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Hepatitis C Virus Is Primed by CD81 Protein for Low pH-dependent Fusion*§

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Hepatitis C virus (HCV) entry into permissive cells is a complex process that involves interactions with at least four co-factors followed by endocytosis and low pH-dependent fusion with endosomes. The precise sequence of receptor engagement and their roles in promoting HCV E1E2 glycoprotein-mediated fusion are poorly characterized. Because cell-free HCV tolerates an acidic environment, we hypothesized that binding to one or more receptors on the cell surface renders E1E2 competent to undergo low pH-induced conformational changes and promote fusion with endosomes. To test this hypothesis, we examined the effects of low pH and of the second extracellular loop (ECL2) of CD81, one of the four entry factors, on HCV infectivity. Pre-treatment with an acidic buffer or with ECL2 enhanced infection through changing the E1E2 conformation, as evidenced by the altered reactivity of these proteins with conformation-specific antibodies and stable association with liposomes. However, neither of the two treatments alone permitted direct fusion with the cell plasma membrane. Sequential HCV preincubation with ECL2 and acidic buffer in the absence of target cells resulted in a marked loss of infectivity, implying that the receptor-bound HCV is primed for low pH-dependent conformational changes. Indeed, soluble receptor-pre-treated HCV fused with the cell plasma membrane at low pH under conditions blocking an endocytic entry pathway. These findings suggest that CD81 primes HCV for low pH-dependent fusion early in the entry process. The simple triggering paradigm and intermediate conformations of E1E2 identified in this study could help guide future vaccine and therapeutic efforts to block HCV infection.

Hepatitis C virus (HCV) entry into permissive cells is initiated through its E1 and E2 glycoprotein interactions with at least four cellular co-factors as follows: CD81; scavenger receptor class B, type 1 (SR-BI); and two tight junction-resident proteins, claudin-1 and occludin (1–6). CD81 belongs to the tetraspanin family and thus contains two extracellular loops, of which the second larger loop (ECL2) specifically binds to the HCV E2 glycoprotein (7, 8). SR-BI, which is normally involved in the regulation of lipoprotein metabolism and cholesterol trafficking (9), plays a role in early steps of HCV entry (10–12); however, its direct interaction with the virus has not been unambiguously demonstrated. Claudin-1 and occludin also consist of four transmembrane domains but are functionally distinct from tetraspanins. The HCV entry determinants of claudin-1 and occludin have been mapped to the first and the second extracellular loops of these proteins, respectively (4, 13, 14). Considering that all four HCV entry co-factors are expressed in non-liver tissues (1, 5), the narrow tropism of this virus is surprising. It is thus likely that the relative expression levels of entry co-factors, their spatial organization, and/or interactions with each other render a cell permissive to infection (15). An alternative possibility suggested in a recent article (16) is that hepatocytes simply lack an HCV-inhibitory factor that is expressed in most other cell types.

The above considerations demonstrate the importance of understanding the sequence of HCV-receptor interactions and elucidating the mechanism by which the viral glycoproteins are triggered to mediate membrane fusion. Despite extensive efforts, the early steps of HCV entry and fusion are poorly characterized. The initial virus attachment to cells occurs through binding to glycosaminoglycans and the LDL receptor (17, 18). Cell-attached HCV is thought to interact with CD81 and SR-BI (6, 11, 18, 19) and engage the tight junction resident co-receptors, claudin-1 and occludin, at later times (4, 20). However, more recent evidence shows that HCV engages CD81 and claudin-1 early in the entry process, as manifested by the similar HCV binding kinetics (21) and direct interactions between these two receptors in the membrane of permissive cells (14, 15, 21, 22). A very slow constitutive endocytosis of CD81 (21) is thought to necessitate the HCV interaction with and internalization through other co-factors (24–27). Indeed, actin-dependent relocation of CD81 to the cell junction area in the presence of...
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of the soluble E2 ectodomain (28) suggests that CD81- and perhaps SR-BI-bound viruses can be transported to the tight junctions where they engage claudin-1 and occludin.

After interacting with the requisite co-factors, HCV enters permissive cells through clathrin-mediated endocytosis and low pH-dependent fusion with endosomes (24, 25, 29–31). Accordingly, HCV infection is blocked by reagents, such as bafilomycin A1 and NH4Cl, that raise endosomal pH and thereby prevent triggering of E1E2 (12, 24, 25, 30). It is not completely clear, however, whether low pH has a direct effect on native glycoproteins. On one hand, the reactivity of conformation-specific antibodies with E2 is altered after HCV exposure to low pH in the absence of cellular receptors (32, 33), apparently due to dissociation of E1E2, which are expressed and incorporated into the viral membrane as a heterodimer (32). On the other hand, the infectivity of HCV pseudoparticles (HCVpp) and of infectious cell culture-grown virus (HCVcc (34)) is not compromised by low pH pretreatment (Refs. 25, 31 but see Ref. 30) for an opposite result. Furthermore, exposure to low pH reportedly failed to induce virus fusion with the plasma membrane, suggesting the requirement for endosomal entry and the existence of additional virus trafficking-dependent steps that trigger HCV fusion (25).

To elucidate the complex mechanism of HCV entry, it is essential to determine the functional roles of E1 and E2 in this process and identify the factors that trigger their refolding. Knowledge of the mechanism by which E1E2 is activated to mediate fusion is essential for designing urgently needed antiviral strategies, which have been successfully implemented for other viruses (see for example Refs. 35–37). Enveloped viruses have evolved diverse strategies to respond to cellular cues and initiate fusion at desired sites. Viral fusion proteins are usually activated by two principal mechanisms, binding to cognate receptors on the cell surface (either directly or through an accessory protein) or low pH encountered in endosomes (38, 39). Entry of the avian sarcoma and leukemia virus (ASLV) is a well characterized example of the “hybrid” two-step triggering mechanism that involves receptor priming and low pH-induced fusion with endosomes (40, 41). The lack of HCV inactivation at low pH (25, 31) led us to hypothesize that, by analogy to ASLV, native E1E2 glycoproteins do not refold under acidic conditions but acquire the sensitivity to low pH following their interactions with one or several HCV entry factors.

Here, we develop a tractable experimental system for studies of HCV activation and low pH-dependent fusion. We show that HCV pretreatment with an acidic buffer or with the soluble CD81 fragment increased infectivity, whereas sequential incubation with the receptor and low pH in the absence of a target membrane irreversibly diminished the ability of the virus to enter permissive cells. The soluble receptor and low pH applied separately or in combination promoted conformational changes in E1E2, as evidenced by the altered binding of conformation-specific monoclonal antibodies and greatly enhanced association of the E2 ectodomain with liposomes. Importantly, irreversible refolding of the soluble receptor-primed E1E2 upon exposure to an acidic buffer appeared to reflect conformational changes that drive virus fusion. This notion was supported by the significant level of low pH-induced fusion between the receptor-primed HCV and the cell plasma membrane under conditions blocking endosomal entry. Our findings suggest that, although other requisite receptors are essential for HCV entry, CD81 may be both necessary and sufficient to render E1E2 competent for fusion with acidic intracellular compartments.

EXPERIMENTAL PROCEDURES

Cells Culture, Antibodies, and Reagents—Human embryonic kidney 293T/17 cells were obtained from the ATCC (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), penicillin/streptomycin (Sigma), and 0.5 mg/ml geneticin. Huh-7.5 hepatoma cells were obtained from Dr. Charles Rice (Rockefeller University) through the Apath LLC (Brooklyn, NY). The Huh-7.5 cells and Huh-7 cell clone S29, a gift from Dr. S. Emerson (NIAID, National Institutes of Health), were grown in DMEM supplemented with 10% FBS and penicillin/streptomycin. Unless stated otherwise, cell culture media and reagents were purchased from Invitrogen.

The expression vector for HCV (strain H77, genotype 1a) E1E2 was a gift from Dr. T. Dragic (Albert Einstein University). A soluble fragment of the HCV E2 glycoprotein (sE2) fused to His tag was expressed in 293T cells, as described in Ref. 42. Briefly, after transfection of sE2 plasmid into 293T, the medium was refreshed with Opti-MEM. The supernatant was harvested at 24 h post-transfection, passed through a 0.45-μm filter, and used for liposome experiments without further purification. Escherichia coli stocks expressing GST fused to the second (large) extracellular loop (ECL2) of human or mouse CD81 were a kind gift from Dr. S. Levy (Stanford University). The GST-ECL2 fusion proteins (CD81 residues 116–202) were expressed and affinity-purified using a Sepharose 4B column (GE Healthcare), as described previously (8). The proteins were then dialyzed to remove glutathione, frozen in aliquots, and stored at −80 °C. The purity of recombinant proteins was verified by Western blotting (see below).

Human monoclonal antibodies (mAb) against the HCV E2 (CBH-7, CBH-4D, HC11, and CBH-8C) and E1 (H111 and H114) glycoproteins were the kind gifts from Dr. S. Foung (Stanford University). Mouse mAb against HCV E2 (H53) was generously provided by Dr. J. Dubuisson (Institut de Biologie de Lille, France). The anti-E2 antibody H1920-19J was purchased from United States Biologicals (Swampscott, MA). Anti-CD81 JS-81 antibody was purchased from Pharmingen, and 5A6 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-GST goat antibody and Sepharose 4B were from GE Healthcare. Glutathione, bafilomycin A1 (BafA1), NH4Cl, and cholesterol were obtained from Sigma, and protease K was from Qiagen (Valencia, CA). Phospholipids, 1-oleyl-2-palmitoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-di-oleyl-sn-glycero-3-phosphoethanolamine-N’-(cap biotinyl) (biotin-DOPA) were purchased from Avanti Polar Lipids (Alabaster, AL).

Production of HCV Pseudoparticles and Infectious HCV—HCV pseudoparticles (HCVpp) were generated by transfecting 293T/17 cells (1.2·10⁶) with 3 μg of the luciferase reporter vec-
tor pNL4–3.Luc.RE– (from Dr. N. Landau, AIDS Research and Reference Reagent Program, National Institutes of Health (43)) and 5.5 µg of the vector expressing either the G glycoproteins of vesicular stomatitis virus (VSV) or E1E2 glycoproteins of Semliki Forest virus (SFV, a gift from M. Kielland) or HCV (H77 strain). Transfection was carried out using PolyFect transfection reagent (Qiagen, Valencia, CA). The pCDNA3.1 vector was used to generate control pseudoviral particles lacking fusion proteins (referred to as pCDNApp). Cells were transferred into fresh growth medium 16 h post-transfection and further incubated for 2 days. Virus-containing supernatant was collected, passed through a 0.45-µm filter, and stored in aliquots at −80 °C.

The HCVcc luciferase reporter virus was generated by introducing the Renilla luciferase gene (amplified from the plasmid pCMV-Renilla (New England Biolabs, Ipswich, MA)) fused to a sequence encoding the foot and mouth disease virus 2A peptide (44) between the p7 and NS2 coding sequence of the CNS2 infectious clone (previously described in Ref. 45) by PCR. Purified plasmid DNA was linearized, and the remaining overhanging nucleotides were eliminated by mung bean nuclelease digestion (New England Biolabs, Ipswich, MA). After extraction with phenol/chloroform, purified plasmid was precipitated and resuspended in 16 ml of complete growth medium and cultivated for 4 days. Supernatants were harvested, filtered, aliquoted, and stored at −80 °C. To assess levels of infectivity, Huh-7.5 cells were infected with supernatants for 48 h and lysed to test for Renilla expression.

Infection by Cell-bound and Immobilized Viruses—Huh-7.5 cells (2.106) grown on 96-well plates were infected with HCVpp, HCVcc, or control pseudoparticles using a spinoculation protocol (46). Briefly, cells were centrifuged with viruses at 2900 × g for 50 min at 4 °C (Sorvall Legend RT, Kendro). The extent of infection was evaluated based on the luciferase activity 48 h postinfection. The approximate multiplicity of infection estimated using the enhanced GFP-expressing HCVpp was 0.1–0.2 (supplementary Fig. 1A). To infect cells with substrate-immobilized viruses, HCVpp or HCVcc were first adhered to 96-well plates (Costar, Corning, NY) coated with poly-L-lysine. One hundred µl of viral suspension was added to each well, and microplates were centrifuged, as above. Immobilized particles were washed and overlaid with Huh-7.5 cells that were detached from Petri dishes, using a nonenzymatic cell dissociation solution Cell Stripper™ (Cellgro, Manassas, VA), and resuspended in a complete growth medium. To accelerate cell attachment, the plates were centrifuged at 180 × g for 1 min at room temperature and maintained at 37 °C (5% CO2) for 48 h. Cells were lysed, and the luciferase expression was measured with the Bright-Glo luciferase kit (Promega, Madison, WI) for HCVpp and SFVpp or the Renilla luciferase-based kit for infectious HCVcc, as per the manufacturer’s instructions, using a microplate reader (Bio-Tek Instruments, Winooski, VT).

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**Virus Treatment with Low pH, Soluble Receptor, and Entry Inhibitors**—Unless stated otherwise, viruses were allowed to adhere to a polylysine-coated 96-well plate and then blocked with 5% FBS in PBS for 1 h on ice. Immobilized viruses were pretreated with citrate buffer (20 mm citric acid, 150 mm NaCl) adjusted to either pH 5.2 or 7.3. Treatment was carried out at 37 °C for 10 min. Cell-free and cell-attached viruses were exposed to DMEM at pH 7.3 or adjusted to pH 5.2 with a predetermined amount of HCl. The choice of an acidic buffer did not affect the results. Receptor priming was carried out by pre-incubating the viruses with human or mouse ECL2 (hECL2 and mECL2, respectively) for 30 min on ice followed by an additional incubation for 30 min at 37 °C. Viruses were washed to remove unbound ECL2, exposed to pH 5.2 or 7.3 for 10 min at 37 °C, and overlaid with Huh-7.5 cells in growth medium. The luciferase activity was determined at 48 h post-infection.

**HCV Entry at the Plasma Membrane**—Virus suspension (100 µl) was preincubated with 2.5 µg of hECL2 (final concentration 25 µg/ml) for 30 min on ice and 30 min at 37 °C or left untreated. Huh-7.5 cells were incubated in the absence or in the presence of BafA1 (50 nm) for 30 min at 37 °C, and viruses (with or without ECL2) were spinoculated onto cells in the cold. Cells were washed with cold PBS to remove unbound viruses and overlaid with prewarmed pH 7.3 or 5.2 solution (DMEM adjusted to a desired pH with HCl) either immediately or after 1 h at 37 °C. Where indicated, DMEM was supplemented with 50 nm BafA1. The treatment with pH 7.3 or pH 5.2 buffers was carried out for 10 min at 37 °C, after which time, fresh growth medium with or without BafA1 was added, and cells were further incubated at 37 °C for 48 h before measuring the luciferase activity.

**Assay for Productive Virus Endocytosis**—Huh-7.5 cells were grown in 96-well plates (Stripwell™, Corning, NY), and viruses were spinoculated onto cells, as described above. Cells were washed to remove unbound viruses and shifted to 37 °C. After varied times of incubation, virus uptake was stopped by placing cells on ice, and the wells were washed and treated on ice with 50 µg/ml of proteinase K in PBS for 45 min to inactivate/remove viruses on the cell surface. Cells detached after proteinase K treatment were pelleted by centrifugation (2 min at 180 × g in the cold), washed, resuspended in complete growth medium, and re-plated onto 96-well plates. Luciferase activity was measured, as described above.

**Whole Virus ELISA**—HCVpp or HCVcc were immobilized on 96-well plates coated either with poly-L-lysine or with 500 ng of Galanthus nivalis lectin (Sigma) following the published protocol (33) with minor modifications (see supplementary Fig. 4). Ninety-six-well plates (Costar, Corning, NY) were incubated with 0.1 mg/ml poly-L-lysine for 1 h at room temperature and washed three times with PBS. Two hundred µl of viral suspension was added to each well, and the plates were centrifuged at 2900 × g for 50 min at 4 °C to aid virus binding. Despite the low apparent titer of nonconcentrated HCVpp preparations, a large number of E2-positive puncta (~3.104/well) was adhered to a polylysine-coated substrate, as evidenced by an immunofluorescence assay (supplementary Fig. 1B). Adhered viruses were blocked with 5% FBS for 60 min on ice, washed, and incubated with ECL2 (human or mouse) for 30 min on ice and 30 min at
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37 °C. Viruses were then exposed to a citrate buffer at pH 5.2 or pH 7.3 for 10 min at 37 °C, washed, and incubated with 2 μg per well of anti-E2 or anti-E1 antibodies for 60 min at room temperature. Free antibodies were removed by washing, and viruses were incubated with goat anti-human (1:5000 dilution, Thermo Scientific, Rockford, IL) or rabbit anti-mouse IgG-HRP (1:3000). The antibody binding was detected by incubation with the 3,3′,5,5′-tetramethylbenzidine substrate kit, and the absorbance at 450 nm was measured using the FL600 plate reader (Bio-Tek Instruments).

Western Blotting—Affinity-purified GST-hECL2 and GST-mECL2 were resolved by 10% SDS-PAGE, transferred onto a nitrocellulose membrane (Bio-Rad), and probed with mouse anti-CD81 antibody 5A6 followed by an HRP-conjugated rabbit anti-mouse IgG (1:5000 dilution, Zymed Laboratories Inc.). Protein bands were visualized using an enhanced chemiluminescence (ECL) prime Western blotting detection reagent (GE Healthcare). The blots were later stripped and re-probed with goat anti-GST antibody to detect hECL2 and mECL2. Ponceau S (Sigma) and Coomassie staining was also performed to confirm the equal amounts of protein in loaded samples for both ECL2 fragments (data not shown). Soluble E2 (sE2) was detected on blots using the goat anti-E2 antibody from United States Biologicals.

Liposome Binding Assay—E2-liposome binding was evaluated by a liposome co-floation assay described previously (47) with minor modifications. Briefly, mixtures of POPC and cholesterol (~2:1 mol. ratio) were prepared by dissolving 4 mg of POPC with 1 mg of cholesterol in 5 ml of chloroform/methanol. For control experiments, 0.1 mg of biotin-DOPE was added to the POPC/cholesterol mixture. The solvents were removed in a rotary evaporator (Buchi, Switzerland) for 20 min at 37 °C, and residual solvent was evacuated under vacuum for 2 h at room temperature. The dried lipid film was hydrated in 1 ml of PBS for 30 min at room temperature while gently shaking, subjected to three freeze/thaw cycles, and passed 10 times through a liposome extruder (Avanti Polar Lipids) using a 100 nm pore size polycarbonate filter. Liposomes were prepared fresh before each experiment and stored at 4 °C.

Soluble E2 (1 μg) was incubated with 2.5 μg of hECL2 or mECL2 (sE2/ECL2 molar ratio 1:4) on ice for 30 min followed by an additional 30 min at 37 °C in 45 μl of PBS. Next, 5 μl of liposomes containing 25 μg of lipid mixture were added to this mixture, and samples were incubated at 37 °C for 1 h to allow liposome binding. Alternatively, sE2 was diluted in the pH 5.0 citrate buffer and incubated with liposomes for 1 h at 37 °C. For a positive control, 25 μg of liposomes containing ~1 mol % of biotin-DOPE were incubated with 1 μg of streptavidin. To minimize nonspecific electrostatic association between proteins and lipids (48), all samples were additionally incubated for 15 min at 37 °C in the presence of 1 M KCl in a final volume of 200 μl. Next, 300 μl of a 67% sucrose solution was added to samples, bringing the final sucrose concentration to 50%, and the mixture was transferred to a clear polycarbonate ultracentrifuge tube and overlaid with 4 ml of 25% sucrose and with 0.5 ml of 5% sucrose. After centrifugation at 190,000 × g (4 °C for 3 h) in the SW50.1Ti rotor (Beckman Instruments), seven ~0.7-ml fractions were collected from the top of a gradient. Fifty-μl aliquots from each fraction were analyzed by SDS-PAGE for the presence of sE2, streptavidin, or GST-ECL2 proteins, as described above.

Statistical Analysis—Unless indicated otherwise, experiments were performed in triplicate, and the results were compared with appropriate controls, using a two-tailed Student’s test. p values <0.05 are marked by a double asterisk, and p values <0.01 are indicated by triple asterisks on the graphs.

RESULTS

To explore the mechanism of the HCV E1E2 activation, we modified a standard cell inoculation protocol by immobilizing the viruses on a solid substrate, an approach that has been developed by our group for imaging single virus-cell fusion (49, 50). This configuration is ideally suited for multiple exposures to solutions and reagents at desired temperature without causing virus aggregation or adversely affecting the cell viability. In contrast, pretreatment of viruses in suspension with even a single reagent requires a lengthy and often damaging ultracentrifugation step to get rid of a reagent prior to cell inoculation. Importantly, immobilized viruses were entry-competent, as evidenced by efficient infection of permissive cells measured by a luciferase expression-based assay (Fig. 1A). An alternative pretreatment protocol employed in this study involves virus pre-binding to permissive cells in the cold and initializing infection by shifting to 37 °C, as described previously (46). While this configuration simplifies the exchange of solutions/reagents used to pretreat HCV, it limits the duration and the temperature of incubation due the virus internalization and entry under physiological conditions.

Because the immobilized HCV system has not been previously validated, control experiments were performed to ensure that the observed infection resulted from a conventional entry pathway that requires cognate receptors and low pH within endosomes. Similar to the results reported for a standard inoculation protocol (7, 31), the JS-81 antibody against CD81 effectively inhibited infection by immobilized HCVcc (supplemental Fig. 2). Also, infection by cell-attached and immobilized viruses was equally sensitive to BafA1, a specific proton pump inhibitor that blocks HCV infection through raising endosomal pH (see below and data not shown). These results demonstrate that immobilized HCVpp and HCVcc enter susceptible cells through a receptor- and low pH-dependent pathway, thus providing a convenient model to study the function of E1E2. In this study, we compared the cell culture-grown infectious virus (HCVcc) and HCV E1E2-pseudotyped particles (HCVpp) that have been widely employed for studies of HCV entry. Because HCVcc and HCVpp behaved virtually identically in all assays described below, the majority of experiments was done with HCV pseudoparticles.

Exposure to Low pH Transiently Enhances HCV Infectivity—To test the hypothesis that engagement of cognate receptors renders HCV competent for low pH-dependent fusion with endosomes, we examined the pH-sensitivity of native glycoproteins. We reasoned that irreversible acid-induced conformational changes in E1E2 should compromise HCV infectivity, as has been observed for disparate low pH-triggered viruses, such as influenza and SFV. A common confounding factor in interpret-
ing virus inactivation data is that viral particles quickly aggregate at low pH as a result of exposure of hydrophobic domains on their glycoproteins (for example see Refs. 51–53). Massive virus aggregation could reduce the infectious titer without necessarily inactivating the fusion proteins. To avoid potential artifacts and to simplify the HCV pretreatment protocol, we employed the immobilized or cell-attached virus configurations discussed above.

HCVpp (H77 strain) and HCVcc (J6/JFH-1 strain) were immobilized at the bottom of multiwell plates and treated with neutral or acidic buffers prior to overlaying Huh-7.5 cells. Pseudoparticles devoid of fusion proteins (pCDNApp) were used as a negative control. The viruses were exposed to a citrate buffer adjusted to either pH 7.3 or pH 5.2 for 10 min at 37 °C. In control experiments, the pH 5.2 pretreatment was carried out on ice for 30 min. Viruses were then overlaid with Huh-7.5 cells, and the extent of infection was measured after 2 days. ND, not determined.

B, effect of low pH on cell-attached viruses. HCVpp or SFVpp were spinoculated onto Huh-7.5 cells, and unbound particles were removed by washing. Cell-bound viruses were briefly exposed to DMEM adjusted to pH 5.2 or to pH 7.3 for 10 min at 37 °C. Fresh medium was added to cells, and plates were further incubated to determine the luciferase activity. Where indicated, Huh-7.5 cells grown in 96-well plates were preincubated with 50 nM BafA1. The drug was maintained throughout the experiment and removed at 3 h postinoculation. The pH 5.2 pretreatment value is the mean of three independent experiments performed in triplicate. C, immobilized HCVpp were exposed to low pH (10 min, 37 °C) followed by a chase at neutral pH, either at 37 °C (black circles) or on ice (open circles). Huh-7.5 cells were overlaid onto viruses at indicated time points, and the resulting infectivity was measured by a luciferase assay. D, immobilized HCVpp or SFVpp were exposed to pH 5.2 or pH 7.3 and overlaid with Huh-7.5 cells right away (1st two columns). Alternatively, viruses were subjected to a double-pulse protocol that involved the pH 5.2 or pH 7.3 treatment followed by a chase at neutral pH for 1 h at 37 °C and the second exposure to an acidic or neutral pH buffer. Target cells were then overlaid onto viruses to allow infection. In all panels luciferase activity was measured 48 h post-infection and normalized to a control experiment in which viruses were pretreated with pH 7.3. Unless indicated otherwise, experimental points are means from representative experiments conducted in triplicate, and the error bars are means ± S.E. The statistical significance of the virus pretreatment effect is indicated by asterisks above bars.

FIGURE 1. Low pH pretreatment enhances HCVpp and HCVcc infectivity. A, infectious HCV and HCV E1E2- or SFV E1E2-pseudotyped viruses (denoted iHCVcc, iHCVpp, and iSFVpp, respectively) were immobilized on polylysine-coated 96-well plates. Pseudoparticles devoid of fusion proteins (pCDNApp) were used as a negative control. The viruses were exposed to a citrate buffer adjusted to either pH 7.3 or pH 5.2 for 10 min at 37 °C. In control experiments, the pH 5.2 pretreatment was carried out on ice for 30 min. Viruses were then overlaid with Huh-7.5 cells, and the extent of infection was measured after 2 days. ND, not determined.

B, effect of low pH on cell-attached viruses. HCVpp or SFVpp were spinoculated onto Huh-7.5 cells, and unbound particles were removed by washing. Cell-bound viruses were briefly exposed to DMEM adjusted to pH 5.2 or to pH 7.3 for 10 min at 37 °C. Fresh medium was added to cells, and plates were further incubated to determine the luciferase activity. Where indicated, Huh-7.5 cells grown in 96-well plates were preincubated with 50 nM BafA1. The drug was maintained throughout the experiment and removed at 3 h postinoculation. The pH 5.2 pretreatment value is the mean of three independent experiments performed in triplicate. C, immobilized HCVpp were exposed to low pH (10 min, 37 °C) followed by a chase at neutral pH, either at 37 °C (black circles) or on ice (open circles). Huh-7.5 cells were overlaid onto viruses at indicated time points, and the resulting infectivity was measured by a luciferase assay. D, immobilized HCVpp or SFVpp were exposed to pH 5.2 or pH 7.3 and overlaid with Huh-7.5 cells right away (1st two columns). Alternatively, viruses were subjected to a double-pulse protocol that involved the pH 5.2 or pH 7.3 treatment followed by a chase at neutral pH for 1 h at 37 °C and the second exposure to an acidic or neutral pH buffer. Target cells were then overlaid onto viruses to allow infection. In all panels luciferase activity was measured 48 h post-infection and normalized to a control experiment in which viruses were pretreated with pH 7.3. Unless indicated otherwise, experimental points are means from representative experiments conducted in triplicate, and the error bars are means ± S.E. The statistical significance of the virus pretreatment effect is indicated by asterisks above bars.
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prolonged chase at neutral pH. The second acidic pulse exhibited the same infectivity-enhancing effect as the first pulse (Fig. 1D, p > 0.55), implying that disappearance of a low pH-induced state was caused by relaxation of activated E1E2 to their original state. The return of infectivity to a basal level was temperature-dependent, because the enhanced luciferase signal persisted for at least 1 h when the low pH pulse was chased in the cold prior to adding target cells (Fig. 1C).

Importantly, exposure of cell-attached HCVpp to an acidic buffer did not bypass the requirement for endocytic entry, because infection of pH-treated viruses was still blocked by BafA1, which raises endosomal pH and interferes with endosome maturation (Fig. 1B). Thus, the enhanced infectivity of cell-bound viruses upon brief exposure to pH 5.2 at the onset of co-incubation with cells was not caused by virus fusion with the plasma membrane. The above results demonstrate that low pH reversibly elicits a more fusion-competent conformation of E1E2, which does not, however, induce direct fusion with the plasma membrane of permissive cells.

**HCV Infection Is Enhanced by Pretreatment with the CD81 Extracellular Loop**—The lack of HCVpp and HCVcc inactivation following the acidic pretreatment implies that E1E2 becomes competent for low pH-mediated fusion only after interactions with one or several cellular receptors, perhaps similar to the priming step of ASLV envelope glycoprotein by its receptor (41). Following the initial attachment to a target cell, HCV is thought to interact with CD81 and SR-BI and then engage the tight junction resident proteins, claudin-1 and occludin (2, 4, 5). It remains unclear, however, whether CD81 promotes conformational changes in E1E2 or simply serves as an attachment factor to facilitate the virus binding to other receptors, which then confer the sensitivity to low pH. We therefore asked if interactions with CD81 known to occur through the second extracellular loop (ECL2) of this receptor (6–8, 58) can modulate the virus response to low pH.

We expressed and purified the GST fusion proteins with human and mouse CD81 ECL2 (8), hereafter referred to as hECL2 and mECL2, respectively. Unlike the recombinant hECL2 protein, mECL2 does not bind E2 or inhibit HCV infection (59). Recombinant proteins were analyzed by Western blotting with anti-CD81 and anti-GST antibodies (Fig. 2A, top and bottom panels, respectively). In agreement with Ref. 8, the recombinant protein appeared as a single 36-kDa band when probed with anti-CD81 antibodies. Blots probed with anti-GST antibodies under nonreducing conditions showed an additional band perhaps corresponding to free GST aggregates. We also tested whether the purified protein formed correct disulfide bonds, which have been shown to be essential for the proper ECL2-E2 interaction (23). The formation of disulfide bonds was apparent from the binding pattern of anti-human CD81 antibody 5A6 that recognized the native hECL2 but not the mouse ECL2 or the reduced form of hECL2 (Fig. 2A). These results are consistent with the correctly folded GST-hECL2 protein.

The functional activity of hECL2 was tested using the entry inhibition assay, because this protein is known to block HCV infectivity (42, 59). As expected, hECL2 inhibited infection of Huh-7.5 cells in a dose-dependent manner (Fig. 2B), whereas mECL2 was without effect. The hECL2 protein equally efficiently inhibited infection by immobilized HCVpp (Fig. 2B, open square). At high concentrations, hECL2 blocked HCVpp entry irrespective of whether it was allowed to bind to cell-adhered viruses for 30 min on ice prior to shifting to 37 °C or added just before raising the temperature (Fig. 2B, black triangle). The inhibition of infection by hECL2 after the virus attachment to cells is consistent with the initial HCV binding to cells through factors like heparan sulfates prior to engaging CD81 (6, 18, 60).

Having verified the functionality of hECL2, we asked whether its binding to E1E2 can modulate the virus entry. HCVpp were immobilized on plates, treated with hECL2, washed, and overlaid with Huh-7.5 cells. In contrast to the
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Reduced HCV infectivity observed in the continued presence of hECL2 (Fig. 2B), pretreatment of immobilized viruses with hECL2 markedly enhanced the luciferase signal compared with mock-treated or mECL2-treated viruses (Fig. 3A). As expected, neither hECL2 nor mECL2 affected the SFVpp infection. Parallel experiments with immobilized HCVcc also showed more than a 2-fold increase in infectivity of hECL2-pretreated viruses (Fig. 3B). Thus, similar to the low pH pretreatment, a brief exposure to hECL2 enhanced the HCV infectivity, likely by rendering E1E2 more fusion-competent.

hECL2-bound E1E2 Glycoproteins Undergo Irreversible Refolding at Low pH—The augmented infectivity of HCV-hECL2 complexes is indicative of an altered conformation of E1E2 upon exposure to the soluble receptor. We therefore probed whether this more functionally active conformation was sensitive to acid by sequentially exposing the virus to hECL2 and pH 5.2. This pretreatment markedly reduced the HCVpp and HCVcc infectivity (Fig. 3, A and B), whereas viruses pretreated with mECL2 were not sensitive to acidic environment. Inactivation of HCV-hECL2 complexes occurred in an hECL2 dose-dependent manner (Fig. 3A, inset). Importantly, the loss of infectivity caused by a hECL2/acid treatment was irreversible, because the fusion activity did not recover when viruses were incubated at neutral pH prior to overlaying Huh-7.5 cells ( supplemental Fig. 3). In other words, hECL2 rendered E1E2 competent to undergo irreversible refolding upon exposure to low pH in the absence of target membranes. Notably, an inverse order of pretreatment (pH 5.2 followed by hECL2) did not inactivate the virus (Fig. 3A). The latter protocol even somewhat augmented HCV infection, likely due to the effect of either low pH (e.g. Fig. 1A) or hECL2 alone. It thus appears that, unlike native E1E2 dimers, acid-treated E1E2 does not interact with the soluble receptor and that the hECL2-induced conformation is distinct from that mediated by low pH.

We next measured the pH dependence of inactivation of hECL2-primed HCVpp by sequentially incubating the virus with the soluble receptor and buffers of different acidity. The resulting changes in HCVpp infectivity were nearly identical to those of control SFVpp particles (Fig. 3C). The HCVpp infectivity was maximally reduced after exposure to pH 6.2. If the observed loss of E1E2 activity reflects conformational changes leading to membrane fusion, as suggested by the results presented below, the high pH threshold for inactivation would be consistent with HCV fusion in early endosomes (25). Irrespective of whether or not HCVpp inactivation upon hECL2 and acid pretreatment corresponds to fusogenic conformational changes in E1E2, the above results demonstrate the greatly enhanced pH sensitivity of hECL2-primed glycoproteins compared with their native conformations.

Receptor-primed Conformation of E1E2 Forms Slowly and Is Relatively Short Lived—Receptor-mediated conformational changes in viral fusion proteins are temperature-dependent

hECL2, as described above, or left untreated (open circles). Viruses were then exposed to solutions of varied acidity for 10 min at 37 °C, using DMEM titrated with HCl. Viruses were washed and overlaid with Huh-7.5 cells. The resulting luciferase activity was measured and normalized to the pH 7.3-treated control. The statistical significance of the virus pretreatment effect is indicated by asterisks above bars.
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(41, 61–63). Consistent with this notion, pretreatment of immobilized HCVpp with hECL2 on ice neither enhanced the infectivity of viruses kept at neutral pH nor compromised the infectivity of those additionally exposed to an acidic buffer prior to overlaying with cells (Fig. 3A). We found that, surprisingly, receptor priming occurred slowly even at an optimal temperature (Fig. 4A, left panel). Here, we used the boost in infectivity and the parallel loss of infectivity of hECL2-treated HCVpp exposed to pH 7.3 or pH 5.2, respectively, to deduce the kinetics of E1E2 priming. Indeed, these changes in infectivity occurred at similar rates (Fig. 4A, left panel, open and black circles). The calculated half-times of 21 ± 9 and 12.2 ± 0.4 min were not significantly different (p > 0.366), supporting the notion that both events were manifestations of the same underlying process, E1E2 priming. We found that neither the hECL2-mediated enhancement nor the hECL2/low pH-induced loss of infectivity reached a plateau within 30 min at 37 °C, demonstrating that a receptor-primed conformation of E1E2 formed slowly. In contrast, the receptor-mediated priming of ASLV envelope glycoprotein has been shown to reach completion within 5 min (61, 63).

We then asked if an hECL2-primed conformation of E1E2 is stable once formed. Immobilized HCVpp were exposed to hECL2 as above, washed, and overlaid with target cells after varied times of incubation at neutral pH. The luciferase signal from hECL2- and hECL2/low pH-treated viruses returned to the level of an untreated control within ~40 min (Fig. 4A, right panel, open and black circles), showing that a receptor-primed state of E1E2 was not long lived in the absence of a target membrane. Similar to the priming process, the return to a basal level, both from an enhanced and compromised infectivity states, followed the same time course (Fig. 4A, right panel, gray versus black smooth lines). Importantly, a primed E1E2 conformation formed and disappeared with a similar time course (Fig. 4A, left versus right panel).

The return to a basal infectivity level was not due to a partial inactivation of the receptor-primed HCVpp, because both the acid sensitivity and the enhanced infection phenotype recovered upon the second exposure to hECL2 after a 60 min-chase at neutral pH (Fig. 4A, right panel, black square and black diamond). It is therefore likely that the disappearance of a primed state reflects the hECL2 dissociation from E1E2 at physiological temperature. By contrast, this metastable receptor-primed state reflects the hECL2 dissociation from E1E2 at physiological temperature (Fig. 4A, left panel, open and black square). Here, we used the boost in infectivity and the parallel loss of infectivity of hECL2-treated HCVpp exposed to pH 7.3 or pH 5.2, respectively, to deduce the kinetics of E1E2 priming. Indeed, these changes in infectivity occurred at similar rates (Fig. 4A, left panel, open and black circles). The calculated half-times of 21 ± 9 and 12.2 ± 0.4 min were not significantly different (p > 0.366), supporting the notion that both events were manifestations of the same underlying process, E1E2 priming. We found that neither the hECL2-mediated enhancement nor the hECL2/low pH-induced loss of infectivity reached a plateau within 30 min at 37 °C, demonstrating that a receptor-primed conformation of E1E2 formed slowly. In contrast, the receptor-mediated priming of ASLV envelope glycoprotein has been shown to reach completion within 5 min (61, 63).

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The above findings show that low pH promotes irreversible refolding of the receptor-primed E1E2, a conformation that is otherwise prone to return to an initial state. We therefore assessed the stability of the hECL2-primed E1E2 dimers at low pH by comparing the rates of HCVpp and SFVpp inactivation. Acidic environment destabilized a primed conformation, as evidenced by a marked loss of E1E2 function. However, a considerably longer exposure to low pH was required to inactivate hECL2-HCVpp complexes than to inhibit the SFVpp infectivity (Fig. 4B). Whereas SFVpp lost activity at low pH in less than 1 min, hECL2-pretreated HCVpp required at least 5 min for a maximal reduction in infectivity, suggesting the existence of a significant activation barrier for acid-induced refolding of primed HCV glycoproteins in the absence of a target membrane.

Together, our results demonstrate that E1E2 dimers undergo reversible temperature-dependent conformational changes at neutral pH following a brief exposure to the CD81 ECL2. In the absence of target membranes primed E1E2 returns to a more stable initial conformation (apparently by releasing the bound hECL2) following a neutral pH chase, while irreversibly refolding into a fusion-incompetent structure at low pH.
Conformational Changes in E1E2 Induced by Low pH and by Soluble CD81 Are Distinct—We sought to verify that exposure to hECL2 and/or to low pH promotes conformational changes in E1E2. Structural changes in E1E2 were probed by testing the reactivity of a panel of conformation-specific monoclonal antibodies against these glycoproteins, using a virus-based ELISA. HCVpp and HCVcc were captured onto multiwell plates coated with polylysine (or with G. nivalis lectin, as described in supplemental Fig. 4 and Ref. 33). Pseudoparticles lacking E1E2 (pCDNApp) were used as a negative control. Viruses were then treated with hECL2 and/or pH 5.2 or left untreated and probed with antibodies. Most human anti-E2 antibodies used in these experiments have been shown to block the E2 binding to CD81 or hECL2 but to also recognize preformed E2/H18528CD81 complexes (64), suggesting that their binding epitopes are adjacent to the CD81-binding site. Therefore, the altered recognition of E2 by these mAbs after the hECL2 pretreatment should be due to conformational changes in E2, as opposed to the occlusion of an antibody-binding site by the soluble receptor.

All tested antibodies against different antigenic domains of E1 and E2 recognized native heterodimers on HCVpp and HCVcc but exhibited distinct binding patterns to pretreated viruses (Fig. 5). The only exception was the mouse H-53 antibody that bound equally to E2 on HCV pseudoparticles irrespective of the pretreatment protocol (supplemental Fig. 4D). The human E2 antibodies, CBH-4D and CBH-7, exhibited most marked changes in binding (Fig. 5, A and B), but their reactivity patterns were distinct. The CBH-4D reactivity with acid-treated viruses was compromised (but see Ref. 33 for an opposite result), whereas pretreatment with hECL2 enhanced the antibody binding (Fig. 5A). By comparison, the CBH-7 binding (as well as the HC-11 binding shown in supplemental Fig. 4B) was not affected by low pH alone but was reduced upon hECL2/pH 7.2 pretreatment (Fig. 5B), which resulted in virus inactivation. Changes in antigenic properties of E1 were tested using anti-E1 antibody H-111 (65). The binding of this antibody was modestly but significantly reduced by low pH pretreatment alone whereas exposure to hECL2 was without effect (Fig. 5C). A similar reactivity pattern was observed for another E1 antibody, H-114 (supplemental Fig. 4E). As expected, the binding of all tested antibodies was not significantly affected upon HCV preincubation with mECL2. Notably, the antibody recognition patterns were similar for HCVcc and HCVpp (Fig. 5, black versus open bars) and were independent of the virus capture method (supplemental Fig. 4B).

The above findings show that pretreatment with hECL2 and/or an acidic buffer elicits conformational changes in E1E2. We took advantage of the altered recognition of E2 glycoprotein to test whether conformational changes induced by low pH were reversible, as suggested by functional experiments. The diminished binding of CBH-4D after pretreatment with an acidic buffer (Fig. 5A) returned to the level of an untreated control following a brief chase at neutral pH (Fig. 5D). These antigenic changes in E1E2 and the loss of low pH-enhanced infectivity (Fig. 1C) exhibited similar kinetics, further validating the use of functional methods to study HCV fusion intermediate.

**FIGURE 5.** Soluble CD81 fragment and low pH promote conformational changes in E1 and E2. HCVpp (open bars) or HCVcc (black bars) immobilized on polylysine-coated plates were preincubated with 25 μg/ml of hECL2 or mECL2 (30 min on ice and 30 min at 37 °C) or left untreated. Viruses were then washed and exposed to pH 5.2 or 7.3 for 10 min at 37 °C. Virus-like particles lacking fusion glycoproteins (pCDNApp, hatched bars) were used as a negative control (for an additional negative control, see supplemental Fig. 4B). Next, plates were incubated with human antibodies CBH-4D against an antigenic domain A of E2 (A and D), CBH-7 against an antigenic domain C (B), or with the anti-E1 mAb H-111 (C) for 1 h at room temperature followed by incubation with HRP-conjugated goat anti-human IgG. D, longevity of a low pH-induced E1E2 conformation on immobilized HCVpp was examined by chasing the pH 5.2-pulse at neutral pH either at 37 °C (open circles) or on ice (filled circles). The CBH-4D mAb was added either immediately or following an indicated chase period. The relative binding efficiency was calculated by normalizing data to the pH 7.3-treated control (set to 100%). The statistical significance is indicated by asterisks above bars in A–C.
ates. Also in line with the functional data, the reduced CBH-4D reactivity caused by the virus exposure to an acidic buffer persisted at neutral pH in the cold (Fig. 5D).

To conclude, the antibody recognition pattern and functional data discussed above show that acid- and hECL2-induced E1E2 conformations are distinct. For instance, the binding site for CBH-4D was better exposed on a receptor-primed conformation, although the low pH treatment (with or without prior exposure to hECL2) partially destroyed or masked this epitope. The low pH- and hECL2-induced intermediates were also differently recognized by CBH-7, which bound especially poorly to an inactivated form of E1E2 obtained by a sequential exposure to hECL2 and pH 5.2. We thus identified two new conformations of E1E2 that could prove useful for understanding the mechanism of HCV fusion and for eliciting novel neutralizing antibodies.

hECL2 and Acid Treatment Promote E2 Binding to Liposomes—Receptor-mediated conformational changes in viral fusion proteins are usually accompanied by exposure of cryptic hydrophobic regions and their insertion into target membranes. This association of fusion proteins or viruses with target membranes can be monitored by a liposome co-flotation assay (61, 63, 66). Liposome binding is evidenced by co-migration of proteins/viruses with liposomes to the top of a sucrose gradient under centrifugal force, although free proteins/viruses remain in the bottom fraction containing 40–50% sucrose. We employed this assay to determine whether receptor priming or low pH can alter the membrane tropism of HCV glycoproteins. Co-flotation assay was first validated using streptavidin, anti-E2, or anti-GST (G and H) antibodies. Blots were incubated with HRP-conjugated secondary antibodies and visualized by ECL.

FIGURE 6. hECL2 but not mECL2 induces soluble E2-liposome binding at neutral pH. A and B, streptavidin-biotin binding was used as a positive control. One μg of streptavidin was incubated with 25 μg of POPC/cholesterol (Chol) liposomes either supplemented (B) or not supplemented (A) with a small amount of biotin-DOPE lipid. sE2 binding to liposomes at low pH was carried out by incubating 1 μg of protein with 25 μg of unilamellar POPC/cholesterol liposomes in a pH 7.3 (C) or pH 5.0 (D) citrate buffer for 1 h at 37 °C. Receptor priming was performed by preincubating 1 μg of sE2 with 2.5 μg of human (E) or mouse (F) ECL2 for 30 min on ice and 30 min at 37 °C. The mixture was then incubated with 25 μg of POPC/cholesterol liposomes for 1 h at pH 7.3, 37 °C. All samples containing protein/liposome mixtures were brought to 1 M KCl and incubated for 15 min before adjusting to 50% sucrose and placed at the bottom of a sucrose gradient. After ultracentrifugation, seven fractions were collected from each sample and analyzed by Western blotting under reducing conditions. The presence of proteins in different fraction was visualized by probing with anti-streptavidin, anti-E2, or anti-GST (G and H) antibodies. Blots were incubated with HRP-conjugated secondary antibodies and visualized by ECL.
ECL2. Whereas sE2-liposome binding was not observed at neutral pH, a large fraction of this protein was found at the top of a gradient following the low pH exposure (Fig. 6, C and D). To assess whether receptor priming also promotes binding to liposomes, sE2 was pretreated with hECL2 and further incubated with POPC/cholesterol liposomes at neutral pH. Under these conditions, sE2 co-migrated with vesicles to the top of the gradient, whereas preincubation with mECL2 did not promote liposome binding (Fig. 6, E and F). Interestingly, virtually all hECL2 was found at the bottom of the gradient, as shown by Western blotting with anti-GST antibodies (Fig. 6G). This result further supports the notion that this receptor fragment dissociated from sE2 in the presence of liposomes despite the reported high affinity sE2-hECL2 binding (23, 67).

The above results demonstrate that transient E2 conformational changes induced by hECL2 or low pH stably associate with liposomes, withstanding high ionic strength and prolonged ultracentrifugation. By analogy to the ASLV-liposome binding in the presence of soluble receptor (61, 63, 66), the above results suggest that target membranes devoid of receptors can stabilize a post-priming conformation of E2 and that this liposome-bound conformation is formed upon shedding the soluble receptor.

hECL2 Supports Infection of CD81-negative Cells—Next, we asked whether the soluble CD81 fragment can support productive entry of HCV. Ectopic expression of CD81 has been shown to restore the ability of HCV to infect cells lacking this receptor but expressing other three entry factors (e.g. HepG2 cells (59, 68)). Moreover, a CD9 chimera bearing the ECL2 of CD81 expressed in HepG2 cells rescued infection (68), demonstrating that hECL2 is the main CD81 determinant of HCV entry. It has not been tested, however, whether hECL2 must be anchored to a target membrane to support infection. To address this question, we utilized the S29 clone of Huh-7 cells that expresses very little (if any) CD81 and thus does not support HCV infection (69). S29 cells were infected by immobilized HCVpp or VSVpp in the absence or in the presence of hECL2 or mECL2 (Fig. 7A). VSVpp, but not HCVpp, infected S29 cells when hECL2 was not included in the medium or when medium contained mECL2. However, in the presence of hECL2, HCVpp infected S29 cells with efficiency comparable with infection of Huh-7.5 cells in the absence of soluble receptor. Similar results were obtained with cell-attached viruses (Fig. 7B). This configuration also allowed HCV entry in the presence of hECL2. To conclude, the soluble CD81 fragment can substitute for the membrane-expressed receptor in supporting HCV entry, similar to the ability of soluble receptor-loaded ASLV to infect receptor-naive cells (70).

ECL2-primed HCV Can Undergo Fusion at Low pH—To assess whether inactivation of the receptor-primed E1E2 at low pH in the absence of a target membrane represents fusogenic conformational changes, we checked if HCV can fuse with a target membrane under these conditions. HCVpp pretreated with hECL2 or left untreated were spinoculated onto Huh-7.5 cells in the cold to minimize the virus uptake and interactions with entry co-factors. Cells were washed to remove free viruses, shifted to 37 °C, and briefly exposed to pH 5.2 either immediately or after 1 h. Endosomal entry pathway was blocked by preincubating the cells with BafA1 and maintaining the drug throughout the first 3 h of inoculation with viruses. Early exposure of untreated (Fig. 8A) or mECL2-pretreated (data not shown) viruses to low pH did not promote detectable infection. However, lowering the pH after 60 min at 37 °C resulted in a modest but highly significant increase in the luciferase signal (Fig. 8A, green bar), whereas SFVpp failed to fuse at the cell surface irrespective of preincubation history. The acquired fusion competence likely occurred through accumulation of receptor-primed HCV on the cell surface because of the faster rate of CD81 binding compared with the rate of virus uptake (Fig. 8B) (7, 25). Most importantly, HCVpp primed with hECL2 exhibited considerable infectivity when low pH was applied either immediately upon shifting to 37 °C or at a later time (Fig. 8A, yellow and blue bars). The ability of hECL2 priming to bypass a prolonged virus-cell preincubation indicates that this receptor fragment alone can render HCV competent to fuse directly with the plasma membrane at low pH. Our data also show that the HCVpp priming by CD81 alone or in combination with other co-receptors occurs relatively slowly.

It is worth stressing that the actual extent of HCVpp fusion mediated by low pH in the presence of BafA1 is higher than it appears on the graph (~16% of the pH 7.3-treated control in the

**FIGURE 7.** Soluble CD81 fragment rescues the ability of HCVpp to infect CD81-deficient cells. A, immobilized HCVpp, VSVpp, or control particles lacking E1E2 (pCDNAp) were preincubated with 25 μg/ml of hECL2 or mECL2 or left untreated. Viruses were then added to S29 cells in the presence or in the absence of ECL2, respectively, and cultivated for 2 days prior to measuring the luciferase activity. B, pseudoparticles were spinoculated onto S29 cells, and cells were washed and incubated in the presence or in the absence of mECL2 or hECL2 at 4 °C for 30 min. Cells were cultivated for 2 days to allow luciferase expression. Data points in both panels were normalized to the signal from Huh-7.5 cells infected under identical conditions with untreated viruses.
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A

![Graph A](image)

**FIGURE 8.** Receptor-primed HCVpp undergoes low pH-dependent fusion with the cell plasma membrane but maintains fusion competence in pH-neutral endosomal compartments. A, Huh-7.5 cells grown in 96-well plates were preincubated with medium containing 50 mM BafA1 for 30 min at 37 °C, washed, and chilled on ice for 5 min. HCVpp, SFVpp, or pCDNApp preincubated with hECL2 or left untreated were then spinoculated onto cells at 4 °C, washed, and chilled on ice for 5 min. HCVpp, SFVpp, or pCDNApp preincubated with hECL2 or left untreated were then spinoculated onto cells at 4 °C, washed, and exposed to pH 5.2 for 10 min at 37 °C in the presence of BafA1 either immediately (0 min) or after a 1-h incubation at 37 °C (60 min). Virus-cell incubation at 37 °C was carried out for 3 h, after which time the medium was changed to remove BafA1. To control for the possible adverse effect of BafA1 on infectivity and cell viability, the drug was added after 3 h of virus-cell incubation and maintained for an additional 3 h before washing (pink bars). Cells were incubated for 48 h and lysed, and the luciferase activity was recorded and normalized to the pH 7.3-treated control in the absence of BafA1 (set to 100%). B, evolution of the HCVpp sensitivity to low pH and the kinetics of productive uptake leading to infection. HCVpp or SFVpp were spinoculated in the cold onto Huh-7.5 cells that were either pretreated with BafA1 (black circles) or left untreated (red circles). Unbound viruses were removed by washing, and entry of cell-attached particles was initiated by shifting to 37 °C (in the presence or absence of BafA1). At the indicated time points, cells were briefly (10 min, 37 °C) exposed to pH 5.2. The resulting infectivity was normalized to control cells exposed to a pH 7.3 buffer in the absence of BafA1. For the protease K experiments (blue triangles), cells were grown on stripwell plates, allowed to bind the virus, as above, and shifted to 37 °C. To inactivate surface-accessible viruses after varied times of incubation, cells were chilled and treated with 50 μg/ml of protease K for 45 min on ice. Cells were washed, and the luciferase signal was measured at 48 h post-infection.

C

![Graph C](image)

Relative infectivity (%) of HCVpp and VSVpp to the NH4Cl block. Huh-7.5 cells were grown on stripwell plates, allowed to bind the virus, as above, and shifted to 37 °C. Infection in cell viability upon treatment with BafA1 (see supplemental Fig. 5), which diminished the luciferase signal for both HCVpp and VSVpp more than 2-fold (Fig. 8A, pink bars). Factoring in the adverse effect of BafA1 yields ~35% for the efficiency of low pH-mediated fusion of hECL2-primed HCVpp and of control viruses after a 1-h incubation. In addition, for viruses co-incubated with cells for 60 min at 37 °C, the results must be corrected for the fraction of internalized viruses that are no longer accessible to low pH. Through inactivating the surface-accessible HCVpp by protease K (see also Ref. 25), we found that ~60% of pseudoparticles were protected from the protease after 1 h at 37 °C and were thus internalized (Fig. 8B, blue triangles). Therefore, the real fraction of viruses that fused in the presence of BafA1 at this time should be as high as 87%. Note, however, that Meertens et al. (25) have not detected HCVpp fusion at the cell surface under similar experimental conditions, perhaps due to differences in HCVcc and HCVpp preparations and/or the choice of the virus strain.

To evaluate the rate of receptor priming of untreated HCVpp as it occurs on the cell surface, we measured the effect of the pH 5.2 pulse applied at varied times of virus-cell incubation. As observed above (Fig. 1B), immediate exposure to acidic pH enhanced infectivity (Fig. 8B, red circles). Strikingly, low pH pulses applied within the next 10 min of incubation resulted in a marked loss of signal, which then fully recovered by the 60-min time point. In comparison, the apparent resistance of SFVpp to low pH increased gradually over time (Fig. 8B, gray circles), likely as a result of virus endocytosis. The HCVpp escape from acid inactivation occurring between 10 and 60 min could be due to the reversal of the receptor-primed conformation of E1E2, as observed in experiments with hECL2 (Fig. 4A), or due to the acquisition of pH resistance, perhaps through engaging entry co-factors other than CD81. An alternative mechanism of the HCVpp escape from acid inactivation is through fusion at the cell surface. To test this possibility, we measured the extent of infection in the presence of BafA1 as a function of time of the low pH application. The resulting infectivity was corrected for the BafA1-mediated reduction in the luciferase signal and for the amount of internalized viruses (Fig. 8B, black circles), as discussed above. Comparison of the HCVpp response to acid treatment in the presence and in the absence of BafA1 (Fig. 8B, red versus black circles) revealed that, to a large extent, the virus escape from acidic pH occurred through the HCVpp fusion at the cell surface but not through its inactivation or reversal to an initial pH-resistant conformation.

The above acid treatment protocol revealed a complex dynamics of HCV on the surface of permissive cells, from potentiating of entry at the onset of co-incubation to inactivation and, finally, to fusion with the plasma membrane. Our results are consistent with the hypothesis that initial interac-

absence of BafA1). These data must be corrected for the reduction in cell viability upon treatment with BafA1 (see supplemental Fig. 5), which diminished the luciferase signal for both HCVpp and VSVpp more than 2-fold (Fig. 8A, pink bars). Factoring in the adverse effect of BafA1 yields ~35% for the efficiency of low pH-mediated fusion of hECL2-primed HCVpp and of control viruses after a 1-h incubation. In addition, for viruses co-incubated with cells for 60 min at 37 °C, the results must be corrected for the fraction of internalized viruses that are no longer accessible to low pH. Through inactivating the surface-accessible HCVpp by protease K (see also Ref. 25), we found that ~60% of pseudoparticles were protected from the protease after 1 h at 37 °C and were thus internalized (Fig. 8B, blue triangles). Therefore, the real fraction of viruses that fused in the presence of BafA1 at this time should be as high as 87%. Note, however, that Meertens et al. (25) have not detected HCVpp fusion at the cell surface under similar experimental conditions, perhaps due to differences in HCVcc and HCVpp preparations and/or the choice of the virus strain.

To evaluate the rate of receptor priming of untreated HCVpp as it occurs on the cell surface, we measured the effect of the pH 5.2 pulse applied at varied times of virus-cell incubation. As observed above (Fig. 1B), immediate exposure to acidic pH enhanced infectivity (Fig. 8B, red circles). Strikingly, low pH pulses applied within the next 10 min of incubation resulted in a marked loss of signal, which then fully recovered by the 60-min time point. In comparison, the apparent resistance of SFVpp to low pH increased gradually over time (Fig. 8B, gray circles), likely as a result of virus endocytosis. The HCVpp escape from acid inactivation occurring between 10 and 60 min could be due to the reversal of the receptor-primed conformation of E1E2, as observed in experiments with hECL2 (Fig. 4A), or due to the acquisition of pH resistance, perhaps through engaging entry co-factors other than CD81. An alternative mechanism of the HCVpp escape from acid inactivation is through fusion at the cell surface. To test this possibility, we measured the extent of infection in the presence of BafA1 as a function of time of the low pH application. The resulting infectivity was corrected for the BafA1-mediated reduction in the luciferase signal and for the amount of internalized viruses (Fig. 8B, black circles), as discussed above. Comparison of the HCVpp response to acid treatment in the presence and in the absence of BafA1 (Fig. 8B, red versus black circles) revealed that, to a large extent, the virus escape from acidic pH occurred through the HCVpp fusion at the cell surface but not through its inactivation or reversal to an initial pH-resistant conformation.

The above acid treatment protocol revealed a complex dynamics of HCV on the surface of permissive cells, from potentiating of entry at the onset of co-incubation to inactivation and, finally, to fusion with the plasma membrane. Our results are consistent with the hypothesis that initial interac-
tions with CD81 occurring within the first minutes of co-incubation make the virus vulnerable to acid inactivation, as also observed for cell-free viruses primed with hECL2 (Figs. 3 and 4). Next, HCVpp appears to acquire fusion competence with a half-time of ~16 min (Fig. 8B, red circles), in agreement with the reported rate of HCV-CD81 binding on Huh-7.5 cells (7). This second step could be driven by the HCV binding to additional co-receptors and/or by direct insertion of hydrophobic E2 domains into the target membrane (see under “Discussion”). To summarize, CD81 appears to play a critical role in rendering the HCV competent for low pH-dependent fusion.

Receptor-primed HCV Is Stable in pH-neutral Intracellular Compartments—The return of infectivity of hECL2-primed HCVpp to a basal level (Fig. 4A) shows that this conformation of E1E2 is relatively short lived in the absence of a target membrane. The transient nature of hECL2-HCV interactions is also apparent from dissociation of the soluble CD81 fragment from sE2 in the presence of liposomes (Fig. 6, E and F). However, to fuse with acidic endosomes, HCV needs to sustain its fusion competence throughout the slow internalization process (Fig. 8B) (7, 25). We therefore asked whether the receptor-primed state is more stable when HCV enters Huh-7.5 cells through a normal endocytic pathway. Cells were inoculated with HCVpp in the presence of NH4Cl to permit virus uptake but block normal endocytic pathway. The high stability of E1E2 in neutral intracellular compartments is reminiscent of the stability of internalized ASLV that remains infectious for several hours in the presence of NH4Cl (41). These results show that in contrast to the hECL2-primed E1E2, which appears to revert back to a native state in the absence of target membrane, HCV-receptor complexes formed through a conventional entry pathway into target cells remain fusion-competent for a long time. Considering a stable association of the HCV sE2 (as well as ASLV (61, 63, 66)) with liposomes in the presence of the soluble receptor, it is conceivable that a reversible CD81-primed conformation of E1E2 is stabilized through insertion of the hydrophobic fusion peptides/loops into a cell membrane.

DISCUSSION

The HCV E1E2-mediated fusion is a multistep process that, at least in part, determines the narrow cell tropism of this virus. The exact roles of the four cellular factors required for HCV entry and the spatiotemporal regulation of E1E2-mediated fusion have not been fully elucidated. Experimental findings of this study reveal that, contrary to the view that CD81 is a secondary HCV receptor, this protein plays a central role in entry of this virus. Arguments against CD81 as the main HCV receptor include its extremely slow constitutive endocytosis and the fact that anti-CD81 antibodies block infection but not HCV attachment to permissive cells (10, 18, 23, 59). We provided evidence that CD81 promotes conformational changes in E1E2, rendering these glycoproteins competent to mediate low pH-dependent fusion. This conclusion is based on the observations that HCV pretreatment with hECL2 results in the following: (i) enhanced infectivity; (ii) altered recognition by conformation-specific antibodies to E1 and E2; (iii) E2 binding to liposomes; (iv) irreversible refolding/inactivation at low pH in the absence of a target membrane, and (v) fusion with the plasma membrane of permissive cells at acidic pH.

The low pH- and hECL2-induced E1E2 conformations identified in this work exhibited common features, such as: (i) the tendency to revert back to an original state in the absence of target membranes; (ii) binding to liposomes, and (iii) enhanced infectivity compared with untreated viruses. However, as evidenced by the distinct patterns of antibody binding and by the specific order of exposure to hECL2 and pH 5.2 required for the HCV inactivation, these two conformations appear to be different. The acid-induced E2-liposome binding can explain the puzzling observation that low pH promotes HCV fusion with lipid vesicles in the absence of receptors (71). It is feasible that E1E2 dimers dissociate at low pH (32) permitting the E2 binding to liposomes and, ultimately, fusion. The liberation of a fusion protein and its interaction with a target membrane is also consistent with the enhanced infectivity of acid-pretreated HCV, although this pretreatment does not mediate fusion with the plasma membrane of permissive cells (Fig. 18).

The effects of hECL2 on HCV infectivity are in good agreement with published data. A dose-dependent inhibition of HCV entry by the CD81 extracellular loop has been reported previously (59). These authors have also observed a modest infectivity increase of hECL2-pretreated viruses when the soluble receptor was diluted prior to inoculating the cells. Here, we discovered that a combination of hECL2 and low pH caused irreversible conformational changes in E1E2 leading to loss of infectivity. Although inactivation of fusion proteins upon receptor binding and exposure to low pH has been described for unrelated viruses (41, 72), the sensitization of hECL2-primed HCV to low pH is surprising because three additional cellular factors are required for productive entry. It is therefore possible that the hECL2/pH 5.2 treatment promotes off-path conformational changes that do not lead to fusion. However, direct association of hECL2-primed sE2 with liposomes (Fig. 6) and fusion of hECL2-primed HCVpp with the plasma membrane at low pH (Fig. 8) argue against this possibility.

The inhibitory effect of hECL2 present throughout the HCV entry (Fig. 2B) and the infectivity-enhancing effect of a brief pretreatment with this protein (Fig. 3, A and B) appear paradoxical. The former effect is apparently due to the competition between CD81 and hECL2 for binding to E2 and/or due to disruption of CD81 dimerization or its interactions with other HCV entry co-factors (14, 73). This notion is consistent with efficient infection of S29 cells in the presence of hECL2. The favorable effect of hECL2 pretreatment on infectivity may reflect more efficient interactions of a pre-triggered E2 with...
CD81. We envision that, by analogy to the HCV isolate capable of utilizing the mouse CD81 for entry (74), hECL2 binding and dissociation from E2 could leave this protein in a transiently activated state that could more readily engage CD81. The infectivity-enhancing effect of low pH pretreatment is also consistent with the notion that a stable native conformation needs to be disrupted, perhaps through dissociation of E1E2 dimers, to more efficiently promote fusion.

The notion that E1E2 dimers could be exceedingly stable is supported by the slow HCVpp priming with hECL2 (half-time ~16 min; Fig. 4A). Moreover, primed E1E2 returned to an original state with the same time course, while shedding the soluble receptor. The loss of the soluble receptor is surprising, considering the high binding affinity and the very long half-life of hECL2-sE2 complexes (23, 67). Note that the low pH-induced conformation was even less stable, as evidenced by a shorter relaxation time of 3–6 min (Figs. 1D and 5B). Reversible E1E2 intermediates are indicative of comparable free energies of activated and native conformations. A possible explanation for the high stability of the hECL2-sE2 complexes reported in the literature compared with the complex with a native E1E2 dimer is that a native form could be stabilized by the transmembrane domains. These domains contain a dimerization motif that would disfavor the E1E2 dissociation that might be essential for initiating the fusion reaction (75, 76). The slow and reversible priming of E1E2 by hECL2 indicates that additional entry co-factors may be needed to stabilize this potentially fusion-competent intermediate. However, stable binding of the soluble receptor-primed sE2 with liposomes at neutral pH and concomitant shedding of hECL2 (Fig. 6) show that a target membrane can capture a post-primed conformation of E2 without the need for additional receptors.

It remains to be determined whether the hECL2-primed HCV can engage liposomes or the cell plasma membrane in a manner similar to sE2. Such direct binding would be consistent with the slow acquisition of fusion competence on the surface of Huh-7.5 cells (Fig. 8). However, this notion is difficult to reconcile with the HCV reliance on other entry co-factors, unless this pre-hairpin-like intermediate interacts with or somehow depends on these co-factors. It is therefore possible that HCV interactions with additional entry co-factors can account for the stabilization of a fusion-competent conformation of E1E2 on the surface of permissive cells. However, the arguments given below seem to support the notion that CD81 alone can prime E1E2 for fusion and that this conformation may be stabilized by the insertion of hydrophobic E2 domains into a cell membrane.

First, as discussed above, liposomes seem to trap a post-primed conformation of sE2. Second, whereas cell-free viruses are inactivated upon hECL2 and acid treatment, cell-bound HCVpp-hECL2 complexes fuse directly with the plasma membrane at low pH under conditions disfavoring the HCV binding to entry co-factors (pre-binding to cells at 4 °C). This shows that the hECL2 priming can bypass the slow receptor-priming step(s) required for low pH-dependent fusion of untreated viruses (Fig 8) (31). Third, the HCV variant adapted to use mouse CD81, but not the wild-type virus, could fuse with the plasma membrane at low pH without preincubation with Huh-7.5 cells at 37 °C (74). The ability of this mutant to quickly acquire fusion competence correlated with the increased exposure of the CD81-binding site on E2 and enhanced sensitivity to neutralization by anti-E2 antibodies. Fourth, the priming of immobilized HCVpp by hECL2 (Fig. 4A), the HCVpp binding to CD81 on the cell surface (4, 7), and the acquisition of competence for low pH-dependent fusion for cell-associated viruses (Fig. 8B) all occur within a similar time frame, perhaps reflecting the same underlying process.

In summary, our results show that HCV-CD81 binding can confer fusion competence upon encountering low pH. This raises an intriguing possibility that other HCV entry co-factors can play regulatory roles, perhaps stabilizing the fusion intermediates and/or guiding the virus uptake and trafficking to acidic compartments. This study may also have a broader impact on understanding the HCV entry and fusion. Although it is well established that the E2 glycoprotein bears receptor binding functions, its role in membrane fusion remains controversial (77–81). The sE2-liposome binding shows that this protein undergoes substantial conformational changes in response to the hECL2 binding and directly engages a target membrane while shedding the soluble receptor, suggesting that E2 is the fusion protein of HCV. Finally, intermediate conformations of E1E2 identified in this work could be useful for vaccine therapeutic efforts. Further functional characterization of HCV fusion should elucidate the critical steps of this process and suggest novel therapeutic strategies to battle infection.

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