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Hepatitis C Virus (HCV) Envelope Glycoproteins E1 and E2 Contain Reduced Cysteine Residues Essential for Virus Entry*

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The HCV envelope glycoproteins E1 and E2 contain eight and 18 highly conserved cysteine residues, respectively. Here, we examined the oxidation state of E1E2 heterodimers incorporated into retroviral pseudotyped particles (HCVpp) and investigated the significance of free sulfhydryl groups in cell culture-derived HCV (HCVcc) and HCVpp entry. Alkylation of free sulfhydryl groups on HCVcc/pp with a membrane-impermeable sulfhydryl-alkylating reagent 4-(*N*-maleimido)benzyl-α-trimethylammonium iodide (M135) prior to virus attachment to cells abolished infectivity in a dose-dependent manner. Labeling of HCVpp envelope proteins with EZ-Link maleimide-PEG2-biotin (maleimide-biotin) detected free thiol groups in both E1 and E2. Unlike retroviruses that employ disulfide reduction to facilitate virus entry, the infectivity of alkylated HCVcc could not be rescued by addition of exogenous reducing agents. Furthermore, the infectivity of HCVcc bound to target cells was not affected by addition of M135 indicative of a change in glycoprotein oxidation state from reduced to oxidized following virus attachment to cells. By contrast, HCVpp entry was reduced by 61% when treated with M135 immediately following attachment to cells, suggesting that the two model systems might demonstrate variations in oxidation kinetics. Glycoprotein oxidation was not altered following binding of HCVpp incorporated E1E2 to soluble heparin or recombinant CD81. These results suggest that HCV entry is dependent on the presence of free thiol groups in E1 and E2 prior to cellular attachment and reveals a new essential component of the HCV entry process.

An obligate stage of viral entry for enveloped viruses is the process of membrane fusion between the viral membrane and a target cell membrane. This event is required to deliver the viral nucleocapsid into target cells for the initiation of productive infection. Membrane fusion is mediated by viral fusion proteins present on the surface of enveloped viruses that are characterized by the presence of at least two hydrophobic sequences, including a fusion peptide and a transmembrane domain. Fusion proteins are initially expressed as inactive precursors that remain trapped in a metastable conformation, shielding the fusion peptide from the external environment. A specific biochemical trigger activates the glycoprotein complex such that the fusion peptide is exposed and inserted into the target cell membrane, initiating the fusion reaction. Activation triggers include low pH (1), receptor binding and low pH (2), endosomal cleavage (3), or thiol isomerization (4, 5). Following insertion of the fusion peptide into the target cell membrane, the fusion protein refolds into a lower energy conformation, thereby drawing the viral and cellular membranes together. Fusion then proceeds through three distinct phases: lipid mixing, pore formation, and pore expansion (6).

Hepatitis C virus (HCV)[3] is a member of the Flaviviridae family of enveloped positive strand RNA viruses and displays two envelope glycoproteins, E1 (polyprotein residues 191–383) and E2 (E2; residues 384–746), on the virus surface. These type I transmembrane proteins contain heavily glycosylated N-terminal ectodomains and C-terminal transmembrane domains. Glycoproteins E1 and E2 contain eight and 18 cysteine residues, respectively, and have been detected on the surface of virions as both covalently and noncovalently associated heterodimers (E1E2) (7–9). The function of E1 during virus entry is not precisely known, whereas glycoprotein E2 attaches virions to cell surface receptors. A number of membrane interactive sequences have been identified in both E1 and E2; however, the exact location of the fusion peptide remains unclear (10–12).

The N-terminal region of E2 (residues 384–661) can be expressed independently of the polyprotein and folds into a structure capable of binding cellular receptors. This region is referred to as the receptor-binding domain (13, 14). The receptor-binding domain is connected to the transmembrane domain via a hydrophobic heptad repeat (residues 675–699), which is essential for E1E2 heterodimerization and infectivity (15). The secondary structure and function of this region is analogous to the stem region of the flaviviral glycoprotein E, a well characterized class II fusion protein (16). Recently, circular dichroism and infrared spectroscopy analyses of a recombinantly expressed E2 ectodomain indicated the protein to contain ~28% β-sheet and is consistent with the expected secondary structure content of a class II fusion protein (17). The disulfide bonding arrangement of the 18 conserved cysteine residues of expressed E2 ectodomain has been mapped using mass spectrometry following trypsin digestion. This informa-

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[3] The abbreviations used are: HCV, hepatitis C virus; HCVpp, HCV-pseudotyped particles; HCVcc, cell culture-derived HCV; M135, 4-(*N*-maleimido)-benzyl-α-trimethylammonium iodide; maleimide-biotin, EZ-Link maleimide-PEG2-biotin; NHS-biotin, Sulfo-NHS-LC-Biotin; VSVGpp, retroviral particles pseudotyped with VSV G protein; LEL, large extracellular loop.

tion in combination with antibody and CD81 binding site studies (18–20) has allowed modeling of expressed E2 ectodomain onto the three domains of a class II fusion protein scaffold (17). Sixteen cysteines were found to be engaged in eight disulfide bonds, with two cysteines (Cys-597 and Cys-620) not definitively assigned (17). Due to the difficulty in expressing E1 alone or as a soluble E1E2 heterodimer, the disulfide arrangement of the eight conserved cysteines of E1 has not yet been determined.

HCV entry into hepatocytes is dependent on an initial attachment event with glycosaminoglycans (21, 22). Other cellular receptors or entry cofactors for HCV include CD81 (13), scavenger receptor B1 (SR-B1) (13, 14, 23), and tight junction proteins claudin-1, -6, or -9 (24–26) and occludin (27). Transduction of murine cells with CD81, SR-B1, claudin-1, and occludin confers permissiveness to HCV entry, confirming the essential role that these four proteins play in HCV entry (27, 28). Other co-factors implicated in HCV entry include epidermal growth factor receptor, ephrin receptor A2 (29), and the low density lipoprotein receptor (30, 31). Following attachment, HCV undergoes clathrin-mediated endocytosis and membrane fusion within the early endosome (32, 33). This entry process requires the presence of both E1 and E2 (7, 34, 35). Although fusion of effector cells expressing E1E2 and Huh7 target cells displaying the HCV receptors has been demonstrated after exposure to low pH (11, 36), low pH does not efficiently induce HCV viral fusion at the plasma membrane of cells treated with bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase, which neutralizes the pH of the endosome (32). At present, the form of E1E2 that is responsible for virus infectivity, and the structural changes occurring in E1E2 during virus entry have not been elucidated.

In this study, we investigated whether the redox state of E1 and E2 is an essential factor in regulating virus entry. We show here that entry of cell culture-derived HCV (HCVcc) and retroviral pseudotypes incorporating E1E2 (HCVpp) is dependent on the presence of free thiol groups and that HCV glycoproteins are likely to switch from a reduced to an oxidized state following cellular attachment.

EXPERIMENTAL PROCEDURES

Vectors and Antibodies—Construction of pE1E2H77c has been described previously (7). pNL4–3. LUC.R$^-$E$^-$ was obtained from Dr. N. Landau though the National Institutes of Health AIDS Research and Reference Reagent Program (37). The vector pJC1FLAG2(p7-NS-GLUC2A), was a kind gift from Prof. Charles Rice (The Rockefeller University) and comprises the structural region (core-p7) of HCV-J6 (genotype 2a) and the nonstructural region (NS2A-NS5B) of JFH1 (genotype 2a) (38). The plasmid encodes a FLAG epitope tag at the N terminus of E2 and Gaussia luciferase that is secreted from infected or transfected cells. Plasmid pHEF-VSVG was obtained from Dr. Lung-Ji Chang though the National Institutes of Health AIDS Research and Reference Reagent Program (39).

mAbs A4 and H52 (40, 41) were kind gifts from Dr. Jean Dubuisson (Institut Pasteur de Lille, Lille, France). mAb 183 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (42). The monoclonal anti-FLAG M2 antibody was purchased commercially (Sigma-Aldrich).

Cells and Cell Lines—Human embryonic kidney cells (HEK 293T) were maintained in Dulbecco's modified eagle medium (DMEM) (Invitrogen) with 10% fetal calf serum and 2 mM L-glutamine (DMF10). Human hepatoma-7.5 (Huh7.5) cells were a kind gift of Professor Charles Rice (The Rockefeller University) and were maintained in DMF10 supplemented with 0.1 mM nonessential amino acids (DMF10NEA).

Production of HCVcc—HCV RNA was transcribed *in vitro* from XbaI-linearized pJC1FLAG2(p7-NS-GLUC2A) DNA using Ampliscribe^{TM} T7 High Yield Transcription Kit (Epicenter Biotechnologies). RNA was purified, either by standard phenol/chloroform extraction and ethanol precipitation, or using the RNeasy Mini Kit (Qiagen). Purified RNA was stored at −80 °C.

Huh7.5 cells were seeded at 350,000 cells/well in six-well plates 18 h prior to transfection in antibiotic-free DMF10NEA. Cells were transfected with 6 μg of RNA using 8 μl of DMRIE-C reagent (Invitrogen) in Opti-MEM (Invitrogen). Fresh Opti-MEM was added to cells 4 h post-transfection. Seventy-two h later, virus in the tissue culture supernatant was filtered (0.45 μm) and buffer-exchanged via ultrafiltration (100 kDa molecular weight cut-off; Amicon) into TN-Ca^{2+} (14 mM Tris, 12 mM HEPES, 150 mM NaCl, 1.8 mM Ca^{2+}, pH 6.8). Virus was stored at −80 °C until further use. The $TCID_{50}$/ml was determined according to the method of Lindenbach *et al.* (43) using the Reed-Muench calculator (44).

HCVcc Infectivity Assays—Huh7.5 cells were seeded at 30,000 cells/well in a 48-well plate format. Buffer-exchanged HCVcc was diluted to 1210 $TCID_{50}$/ml in TN-Ca^{2+} and then alkylated with 0 to 1 mM 4-(*N*-maleimido)benzyl-α-trimethylammonium iodide (M135) at 37 °C for 50 min. Reactions were quenched by addition of DMF20NEA. Alkylated virus was incubated with cells at 37 °C for 2 h. Cells were washed once with TN-Ca^{2+}, and fresh DMF10NEA was added to each well. Luciferase activity in the tissue culture supernatant was assayed 72 h later using the *Renilla* luciferase kit (Promega) and quantified on a Fluostar Optima fitted with luminescence optics (BMG LABTECH). Alternatively, 1210 $TCID_{50}$/ml of HCVcc in TN-Ca^{2+} was incubated with cells at 4 °C for 2 h, followed by removal of unbound virus and addition of 0–5 mM M135 in TN-Ca^{2+} at 37 °C for 50 min. Rates of infection were measured 72 h later as described above.

In assays where DTT was added to alkylated virus, 1210 $TCID_{50}$/ml of HCVcc in TN-Ca^{2+} was incubated with M135 for 50 min at 37 °C and then added to cells for 2 h at 4 °C. DTT in TN-Ca^{2+} was then added to virus-bound cells for 1 h at 37 °C.

To test M135 toxicity, Huh7.5 cells were directly incubated with 0 to 5 mM M135 for 2 h at 37 °C. Cells were washed and fresh DMF10NEA was added. Seventy-two h later, cell viability was determined by trypan blue staining.

HCVpp Infectivity Assays—HEK 293T cells were seeded at 350,000 cells/well in a six-well plate. Eighteen h later, cells were co-transfected with pE1E2H77c or pHEF-VSVG along with pNL4–3. LUC.R$^-$E$^-$ using FuGENE 6 (Roche Applied Science). Seventy-two h post-transfection, culture supernatants

were filtered (0.45 μm). Particles were pelleted by ultracentrifugation (SW 41 rotor, 16,000 rpm, 4 °C, 2 h) and then resuspended in TN-Ca^{2+}. Particles were alkylated with 0 to 5 mM M135 pre- or post-binding to Huh7.5 cells as described for HCVcc. Infection of Huh7.5 cells was determined 72 h later by luciferase activity in the cell lysate.

Recombinant CD81—The expression and purification of a chimera composed of maltose-binding protein linked to CD81 large extracellular loop (residues 113–201) (MBP-LEL(113–201)) has been described previously (45). Solid phase CD81 binding assays using HCVpp were performed as described previously (18).

Heparin Binding Assay—Partially purified HCVpp were either untreated or alkylated with 5 mM M135 for 30 min then quenched with 500 mM cysteine for 30 min at room temperature. Virions were pelleted for 2 h at 14,000 × *g* and lysed in PBS containing 1% Triton X-100 and 1 mM EDTA. One quarter of the lysate was run directly in reducing SDS-PAGE, whereas the remaining lysate was diluted into heparin binding buffer (0.15 M NaCl, 0.02 M Tris/HCl (pH 7.4)) and added to heparin-Sepharose (Sigma) overnight at 4 °C (46). Beads were washed three times in binding buffer and subjected to reducing SDS-PAGE and Western blotting.

Maleimide Biotinylation—HEK 293T cells were seeded at 500,000 cells/well in a six-well plate. Five h later, cells were co-transfected with pE1E2H77c or cDNA4 empty vector along with pNL4–3. LUC.R^-E^- using FuGENE 6 (Roche Applied Science). Seventy-two h post-transfection, culture supernatants were filtered (0.45 μm). HCVpp were pelleted by ultracentrifugation (SW 41 rotor, 25,000 rpm, 4 °C, 2 h) over a 25% sucrose cushion. Pelleted particles were resuspended in PBS (pH 7.2) containing 1.25 mM EZ-Link maleimide-PEG2-biotin (maleimide-biotin; Thermo Scientific) or Sulfo-NHS-LC-Biotin (NHS-biotin; Thermo Scientific) and incubated at room temperature for 30 min. Reactions were quenched with 100 mM cysteine or glycine, respectively, for 30 min at room temperature. Particles were pelleted by centrifugation (14,000 × *g*, 2 h, 4 °C) and then lysed (0.6 M PBS, 1 mM EDTA, 0.02% sodium azide, 1% Triton X-100). Particle lysates were reduced with 100 mM DTT. High capacity streptavadin-agarose resin (Thermo Scientific) was used to isolate biotinylated proteins. Samples were run on reducing SDS-PAGE and Western blotted with mAbs directed toward E2, E1, and HIV-1 capsid protein p24 (H52, A4, and 183, respectively). For samples preincubated with M135, HCVpp were pelleted by ultracentrifugation, then resuspended in 0 to 1 mM M135 and incubated for 30 min at room temperature prior to biotinylation as described above.

In maleimide biotinylation assays where HCVpp were prebound to CD81 or heparin, 500 μg of wild type or F186S mutant MBP-LEL(113–201), or 1 mg/ml heparin sodium salt (from porcine intestinal mucosa) were added to particles and incubated at room temperature for 1 h prior to ultracentrifugation and labeling as described above.

SDS-PAGE and Western Blotting—Samples were run on 10% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane (0.22 μ; Amersham Biosciences, GE Healthcare). E1, E2, and HIV-1 proteins p24 or $Pr55^{Gag}$ were visualized by mAbs A4, H52, and 183, respectively, and Alexa Fluor 680 goat anti-mouse (Invitrogen). Blots were imaged using an Odyssey Infrared Imaging System at 680 nm (LI-COR Biosciences).

RESULTS

Requirement for Free Sulfhydryl Groups in Envelope Proteins during HCV Entry—To investigate whether the redox state of the viral E1E2 complex is a determinant of infectivity, we examined the effects of treating HCVcc and HCVpp with the membrane impermeable sulfhydryl akylating agent M135. Infectious HCVcc were produced by transfection of Huh7.5 cells with RNA transcribed from the full-length chimeric HCV genomic construct JC1FLAG2(p7-NS-GLUC2A) (38). Supernatants containing cell-free HCVcc were exchanged into TN-Ca^{2+} buffer by ultrafiltration to remove sulfhydryl-containing contaminants. We also examined the effect of M135 alkylation in an independent model of HCV entry where E1E2 is displayed on the surface of retroviral particles that are capable of mediating a single cycle of entry into hepatocytes, HCVpp. This system, which has been used extensively to identify and study the role of cellular factors in HCV entry (47), enables visualization of E1E2 by Western blotting and immunoprecipitation, as well as measurement of CD81 binding activity. For HCVpp, particles bearing E1 and E2 from prototype strain H77c were produced in HEK 293T cells. Seventy-two h post-transfection, HCVpp were pelleted by ultracentrifugation to deplete sulfhydryl contaminants present in the medium and resuspended in TN-Ca^{2+}. Preparations of HCVcc/pp were treated with M135 in TN-Ca^{2+} buffer for 50 min at 37 °C. Reactions were quenched by addition of DMF20NEA, and then alkylated virus was applied to Huh7.5 cells for 2 h at 37 °C. Excess virus was removed, and cells were incubated at 37 °C for 3 days prior to luciferase assay.

Significant inhibition of HCVcc entry was observed in a dose-dependant manner when virus was incubated with 0.2, 0.5, and 1 mM M135 ($p < 0.05$, Student's *t* test) compared with untreated virus (Fig. 1*A*). Complete inhibition of entry occurred at 0.5 and 1 mM M135. Inhibition by M135 was virus dose-dependent with an HCVcc titer of 2420 $TCID_{50}$/ml sufficient to overcome the inhibitory effect of 1 mM M135, whereas complete inhibition was achieved with 1210 $TCID_{50}$/ml or less (Fig. 1*B*). When HCVpp was alkylated with 0.5, 1, 2, and 5 mM M135, entry was significantly lowered compared with untreated virus ($p < 0.05$, Student's *t* test) (Fig. 1*C*). In contrast to the complete inhibition of entry observed for HCVcc, HCVpp required higher concentrations of M135 to achieve 80% inhibition. Increasing the concentration of M135 used to alkylate HCVpp to 10 mM did not result in >80% inhibition of entry (data not shown), suggesting that a component of E1E2 incorporated into HCVpp cannot be inhibited by M135.

To establish the specificity of M135 alkylation, we examined whether vesicular stomatitis virus (VSV) envelope protein G (VSVG) mediated entry was sensitive to M135 alkylation. The VSV G protein contains 12 Cys residues engaged in 6 disulfide bonds in both pre- and post-fusion conformations and there have been no reports of this virus being sensitive to alkylating agents (48, 49). Retroviral particles pseudotyped with VSV G protein (VSVGpp) were produced and prepared as described

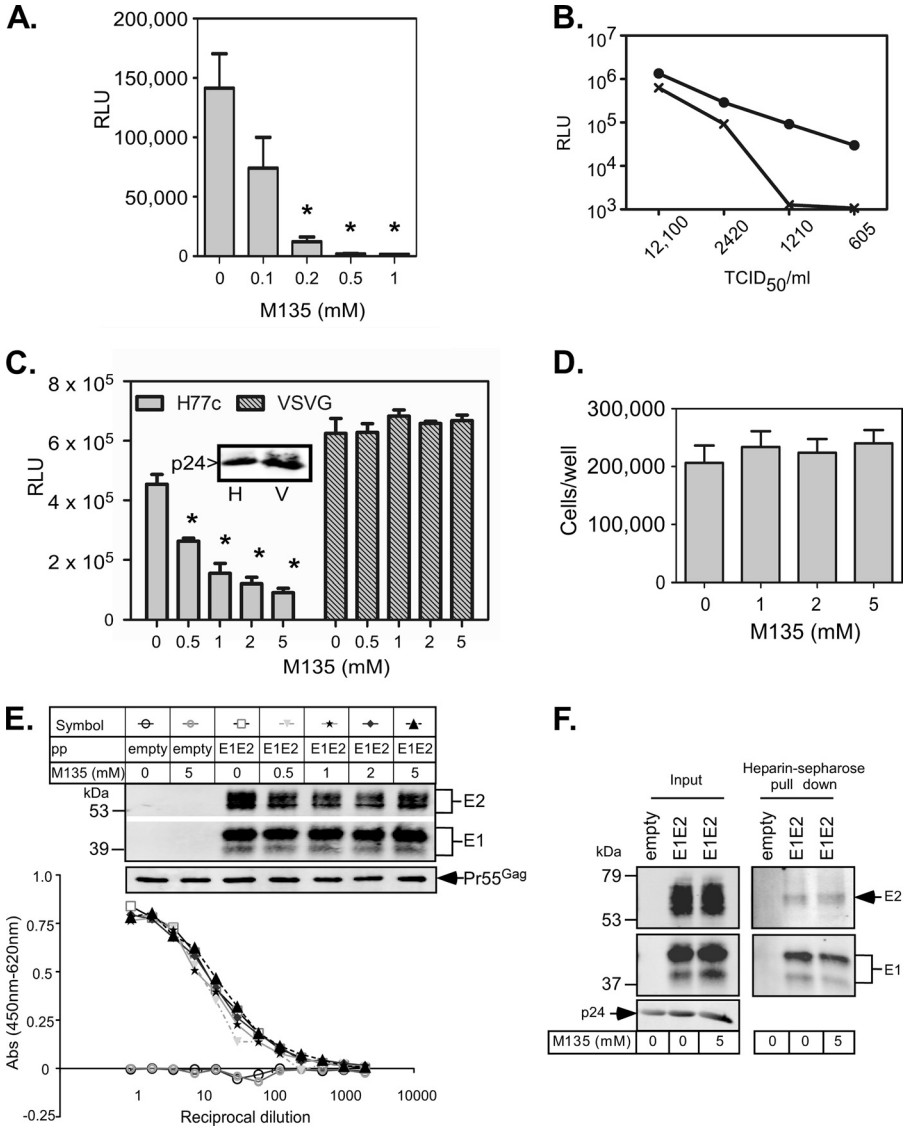

FIGURE 1. **HCV entry is dependent on the presence of free sulfhydryl groups.** *A*, preparations of HCVcc were alkylated with M135 prior to addition to Huh7.5 cells. Luciferase activity in the culture supernatant was measured 72 h later. *RLU*, relative light units. Data are the mean and S.E. of four independent experiments, each consisting of six replicates. *Asterisks* indicate significantly different entry activity relative to untreated virus ($p < 0.05$, Student's *t* test). *B*, increasing concentrations of HCVcc were incubated with 0 (●) or 1 mM M135 (×), and luciferase activity in the culture supernatant was measured 72 h later. Data shown are a representative assay with each point the average of quadruplet wells. *C*, preparations of HCVpp or VSVGpp were alkylated with M135 prior to addition to Huh7.5 cells. Luciferase activity in the cell lysate was measured 72 h later. Data are the mean and S.E. of three independent experiments, each consisting of four replicates. *Asterisks* indicate significantly different entry activity relative to untreated virus ($p < 0.05$, Student's *t* test). Western blot analysis of HIV-1 structural protein p24 shows similar virus input levels for HCVpp (*H*) and VSVGpp (*V*) (*inset*). *D*, Huh7.5 cells were incubated with increasing concentrations of M135 for 2 h at 37 °C. Cell viability was determined 72 h later by trypan blue exclusion. *E*, the ability of E1E2 from HCVpp (H77c) to bind CD81 following M135 alkylation was determined by a direct binding immunoassay using recombinant CD81 MBP-LEL(113–201). Input E1, E2, and HIV-1 Gag ($Pr55^{Gag}$) were determined by Western blot for each sample applied to the binding assay (probed with mAbs A4, H52, and 183, respectively). *F*, HCVpp were untreated or treated with 5 mM M135 prior to lysis and immunoprecipitated with heparin-Sepharose. Immunoprecipitated glycoproteins were detected in reducing SDS-PAGE followed by Western blotting with A4 (E1) and H52 (E2). Input amounts of HCVpp were detected directly without immunoprecipitation in reducing SDS-PAGE followed by Western blotting with mAbs A4 (E1) and H52 (E2) and 183 (p24).

for HCVpp. Entry of VSVGpp remained unaffected following incubation with up to 5 mM M135 (Fig. 1*C*). Given that VSVG contains no free sulfhydryl groups with which M135 can react, this finding indicates that the inhibition of entry observed for M135-alkylated HCV is mediated via free sulfhydryls.

To confirm that the inhibitory effect of M135 on virus entry was not due to cellular toxicity, Huh7.5 cells were incubated with increasing concentrations of M135 for 2 h at 37 °C. Seventy-two h later, cell viability was determined by trypan blue staining. It was found that incubation of Huh7.5 cells with up to 5 mM M135 did not affect cell survival (Fig. 1*D*).

CD81 is an essential attachment factor required for HCV infection of liver cells (13) and is mediated through a direct interaction between E2 and the large extracellular loop (LEL) of CD81 (15, 17–20, 50). To determine whether M135 alkylation of HCV can affect the ability of the virus to engage the CD81 receptor, a CD81 binding assay was used. This assay employs a recombinant protein consisting of the LEL of CD81 (residues 113–201) N-terminally fused to maltose binding protein (MBP-LEL(113–201)). This construct has been used extensively to characterize CD81-E2 interactions (15, 18, 45, 51–53), demonstrates binding to virion-incorporated E2 (10, 18), and is

thought to mimic the native form of CD81 by interacting with claudin-1 (54). In addition, virion-incorporated E2 can be examined directly for its ability to bind CD81 (10, 18). Due to the low titer of HCVcc produced, CD81 binding analyses were performed with HCVpp only. It was found that incubation of HCVpp with up to 5 mM M135 had no effect on the ability of HCVpp-derived E2 to bind MBP-LEL(113–201) (Fig. 1*E*). Western blot analysis of HCVpp using mAbs directed toward E1, E2, and HIV-1 $Pr55^{Gag}$ (mAb A4, H52, and 183, respectively) confirmed that equivalent concentrations of HCVpp bearing comparable amounts of E1 and E2 were applied to the CD81 binding assay (Fig. 1*E*). These data indicate that abrogation of HCV entry following incubation with M135 is not likely to be due to an inability of alkylated virus to bind CD81. The finding that CD81 binding is unaffected by alkylation of E2 also suggests that the overall structure of the E2 receptor binding domain is intact as the CD81 binding site is comprised of at least four discontinuous sequences within E2 located in two separate subdomains (17). Finally, similar amounts of alkylated and unalkylated HCVpp were immunoprecipitated using heparin-Sepharose suggesting that the ability of alkylated HCVpp to bind glycosaminoglycans was maintained (Fig. 1*F*). Together, these data indicate that the surface proteins of HCV are sensitive to sulfhydryl alkylation and that the viral glycoproteins must be in a reduced state prior to cellular attachment to mediate virus entry.

Detection of Free Thiols in E1 and E2 Using Thiol-specific Biotinylation Reagent—We next asked whether free sulfhydryls could be visualized in the E1E2 complex. Due to the technical difficulties associated with obtaining HCVcc in sufficient amounts and purity to enable robust biochemical analyses, assays were performed using HCVpp only. The HCV glycoproteins present in retroviral particles have been extensively characterized, and both HCVpp and HCVcc are known to utilize the same cellular receptors in virus entry and mediate entry in a pH-dependent manner (7, 8, 27, 35, 36). Furthermore, our studies indicate that free sulfhydryl groups are essential for both HCVcc and HCVpp entry.

HCVpp were partially purified through a 25% sucrose cushion then labeled with a sulfhydryl-specific biotinylating agent, maleimide-biotin (1.25 mM). As a control, HCVpp were also labeled with the amine-specific biotinylating agent, NHS-biotin (1.25 mM). Excess labeling reagent was quenched with 100 mM cysteine (for maleimide-biotin) or glycine (for NHS-biotin). The HCVpp were lysed and then reduced with 100 mM DTT to separate heterodimeric E1E2. The biotinylated proteins were isolated by binding to high capacity streptavidin-agarose resin and then run under reducing conditions on SDS-PAGE. Western blot analysis of the reduced streptavidin-agarose-precipitated proteins using anti-E1 mAb (A4) and anti-E2 mAb (H52) (40, 41) demonstrated that E1 and E2 contain at least one free sulfhydryl group each (Fig. 2*A*). Alkylation of E1 and E2 with maleimide-biotin was prevented by pretreatment of the HCVpp with M135; by contrast NHS-biotin labeling was not affected (Fig. 2, *A*–*C*). These data confirm the specificity of the reactions targeting thiols and verify that the observed inhibition of HCVcc/pp infectivity by M135 is due to sulfhydryl alkylation events on E1 and E2. These results indicate

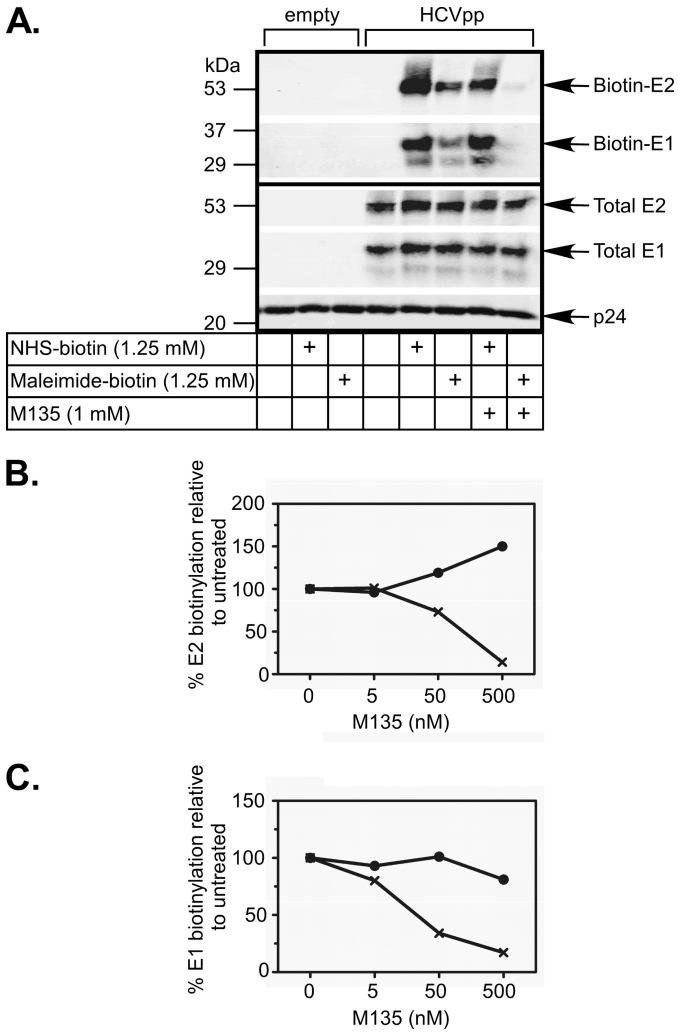

FIGURE 2. **Free sulfhydryl groups are present on both E1 and E2 in HCVpp.** *A*, HCVpp or no envelope control particles (empty) were labeled with maleimide-biotin or NHS-biotin. Particles were lysed and reduced by addition of DTT prior to capture of biotinylated proteins by streptavadin-agarose. For M135-treated samples, HCVpp were incubated with 1 mM M135 followed by labeling with maleimide-biotin or NHS-biotin. Samples were run on reducing SDS-PAGE and Western blotted with anti-E2 and anti-E1 MAbs H52 and A4, respectively. Molecular weight markers are shown to the left. Input E1, E2, and HIV-1 capsid protein p24 were determined by Western blot (probed with mAbs A4, H52, and 183, respectively) for each sample prior to streptavadin-agarose pulldown. *B* and *C*, HCVpp were incubated with 5–500 nM M135 prior to labeling as described above. The resulting Western blots of E2 (*B*) and E1 (*C*) labeled by maleimide-biotin (×) or NHS-biotin (●) were quantified by densitometry analyses (representative assay).

that not all cysteine residues in the full length, prefusion forms of E1 and E2 are involved in intra- or intermolecular disulfide bonds. In fact, free thiol groups in E1 and E2 are essential for HCV infectivity.

Redox State of HCVpp and HCVcc Envelope Proteins Following Attachment to Cells—To examine whether a change in oxidation state occurs in the HCV envelope glycoproteins after cellular attachment, HCVcc or HCVpp were incubated with Huh7.5 cells at 4 °C for 2 h to allow virus attachment but not internalization. Unbound virus was removed, and M135 was immediately applied to cells followed by incubation at 37 °C for 50 min to allow entry events to proceed. The addition of 0.5–5 mM M135 to cell-bound HCVcc had no effect on virus entry,

indicating an absence of free cysteine residues available for alkylation (Fig. 3*A*). A similar trend was observed for HCVpp. However, a significant reduction in entry was observed with 2 or 5 mM M135 ($p < 0.05$, Student's t test) indicating that a component of cell-bound HCVpp remained sensitive to alkylation (Fig. 3*B*). Entry of cell-bound VSVGpp was not affected by treatment with M135, indicating that M135-mediated inhibition of entry is specific for HCVpp. These data suggest that the HCV envelope proteins switch from a reduced state to an oxidized state following receptor engagement.

M135 Block to Infection Is Not Rescued by DTT—If a change in glycoprotein oxidation state does occur during virus entry, the alkylation of E1 and E2 with M135 may induce an isomerization-arrested state where a labile disulfide cannot be reduced by a free thiol due to alkylation. We therefore investigated whether the M135-induced block to HCVcc infectivity could be rescued by addition of exogenous DTT. To examine this, 1210 $TCID_{50}$/ml of HCVcc was M135-alkylated and bound to Huh7.5 cells at 4 °C for 2 h. Following removal of unbound virus, the virus-adsorbed cells were incubated with DTT in increasing concentrations for 1 h at 37 °C. It was found that addition of 0.1–2 mM DTT did not significantly increase the infectivity of alkylated HCVcc ($p > 0.05$, Student's t test) (Fig. 4*A*). The addition of DTT to cell bound prealkylated virus at different time points did not restore virus infectivity either (data not shown). Incubation of Huh7.5 cells with up to 2 mM DTT did not affect cell viability as determined by a trypan exclusion test, indicating the lack of DTT-mediated recovery is not simply due to toxicity (data not shown).

To determine whether DTT directly affects HCVcc infectivity, the virus was combined with various concentrations of reducing agent and the mixture incubated with Huh 7.5 cells for 1 h at 37 °C. Virus infectivity was not significantly reduced following treatment with up to 2 mM DTT (Fig. 4*B*; $p > 0.05$, Student's t test), indicating that this reducing agent does not affect the structural integrity of the HCV virion or the glycoproteins and cellular factors involved in entry. These data suggest that an isomerization-arrested state induced in HCVcc by the M135-alkylating agent cannot be rescued by chemical reduction of disulfides within E1 and/or E2.

Effect of Isolated Cellular Receptors on Redox State of E1 and E2—Current HCV attachment models indicate that glycosaminoglycans mediate an initial virus adsorption event that is followed by ligation of E2 with the LEL of CD81. These models are based on the results of time of addition studies showing that highly sulfated heparan sulfate or soluble heparin can only block infection when included at the time of virus inoculation (21, 23, 55–57), whereas CD81-specific antibodies can inhibit infection when added up to 60 min post-inoculation (24, 57, 58).

To determine whether HCVpp binding to soluble heparin or CD81 induces a change in HCV glycoprotein oxidation, HCVpp were incubated with heparin sodium salt (1 mg/ml; sufficient to inhibit HCVpp entry, data not shown), MBP-LEL(113–201), or the mutant MBP-LEL(113–201)F186S (500 μg) that shows reduced binding to E2 (45), prior to purification and labeling with maleimide- or NHS-biotin as described above. Densitometry analyses of Western blots produced by three independent experiments show that binding of HCVpp to heparin or MBP-LEL(113–201) did not significantly change the degree of labeling of E1 or E2 with either maleimide-biotin or the NHS-biotin control ($p > 0.05$, Student's t test; Fig. 5). This result suggests that cell-free HCVpp-heparin or HCVpp-CD81 binding alone is not sufficient to induce a change in the oxidation state of the HCV glycoproteins.

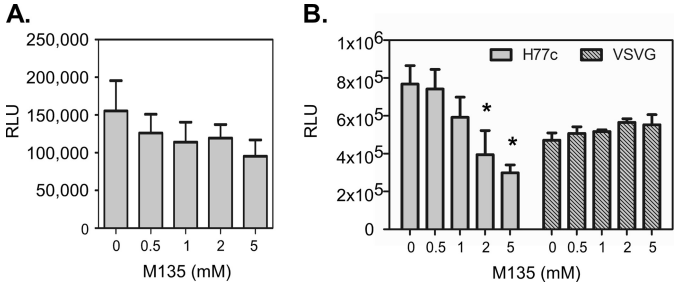

FIGURE 3. **HCVpp but not HCVcc remains sensitive to M135 labeling following attachment to cells.** HCVcc (*A*), HCVpp, or VSVGpp (*B*) were bound to Huh7.5 cells at 4 °C prior to addition of M135 at 37 °C. Luciferase activity was determined 72 h later. *RLU*, relative light units. Data are the mean and S.E. of four (*A*) or three (*B*) independent experiments, each consisting of six (*A*) or four (*B*) replicates. *Asterisks* indicate significantly different entry activity relative to untreated virus ($p < 0.05$ Student's t test). VSVGpp virus stocks were the same as those used in Fig. 1*C*.

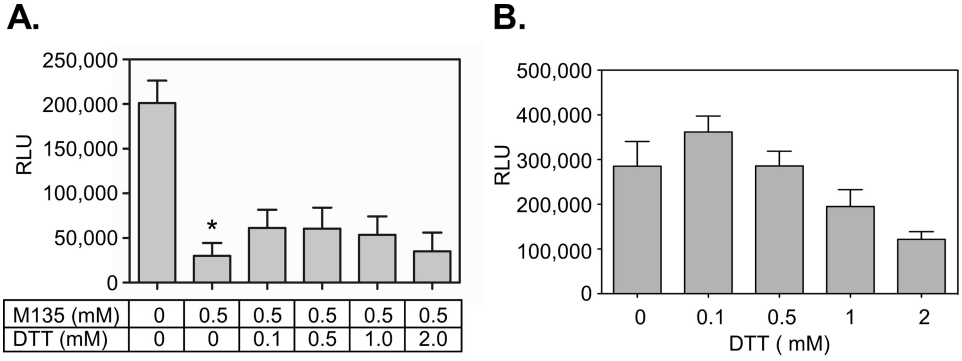

FIGURE 4. **DTT treatment of alkylated HCVcc does not rescue entry.** *A*, M135-alkylated HCVcc were bound to Huh7.5 cells for 2 h at 4 °C, followed by incubation with DTT at 37 °C for 1 h. Luciferase activity in the culture supernatant was assayed 72 h later. *RLU*, relative light units. Data shown are the mean and S.E. of four independent experiments each performed with six replicates. *Asterisks* indicate significantly different entry activity to untreated HCVcc ($p < 0.05$, Student's t test). *B*, various concentrations of DTT were added to HCVcc, and the virus-DTT mixtures were added to Huh 7.5 cells for 1 h at 37 °C. After washing, cells were incubated in fresh medium, and luciferase activity was quantitated 72 h later. Mean ± S.E. of four independent assays.

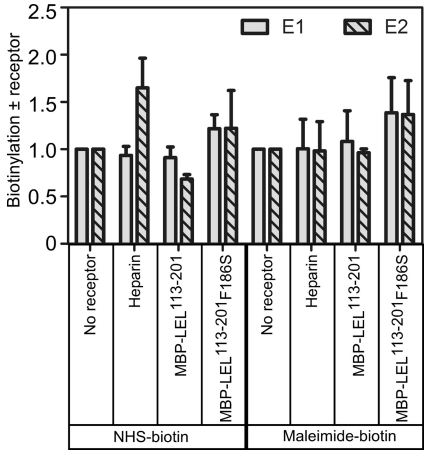

FIGURE 5. **CD81 or heparin binding in isolation does not affect the oxidation state of E1 or E2.** HCVpp were incubated with heparin sodium salt, dimeric MBP-LEL(113–201), or mutant MBP-LEL(113–201)F186S prior to purification and labeling with maleimide- or NHS-biotin. Particles were lysed and reduced by addition of DTT then applied to streptavidin agarose for capture of biotinylated proteins. Samples were run on reducing SDS-PAGE and Western blotted with anti-E2 and anti-E1 mAbs (H52 and A4, respectively). The ratio of maleimide-biotin and NHS-biotin labeling was calculated for E1 and E2 ± heparin, MBP-LEL(113–201), and MBP-LEL(113–201)F186S by densitometry analyses of Western blots produced from three independent experiments (means ± S.E.).

DISCUSSION

In this study, we used the sulfhydryl-reactive agents M135 and maleimide-biotin to reveal that the HCV glycoproteins contain free thiol groups that are essential for virion infectivity. We also showed that E1 and E2 transition from a M135-sensitive to a M135-resistant state following incubation with HCV-permissive cells. We propose that HCV undergoes thiol-disulfide isomerization where free thiol groups present in cell-free HCVcc become engaged in disulfide bonds following interactions with the target cell surface during entry.

Krey *et al.* (17) recently assigned eight of nine disulfides in the monomeric E2 ectodomain; the remaining disulfide (Cys-597–Cys-620) located in domain III was not conclusively identified but was assumed to form. The assignment of all nine disulfide bonds in monomeric recombinant E2 is consistent with the study of Fenouillet *et al.* (60) that determined that the soluble E2 ectodomain contains <0.5 free thiols. Our observation that at least one free thiol group is present in both E1 and E2 and that alkylation of HCVcc prevents infectivity contradicts a scenario where all nine disulfides are formed in the prefusion state. It is possible that the disulfide arrangement identified by Krey *et al.* (17) represents a conformational end point of E2 resulting from thiol-disulfide isomerization induced by receptor interactions. Such isomerization events may enable the E1E2 complex to undergo subsequent acid-induced structural rearrangements, such as E1-E2 dissociation (61), in an endosomal compartment where it mediates virus-cell membrane fusion. Our findings support a scenario where the functional form of viral E1E2 contains free thiol groups that form new disulfides in an isomerization event that occurs during virus entry. Given that E1 and E2 contain an even number of cysteines, our findings are consistent with recent preliminary studies that suggest that the majority of E1E2 in HCVcc is covalently linked (9). Furthermore, HCV entry can proceed even when four to five disulfide groups are reduced suggesting a high degree of tolerance to disulfide reduction during entry (60).

The finding that HCVcc is insensitive to sulfhydryl alkylating agents immediately following cellular attachment suggests that a change in oxidation state has been triggered by binding to an attachment factor involved in a very early stage of entry. Heparan sulfate proteoglycans are involved in early HCV attachment events (23, 55–57) and may directly associate with E2 (22, 55). Antibody inhibition studies suggest that interactions between HCV and CD81, SR-B1, and claudin-1 receptors occur later, 18–60 mins after virus attachment to cells (23, 24, 57, 62). However, neither soluble heparin nor recombinant soluble CD81 altered the oxidation state of E1 or E2. It is possible that only binding events with glycosaminoglycans, CD81, and/or other receptors/cofactors in a cellular context can trigger disulfide rearrangement within the viral glycoproteins.

Following binding to HCV permissive cells, both HCVpp and HCVcc are resistant to low concentrations of M135, suggesting that thiol groups have been converted from a reduced to an oxidized state. However, at the highest M135 concentrations, HCVpp remained somewhat sensitive to sulfhydryl alkylation immediately following cellular attachment. HCVcc particles are thought to bud from the endoplasmic reticulum (63, 64), whereas HCVpp are produced by budding of retroviral particles from the surface of cells, which incorporates a portion of cell surface localized E1 and E2 (7). It is therefore likely that the arrangement of E1 and E2 on the surface of HCVpp is similar to HIV-1 where eight to ten glycoprotein spikes are evident per virus particle (65). In contrast, the arrangement of glycoproteins on HCVcc is predicted to be similar to that of the flaviviruses (61, 67) where a lattice of highly ordered glycoproteins is evident (66, 68). The presence of E1 and E2 in an ordered lattice may provide an environment where oxidation occurs cooperatively between neighboring cysteines, accelerating conformational changes. Furthermore, it is possible that glycoprotein recruitment to the site of receptor attachment is required for HCVpp entry, as has been described for HIV-1 (59). Given that relocalization of glycoproteins through the viral membrane will not occur at 4 °C, a delay in the recruitment and subsequent conformational changes in HCVpp glycoproteins may occur. As such, resistance to M135 may occur later for HCVpp than HCVcc. Attempts to visualize a switch from reduced to oxidized glycoproteins on HCVpp bound to Huh7.5 cells followed by alkylation and whole cell lysis were not successful as we were not able to detect specifically bound HCVpp directly by Western blotting (data not shown).

The role of free thiol groups in the viral E1E2 complex may be to catalyze disulfide reduction and/or isomerization, controlling conformational changes in the glycoproteins during fusion activation. For the retroviruses, human T-cell leukemia virus and Moloney murine leukemia virus, the pre-fusion complex formed by the receptor binding (SU) and fusion (TM) glycoproteins is constrained by a labile disulfide bond mediated by a C*XX*C thiol isomerisation motif in SU and a CX_6CC motif within TM (4, 5). Following receptor binding, the redox-active cysteine in the C*XX*C motif catalyzes reduction of the labile disulfide triggering fusion activation. An isomerization-arrested state can be induced in SU-TM by alkylation with M135

or by Cys-to-Ala substitution of Cys-225 present in the C*XX*C motif, which leads to the formation of a stable SU-TM disulfide. This isomerization-arrested state can be rescued by DTT, which reduces the artificially induced stable disulfide bond. In these cases, the formation of new disulfide bonds in SU or TM does not appear to be required for infectivity. Our data suggest that the HCV glycoproteins undergo disulfide isomerization during virus entry distinct to the mechanism employed by retroviruses. In contrast to the retroviruses, addition of exogenous DTT was unable to rescue the M135 induced isomerization-arrested state of HCVcc. Such a scenario is consistent with a requirement for free thiol groups in E1 and/or E2 (which are alkylated by M135) to participate in new disulfide bonding events. Interestingly, E1 contains a highly conserved C^{226}(V/L)PC motif. However, mutagenesis of either Cys to alanine or serine results in a biosynthesis defect preventing an examination of the role of these Cys residues in virus entry.[4]

In conclusion, this study has identified a critical requirement for free thiol groups in the HCV envelope proteins and implicates a role for thiol isomerization during virus entry.

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