by fluorescence microscopy using a filter with emission spectra of 590 to 650 nm. For visualizing expression of envelope proteins in the fusion assay, cells were fixed and permeabilized as described above and stained with H52, a conformation-independent anti-E2 monoclonal antibody (12), and Alexa Fluor 594-conjugated goat anti-mouse IgG1 as primary and secondary antibodies, respectively. For visualizing VSV-G expression in controls used for fusion, rabbit VSV antiserum (no. 06141; Lee Biomolecular Research, Inc.) was the primary antibody and Alexa Fluor 546-conjugated goat antirabbit IgG the secondary antibody.

Generating pseudoparticles using mutants in HCV E1 and E2 proteins. Subconfluent 293T cells in six-well plates were cotransfected with 2 μg pCMVdelR8.91, 2 μg pCSRW, and 5 μg pcDNA3.1-HE1E2 (containing sequences for either wild-type or mutant E1 and E2 proteins). Supernatant was collected at 48 h posttransfection, filtered through a 0.45-μm Whatman filter, and assayed for reverse transcriptase activity to determine the amount of virions contained (45). Assays were quantified on a PhosphorImager (Molecular Dynamics) using ImageQuant (Amersham Biosciences). Preparations with equal amounts of reverse transcriptase activity were used to transduce Huh-7.5 target cells that were plated in 24-well plates (1×10^5 cells/well) 24 h prior to transduction. Inoculum of pseudoparticles in serum-free DMEM was added to wells for 3 h at 37°C before addition of serum-containing medium. Medium was again replaced 24 h post-transduction, and cells were incubated at 37°C for an additional 72 to 96 h. Transduction efficiency was determined by fluorescence microscopy using filters with emission spectra of 590 to 650 nm.

RESULTS

Cell-cell fusion assay using HCV envelope proteins. We devised an assay where fusion of two cell types depended on the presence of functional HCV envelope proteins on the surface of one cell type, HCV receptors on the other, and a fluorescent reporter system split between the two cell types. The scheme for our fusion assay is shown in Fig. 1A. We used 293T cells, due to their high transfection efficiencies, to express HCV envelope proteins. We used Huh-7.5 liver cells (8) as target cells, since they permit entry of HCV pseudoparticles (25) and must therefore express the HCV receptors. Cocultivation of the cells allowed HCV envelope proteins on the surface of one cell type to bind receptors on the other. Since fusion of many flaviviruses is thought to occur in endosomes (3), we transiently lowered the pH of the coculture to pH 5.0. Many cells fused with each other, resulting in syncytia that fluoresced green due to expression of the reporter. For the reporter, 293T cells contained the GFP sequence downstream of a T7 promoter, and Huh-7.5 cells expressed high levels of T7 polymerase from vaccinia virus via the late promoter (details in Materials and Methods). When the cytoplasm of the two cell types mixed as a result of fusion, green syncytia were formed (Fig. 1B, top panel). Control cells transfected with an empty vector showed few, very faint green cells due to baseline expression from the T7 promoter in the absence of T7 polymerase (Fig. 1B, panel 2). Cells expressing viral envelope proteins that were exposed to an identical buffer but at pH 7.0 also showed very few green cells (not shown). To confirm that fusion was dependent on the presence of HCV envelope proteins on the cell surface, cells were fixed, permeabilized, and subjected to indirect immunofluorescence using antibodies to HCV envelope proteins. Each syncytium showed high levels of expression of HCV envelope proteins, further evidence that the large, green multinucleated cells specifically resulted from fusion mediated by the HCV E1 and E2 proteins. The positive control for fusion consisted of 293T cells expressing the VSV-G protein, which resulted in characteristic large syncytia at pH 5.0 but not at pH 7.0, and these stained with anti-VSV-G antibodies but not with anti-HCV antibodies (Fig. 1B, panel 3).

In contrast to the giant syncytia seen with VSV-G, HCV syncytia were small and typically contained two to four nuclei (see Fig. 1C for several examples). Thus, a typical fusion experiment with VSV-G resulted in a single giant syncytium, inclusive of every cell in the well, while fusion with HCV E1 and E2 resulted in several hundred discrete syncytia. There are at least two differences between VSV-G and HCV envelope proteins that could account for the observed differences in fusion. The VSV-G receptor is likely present in every cell, allowing each cell to be included in the syncytium. By contrast, HCV receptors are present only on target cells; thus, cells expressing E1 and E2 need to be directly adjacent to target cells for fusion to occur. More importantly, the VSV-G protein is expressed at high levels on the surfaces of cells, whereas HCV E1 and E2 contain ER retention signals, and only a small fraction of these proteins is transported to the cell surface (12, 16). To investigate if syncytium formation depended on levels of fusion protein expression, we followed each of our fusion experiments with detection of E1E2 expression levels by immunofluorescence (central images in Fig. 1B). Cells were permeabilized to visualize total cell-associated envelope proteins. In most cases, only cells that produced abundant amounts of E1 and E2 formed syncytia. Moderate to low levels of E1 and E2 expression often resulted in the cells remaining single. This indicated that high amounts of surface E1 and E2 might be important for efficient fusion. Even though the number of syncytia resulting from HCV fusion constituted only 1 to 2% of cells, this number was consistently 5- to 10-fold above the number of green cells seen at neutral pH or at low pH with empty vectors (see Fig. 2). Furthermore, the use of a fluorescent reporter, viz., GFP, allowed unequivocal identification of E1- and E2-mediated syncytia, despite their smaller size.

Characterization of fusion mediated by HCV envelope proteins. (i) pH of fusion. To determine the pH optimum for fusion mediated by the HCV E1 and E2 proteins, we varied the pH of the buffer used for fusion from pH 4.8 to pH 7.0 (Fig. 2A). Fusion was most efficient at pH 5.0 to 5.4, was progressively less efficient as the pH was raised, and was at background levels at pH 7.0. This confirmed that HCV, like other flaviviruses, requires low pH to fuse and that the pH optimal for HCV fusion might be attained in the late endosome (31). Fusion appeared to be less efficient at pH 4.8, but that was likely due to decreased cell viability at this extreme pH.

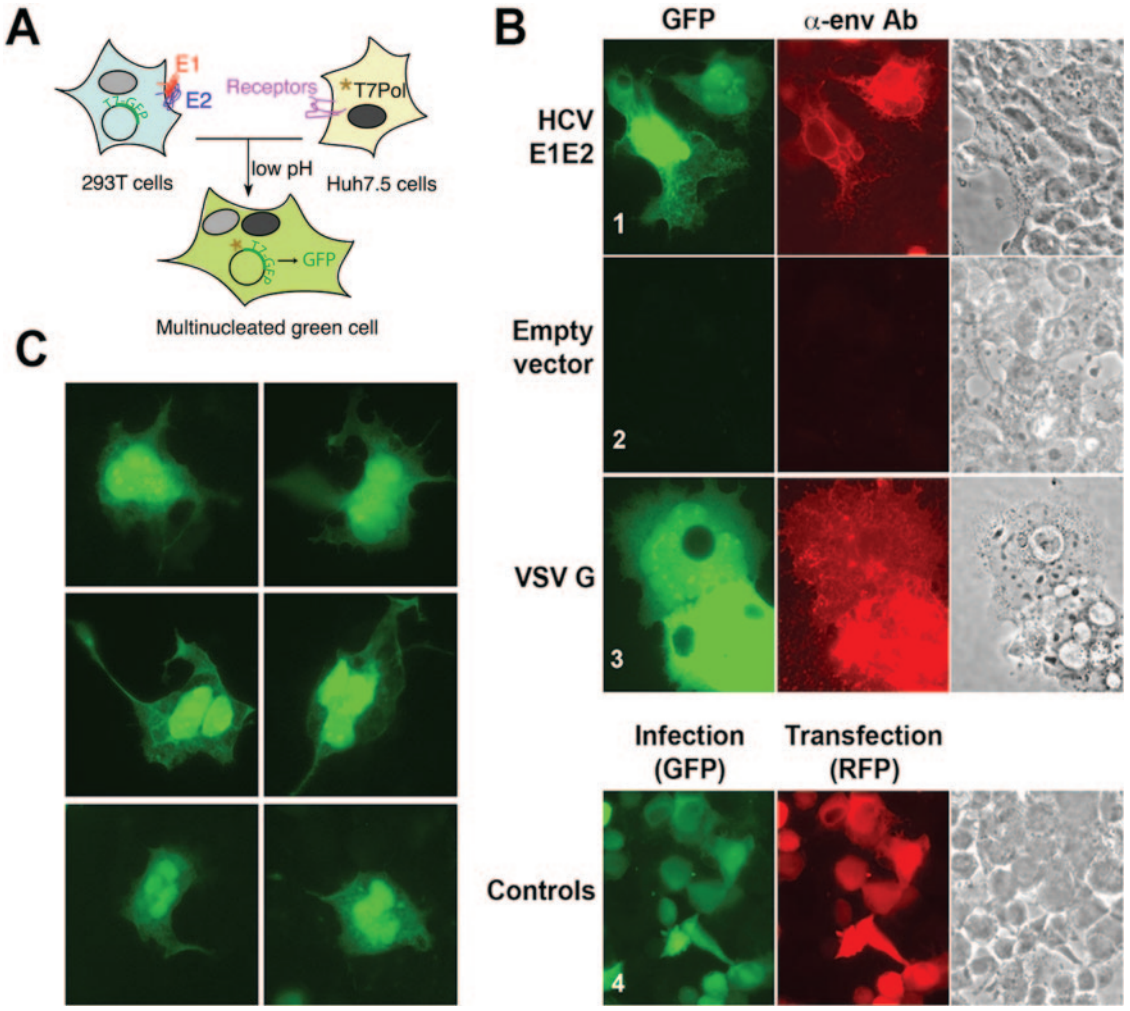

FIG. 1. Cell-cell fusion assay. (A) Scheme for fusion assay. (B) Green syncytia were seen following fusion mediated by HCV E1 and E2 (panel 1 on top) and VSV-G (panel 3) at low pH. Empty vector control is shown in panel 2. On the left of each panel are fluorescent images of the cells showing the GFP expressed upon fusion. In the middle are immunofluorescence images of the same cells (permeabilized and stained with anti-E2 monoclonal antibody H52 in panels 1 and 2 and with anti-VSV-G antibody in panel 3 [α-env Ab]), showing that the syncytia do indeed express the appropriate viral envelope protein. On the right are phase-contrast images of the same cells. Control cells (panel 4) were cotransfected with T7-GFP plasmid and expression construct for RFP and were subsequently infected with vaccinia virus producing T7 polymerase to show both fusion-*in*dependent expression of GFP and efficiency of vaccinia infection (green) and efficiency of transfection alone (red). Note the size of these single cells as opposed to the larger syncytia above. Some size variation due to differences in sizes of the two cell types, 293T and Huh 7.5, is also seen. (C) Examples of close-up views of six syncytia resulting from fusion of cells expressing HCV envelope proteins.

(ii) Target cell specificity. We tested several human hepatocyte- and non-hepatocyte-derived lines for efficiency of HCV envelope protein-mediated fusion (Fig. 2B). Fusion occurred with maximal efficiency with Huh-7.5 cells. Huh-7 cells were also efficient, while Hep3B cells fused with modest efficiency. HepG2 cells did not support fusion, which is corroborated by the finding that they do not support transduction with HCV pseudoparticles (6, 7, 25). However, HepG2 cells expressing the human CD81 receptor, one of the HCV coreceptors, fused efficiently. Another hepatocytic line, FLC4, and nonhepatocytic human lines, such as 293T, supported little to no fusion. All cell lines supported efficient fusion with the VSV-G protein (not shown).

(iii) Envelope protein constructs. We also tested fusion efficiency of HCV envelope proteins expressed from different constructs (Fig. 2C). E1 and E2 proteins from genotypes 1a and 1b, the two most prevalent genotypes of HCV in the United States, were both efficient at cell-cell fusion. Best results were obtained when the E1 and E2 proteins were synthesized as a polyprotein. The C protein contains a hydrophobic signal sequence at its C terminus that translocates the E1 protein into the ER lumen (42). C_{22}E1E2 expressed the terminal 22 amino acids of C and the entire sequences of the E1 and E2 proteins from HCV genotype 1a and was very efficient for fusion. We also tested similar constructs from genotype 1b, containing either the terminal 60 amino acids of the C protein or the entire C protein. Each fused efficiently (data not shown). Native E1 and E2 proteins (as expressed from each of these constructs) are known to be mostly retained in the ER, and we confirmed this by immunofluorescence. We reasoned that

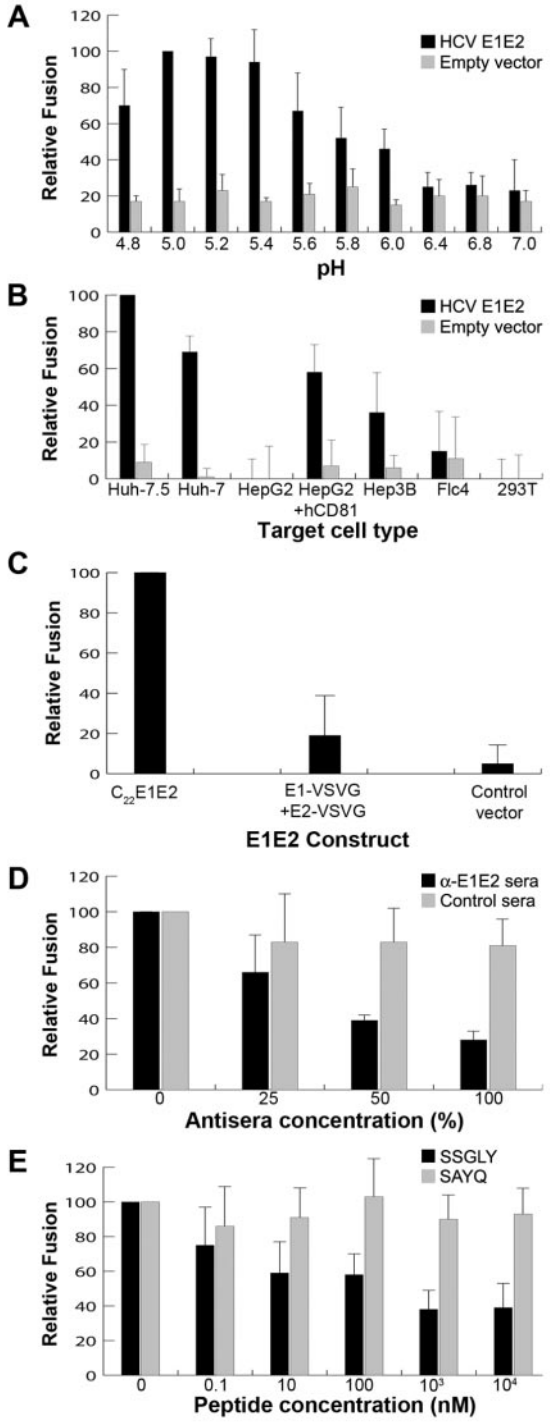

FIG. 2. Characteristics of fusion mediated by HCV envelope proteins. Fusion mediated by HCV E1 and E2 proteins (C_{22}E1E2, genotype 1a) with Huh-7.5 target cells at pH 5.0 was taken as 100% and typically consisted of several hundred syncytia per well. Graphs represent the average of three to four independent experiments. (A) Efficiencies of HCV E1E2-mediated fusion at different pHs. Fusion at pH 5.0 was set to 100%. (B) Target cell specificity for fusion. (C) Use of different expression constructs for E1 and E2 in the fusion assay. C_{22}E1E2(1a) (with 22 amino acids of the C protein and the entire E1E2 sequences from HCV genotype 1a); E1-VSVG plus E2-VSVG contained an equimolar mix of two chimeric constructs, each containing the ectodomains of the HCV E1 or E2 protein (genotype 1b) along with the signal sequence, transmembrane domains, and cytoplasmic tails of the VSV-G protein. (D) Inhibition of HCV E1E2-mediated fusion using anti-HCV antibodies. (E) Inhibition of fusion using peptides contained in HCV E1 sequences. Results were graphed as fractions of fusion obtained in the absence of serum or peptides. For all experiments, except in panel A, data points were normalized by subtracting the amount of fusion that occurred in the control well treated with neutral-pH fusion buffer. In panel A, this neutral-pH control is seen on the extreme right.

higher levels of surface expression might result in more frequent fusion events and perhaps larger syncytia. We tested chimeric E1 and E2 proteins that contained the ectodomains of the HCV E1 and E2 proteins and the signal sequences, transmembrane domains, and cytoplasmic tails of the VSV-G protein. The two envelope proteins were on different constructs and were introduced into cells in equimolar amounts. No HCV C-protein sequences were present. Lacking signals for ER retention (12), the chimeric envelope proteins efficiently reach the cell surface, which we verified by immunofluorescence. These chimeric proteins were previously reported to mediate fusion in a different cell-cell fusion assay (44). However, in our hands, fusion with these chimeric proteins was not robust or reproducible even when the assay was performed with different cell types, different pH, at different times following transfection or infection (to test different levels of protein expression), or by utilizing the constructs, cell lines, and reporter used in the original assay. Removal of the TM domains of E1 and E2 results in loss of both ER retention signals and dimerization sequences (46). Replacement with VSV-G TM domains allows efficient transport to the cell surface, suggesting that the chimeric proteins are not grossly misfolded, but fails to provide a dimerization domain, since VSV-G is a trimer. The absence of this dimerization domain or subtle differences in folding of the chimeric proteins might result in an absence of fusion activity. Taken together, our data suggest that the envelope proteins are most efficient at fusion when expressed in their native conformation. The presence of a portion of the C terminus of the C protein is essential for correct proteolytic processing of the E1 protein, and inclusion of this region appeared to strongly enhance fusion in our assay.

(iv) Fusion inhibitors. We next tested the specificity of the envelope protein requirement for fusion by attempting to inhibit fusion using anti-HCV antibodies (Fig. 2D) or by using peptides derived from sequences in the envelope proteins (Fig. 2E). Human sera pooled from patients that had high levels of antibodies to HCV inhibited fusion by over 70% when bound to cells for an hour before treating cells with pH 5.0 fusion buffer. It is possible that the low-pH treatment resulted in a loss of some antibody binding and therefore less than complete inhibition. Under the same conditions, control human sera had no effect. Neither serum group affected VSV-G-mediated fusion (not shown). Various peptides derived from sequences in HCV E1 and E2 have been demonstrated to inhibit infection with HCV pseudoparticles (23). We examined the effect of two such peptides on fusion when added to cells at various concentrations for an hour before fusion (Fig. 2E). A peptide corresponding to the sequence in the E1 protein between amino acids 197 and 214 (SSGLY) inhibited fusion by ~60% when added to cells at concentrations of 10 nM or above.

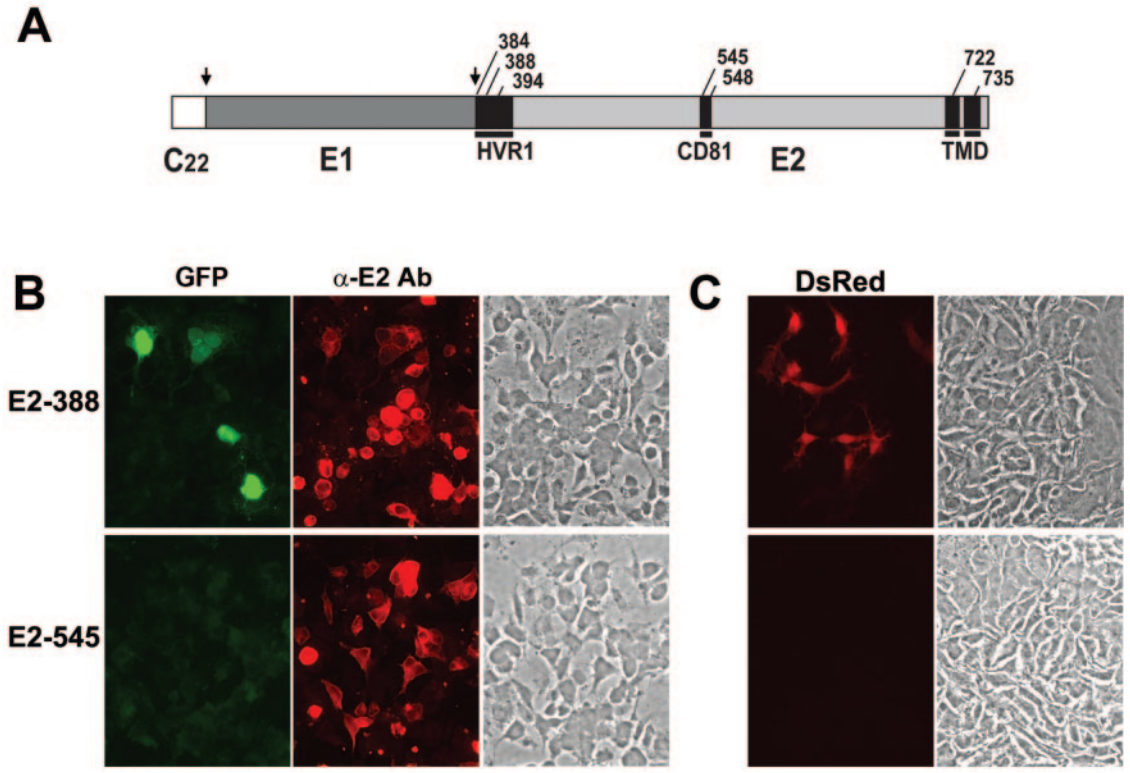

FIG. 3. Location of insertional mutations in HCV E1 and E2 and functional analysis of some mutants. (A) Map of the envelope proteins of HCV genotype 1a with the C-terminal 22 amino acids from the C protein. Insertional mutants are designated by the position of the amino acid located towards the N-terminal end of the insertion (1 is the first methionine of the HCV polyprotein, C is 1 to 192, E1 is 193 to 383, and E2 is 384 to 746). Proteolytic cleavage sites between C and E1 and between E1 and E2 are marked by arrows. Regions of focused mutant analysis are indicated on the map: HVR1, hypervariable region 1; TMD, transmembrane domain of E2; CD81, binding site for CD81. (B) Cell-cell fusion assay performed with mutant E1E2 proteins. Cells expressing mutant E1E2 proteins were fused and processed for immunofluorescence. Fusion-competent mutant E2-388 resulted in green syncytia (top panel), and these expressed HCV E1 and E2 (central). Many cells expressing high amounts of envelope proteins did not form syncytia, since they were not adjacent to receptor-expressing target cells. Mutant E2-545 (bottom panel) did not result in fusion, though it expressed abundant amounts of envelope proteins. α-E2 Ab, anti-E2 antibody. (C) Pseudoparticle infectivity. Pseudotyped particles were generated using the same mutants as shown in panel (B). Particles were harvested, and their amounts were normalized and used to transduce Huh-7.5 cells. Successful transduction resulted in expression of DsRed 3 to 5 days later.

Another peptide (SAYQ) corresponding to partly overlapping sequences (amino acids 190 to 207) had little effect on fusion even at concentrations up to 10 μM. Neither peptide had any effect on VSV-G-mediated fusion (not shown). This effect of peptides corresponds to reports that the peptide SSGLY inhibits transduction with pseudoparticles, while SAYQ does not (23). The assay thus provides an effective and simple means to screen for anti-HCV compounds that specifically inhibit HCV entry into cells.

Generation and functional analysis of mutations in HCV E1 and E2 sequences. Using a Tn*7*-derived transposase, we created a set of insertional mutations in the sequences coding for the E1 and E2 proteins from HCV genotype 1a. Each mutant contained a single insertion of 15 nucleotides at a random location. To begin to test our assay for measuring fusion efficiency of envelope protein mutants, we chose seven mutants from our library whose locations might predict effects on fusion (Fig. 3A). We reasoned that the CD81 binding site might not tolerate insertions, since CD81 is necessary for HCV entry (7, 49). Similarly, the transmembrane domains, with their roles in protein folding and dimerization, may also be essential for fusion. In contrast, the hypervariable region might tolerate insertions of five amino acids, since it varies strikingly among the different genotypes of HCV and even during the course of HCV infection in a given patient (28). As expected, we found that insertional mutations in the CD81 binding site (one at amino acid 545 and another at amino acid 548) prevented fusion, while insertions in the hypervariable region (at amino acids 384, 388, and 394) permitted fusion activity at levels that were approximately 60% of those obtained with wild-type envelope proteins (Fig. 3B and 4A). Insertions in the E2 transmembrane domains (at amino acids 722 and 735) also prevented fusion, as expected. The presence of three separate fusion-competent mutants in the HVR1 demonstrated that this region could tolerate insertions of five amino acids without significant loss of function.

To determine whether the lack of fusion activity was due to a lack of mutant envelope protein expression, we examined envelope protein levels for each mutant by indirect immunofluorescence. We used polyclonal anti-HCV antisera to ensure that all mutant proteins were effectively visualized. We stained both permeabilized and intact cells to determine amounts of envelope proteins in the entire cell and those at the cell surface only. We found that all mutants with read-through insertions

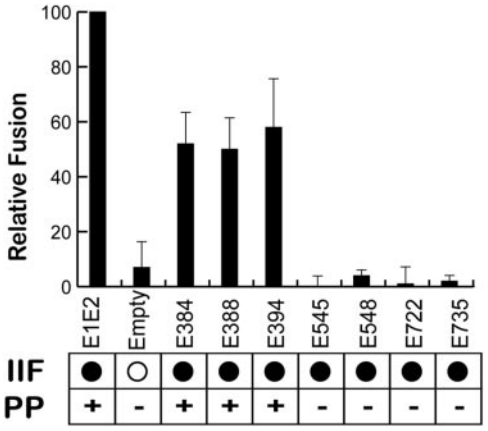

FIG. 4. Quantitative analysis of mutations in the hypervariable region, CD81-binding region, and the transmembrane domains of HCV envelope proteins. Fusion activity of each mutant compared to that of wild-type E1E2 at pH 5.0 is shown in the graph. Results are from three separate experiments. IIF indicates production of envelope proteins by mutants, as visualized by indirect immunofluorescence, using polyclonal sera in permeabilized cells. Filled circles indicate that amount and localization of signal were indistinguishable from those produced by wild-type proteins. Unfilled circle indicates background staining. PP, pseudoparticle assays with the same mutants. A plus sign indicates that the pseudotyped particles generated using the HCV E1E2 glycoproteins from that mutant were successful in transducing Huh-7.5 cells, as seen by DsRed expression. All pseudoparticle preparations were normalized for reverse transcriptase activity before inoculation.

were indistinguishable from the wild type by immunofluorescence, both in the amounts of protein produced and their intracellular location (Fig. 3B and 4). We were unable, however, to determine if any mutants failed to fuse because the amount of protein expressed on the cell surface was too low. Both the E1 and E2 proteins contain signals for retention in the ER (reviewed in reference 17). It is thought that flavivirus viral cores bud into the ER, and the virus is transported outside the cell via the secretory pathway (30) (46). Flow cytometry shows that a very small fraction of total protein escapes ER retention and is expressed on the cell surface (6, 16, 18). We were unable to reliably measure an additional decrease from such a small fraction for any of the mutants.

We proceeded to corroborate our cell-cell fusion assay with a different method that measures HCV envelope protein-dependent viral entry. Pseudoparticles that contain a retrovirus core and HCV envelope glycoproteins were described recently (6, 25). Transduction with these pseudoparticles depends on the presence of functional HCV envelope proteins. Fusion activities of mutants tested above were compared with the infectivities of pseudoparticles containing mutant HCV proteins. These pseudoparticles contained mutant HCV envelope proteins and a gene for the fluorescent marker DsRed in the plasmid containing the retrovirus packaging sequence (described in Methods). Pseudoparticles were harvested and titrated by measuring activity of reverse transcriptase (45). Equal amounts of inoculum from each mutant were used to transduce Huh-7.5 cells as described previously (25). Wild-type E1 and E2 proteins transduced Huh7.5 cells most efficiently, as monitored by fluorescence microscopy. Control transductions with empty vector or with chimeric HCV E1-VSVG and E2-VSVG resulted in rare and faint red cells (not shown). We tested infectivity of pseudoparticles generated using the envelope protein mutants (Fig. 3C and 4). Mutants that were fusion competent also transduced cells efficiently. Mutants that could not fuse were also unable to transduce. This further confirms that the cell-cell fusion assay measures a process that is dependent on HCV entry into cells.

DISCUSSION

The cell-cell fusion assay described here provides a simple, quantitative, and versatile tool to study HCV-mediated fusion. The advantages of this system come from its several features. (i) The ability to isolate the steps of binding and fusion from the rest of the viral life cycle, which allows for focused study of these steps and the means for receptor identification. (ii) The use of GFP as a reporter, which makes the assay highly sensitive and able to detect relatively rare events. By contrast, enzymatic reporters, such as luciferase or β-galactosidase, need lysis of cells and are better suited for studying systems where the majority of cells in the assay fuse. The fluorescent reporter further allows the possibility of high-throughput screens. (iii) The absence of biohazards that accompany studies using infectious viruses is especially relevant for HCV. The HCV isolate that successfully replicated in vitro (29, 47, 50) was very unusual in that instead of causing chronic hepatitis, it caused fulminant hepatic necrosis, a condition that is often rapidly fatal. Studying fusion of this unusually virulent isolate or making an extensive library of mutations in the corresponding infectious clone would require additional safety measures not necessary for our fusion assay. (iv) The assay works with genotypes 1a and 1b of HCV. This is in contrast to the in vitro replication systems that so far work only with an isolate from genotype 2a. The ability to study fusion with genotypes 1a and 1b is especially useful, since these genotypes are the most prevalent, are most likely to cause progression to chronic liver disease, and are most resistant to antiviral therapy. Furthermore, chronic sequelae of HCV, such as cirrhosis and hepatocellular carcinoma, are related to the propensity of the virus to cause chronic inflammation. Fulminant hepatic necrosis, in contrast, is an acute condition and in cases that recover is not followed by chronic liver disease. (v) The ability to inhibit fusion with antibodies and peptides offers an efficient means to screen for antivirals. (vi) The assay is fairly rapid and can be scored within a few hours (as early as 4 to 6 h) after fusion has occurred—in contrast to transduction with pseudotyped particles, where a signal indicating viral entry is seen several days (typically 3 to 5 days) after transduction, when sufficient amounts of retroviral protein synthesis has occurred.

A fusion assay utilizing chimeric E1-VSVG and E2-VSVG proteins, HepG2 cells as target cells, and luciferase as a reporter was previously reported (44). Despite repeated attempts, we were unable to consistently reproduce this, either as originally reported or by using different target cells and GFP as reporter. The great number of variations we tried allowed us to conclude that at least three factors were crucial for our fusion assay to work. The use of a sensitive reporter like GFP was mentioned above. The use of native HCV envelope protein constructs instead of chimeras was also important. The dimerization domains of HCV envelope proteins are in the trans-

membrane regions of E1 and E2 (10, 37). Substitution of this region with the corresponding region from VSV-G not only removes the native dimerization signal but also adds a portion of a protein that is normally a trimer. It is possible that the HCV-VSVG chimeric proteins do not form dimers or do not assume conformations necessary for fusion, leading to a lack of robustness in fusion assays that employ them. This is further corroborated by data from chimeric HCV-influenza virus hemagglutinin proteins, which also do not fuse (21). Finally, we experimented with various human liver cell lines and found that Huh-7.5 cells and the related Huh-7 cells gave the best fusion. Both of these cell lines express high levels of CD81, which is known to be necessary, though not sufficient, to mediate entry of pseudoparticles (25). HepG2 cells, which express little CD81 receptor on their surfaces (25), did not permit fusion in our assay, in contrast to the previously reported HCV fusion assay that utilized HCV-VSVG chimeric proteins (44).

There have been no mutational analyses of HCV envelope proteins that test fusion yet. We have generated an extensive library of mutants in E1 and E2, and this fusion assay provides a convenient method for studying them. Our initial analysis of a few mutants from this library has revealed a region that can tolerate insertions of five amino acids in at least three discrete positions. Our data suggest that the HVR1 might be especially useful to generate epitope-tagged virus for viral entry studies. The recovery of functional mutants also suggests that not all insertional mutants in the HCV E1 and E2 proteins result in folding or transport defects. An analysis of our comprehensive library of mutants is likely to provide a functional map of the HCV envelope proteins.

In summary, the fusion assay allowed us to define important characteristics of HCV fusion. For the first time, we were able to measure the pH of HCV fusion. We found that the envelope proteins needed to be in their native conformation for effective function, and this was aided by including the signal sequence of E1 located at the C terminus of the C protein in the expression construct. The extent of fusion permitted by various cell lines suggested expression levels of HCV receptor(s) in different cell lines. Such information is necessary for using this assay, or other cell-based assays, to identify the HCV receptor(s). We identified a region in the E2 protein that could tolerate insertions and demonstrated how the assay could be used for screening or selecting for functional mutants in HCV envelope proteins. We show that fusion could be effectively inhibited by antibodies and peptides, offering a useful method to screen for antiviral agents. Current anti-HCV therapy consisting of ribavirin and alpha interferon is effective in less than half of all treated patients. There is a need for drugs that target other steps in the viral life cycle. The assay provides for easy application of high-throughput procedures, which would accelerate the discovery of new antiviral agents.

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