Hepatitis C virus (HCV) is an enveloped positive-stranded RNA hepatotropic virus. HCV pseudoparticles infect liver-derived cells, supporting a model in which liver-specific molecules define HCV internalization. Three host cell molecules have been reported to be important entry factors or receptors for HCV internalization: scavenger receptor BI, the tetraspanin CD81, and the tight junction protein claudin-1 (CLDN1). None of the receptors are uniquely expressed within the liver, leading us to hypothesize that their organization within hepatocytes may explain receptor activity. Since CD81 and CLDN1 act as coreceptors during late stages in the entry process, we investigated their association in a variety of cell lines and human liver tissue. Imaging techniques that take advantage of fluorescence resonance energy transfer (FRET) to study protein-protein interactions have been developed. Aequorea coerulescens green fluorescent protein- and Discosoma sp. red-monomer fluorescent protein-tagged forms of CD81 and CLDN1 colocolated, and FRET occurred between the tagged coreceptors at comparable frequencies in permissive and nonpermissive cells, consistent with the formation of coreceptor complexes. FRET occurred between antibodies specific for CD81 and CLDN1 bound to human liver tissue, suggesting the presence of coreceptor complexes in liver tissue. HCV infection and treatment of Huh-7.5 cells with recombinant HCV E1-E2 glycoproteins and anti-CD81 monoclonal antibody modulated homotypic (CD81-CD81) and heterotypic (CD81-CLDN1) coreceptor protein association(s) at specific cellular locations, suggesting distinct roles in the viral entry process.

Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus whose principal reservoir for replication is believed to be hepatocytes within the liver. Viruses initiate infection by attaching to molecules or receptors at the cell surface. Expression and localization of such receptors are often important determinants of a cell's susceptibility to infection and of viral tropism for a particular tissue. The development of retroviral pseudovirions bearing HCV E1-E2 glycoproteins (gps) (i.e., HCVpp) (5, 24, 38) and an infectious system generating HCV pseudoparticles (HCVpp) (5, 24, 38) and an infectious system generating HCV particles in cell culture (HCVcc) (51, 75, 83) has allowed studies on the mechanism of HCV entry and replication.

HCVpp primarily infect liver-derived cells (5, 38, 63), supporting a model in which molecules expressed specifically within the liver act as receptors for the virus and help define HCV tropism. Current evidence suggests that at least three host cell molecules are important for HCV entry in vitro: scavenger receptor class B member I (SR-BI) (6, 33, 39, 64), the tetraspanin CD81 (6, 38, 51, 59), and the tight junction (TJ) protein claudin-1 (CLDN1) (25). HCV gps have been reported to interact with SR-BI and CD81 (reviewed in reference 19). Other factors, such as glycosaminoglycans (2, 3) and low-density-lipoprotein receptor (57), have been implicated in HCV entry, although their role is less well established (reviewed in reference 74). In vivo, SR-BI is present within steroidogenic tissue, macrophages, and liver (44); CD81 is in most tissues (50); and CLDN1 is present in many tissues but is present at high levels in the liver (29). Since these molecules are not uniquely expressed in the liver, their organization or stoichiometry within hepatocytes may explain their viral receptor activity.

SR-BI is a member of the scavenger receptor family and is the major receptor for high-density lipoprotein (44). Antibodies specific for SR-BI have been reported to inhibit HCV infection and overexpression of SR-BI promotes viral infection (6, 14, 33, 39, 80). Experiments to validate an essential role for SR-BI in HCV entry have proven difficult, since all cell types studied to date express SR-BI and since small interfering RNA silencing has been reported to have variable effects on HCVpp infectivity (6, 47, 73, 80).

CD81 is a member of the tetraspanin family of proteins, and experiments demonstrating that expression of CD81 in the CD81-negative HepG2 hepatoma cell line confers viral infec-
tivity support a critical role for CD81 in the viral entry process (6, 27, 54, 81). Recombinant forms of CD81 and antibodies specific for CD81 inhibit infectivity after viral adsorption to the target cell, suggesting that CD81 does not confer an ability for the virus to attach but acts as a coreceptor during the int

CD81 and CLDN1 in cell lines, demonstrating that a subpopu-

lation of CD81 and CLDN1 associate. FRET was independent of the cellular permissiveness to support HCV entry. Treat-

ment of HuH-7.5 hepatoma cells with recombinant HCV E1-E2 gpsi did not modulate the frequency of FRET between CD81 and CLDN1, suggesting that coreceptor complexes exist in the absence of viral proteins. HCV infection and treatment of hepatoma cells with the neutralizing anti-CD81 monoclonal antibody (MAb) reduced the frequency of FRET between CD81-CLDN1 associations but not the frequency of CD81-

CLDN1 associations, highlighting potential antigenic and func-
tional differences between homotypic and heterotypic corecep-

tor protein complexes in the viral entry process.

MATERIALS AND METHODS

Cell lines and reagents. HuH-7.5 cells (11), HepG2, and 293T cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% nonessential amino acids. T84 cells were propagated in a 1:1 mixture of F12 and DMEM with 10% FBS. HuH-7.5, HepG2, and 293T cells were kindly provided by Charles Rice (Rockefeller University, NY), and T84 was provided by Chris Tselepis (University of Birmingham).

The primary antibodies used were anti-CLDN1, JAY.8 (Invitrogen, CA), and 1CS-D9 (Novus); anti-CLDN4 (Invitrogen, CA); anti-CD81 M38 (F. Berditchevski, University of Birmingham, United Kingdom); anti-E1-E2 IJ9/36 (26) and anti-NS5A 9E10 (C. Rice, Rockefeller University). Secondary labeled antibodies were obtained from Invitrogen (Alexa Fluor 488 goat anti-mouse immunoglobulin G [IgG], Alexa Fluor 488 goat anti-mouse IgG2a, Alexa Fluor 633 goat anti-mouse IgG, Alexa Fluor 633 goat anti-mouse IgG1, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 633 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-human IgG, Alexa Fluor 633 goat anti-human IgG, tetramethyl rhodamine isothiocyanate [TRITC] goat anti-mouse IgG, and TRITC goat anti-rabbit IgG).

HCV strain HCV-1 (genotype 1a) E2_H77 (the subscript refers to the final amino acid of the expressed protein within the HCV polyprotein [45]) and E1-E2 were expressed in CHO cells and extracted from extracellular supernatants or intracellular lysates, respectively. Antigens were purified using Galanthus nivalis lectin (Sigma, United Kingdom) chromatography and fast-flow Sepharose cat-

ion-exchange chromatography (Pharmacia) to >90% purity, as previously re-

ported (18).

HCVpp/HCVcc genesis and infection. Pseudoviruses expressing luciferase or enhanced green fluorescent protein (eGFP) reporters were generated by the following protocols. 293T cells were transfected with a 1:1 ratio of plasmids carrying HIV provirus expressing luciferase and HCV strain H77 E1-E2 envelope gpsi, murine leukemia virus (MLV) gpsi, or empty vector (Env-pp), as previously described (38). Alternatively, 293T cells were cotransfected with plas-
mids encoding HIV provirus expressing eGFP (CSW0) (10), HCV polyprotein, and HCV strain H77 gpsi or empty vector in a 1:1:4 ratio as previously described (27). Supernatants were harvested 48 h posttransfection, pooled, and filtered. Virus-

containing medium was added to target cells plated at 1.5 × 10^6 cells/cm^2 and incubated for 8 h. Unbound virus was removed, and the cells were fed again with their respective growth media and incubated at 37°C. After 72 h, infections were terminated and firefly luciferase activity in lysed cells was measured or cellular eGFP was quantified by flow cytometry. Specific pseudotype infectivity was calculated by subtracting the mean Env-pp signal from the HCVpp or MLVpp signals.

HCVcc JFH-1 was generated as previously described (51, 70). Briefly, RNA was transcribed in vitro from full-length genomes by using the Megascript T7 kit (Ambion, Austin, TX) and electroporated into HuH-7.5 cells. At 72 h and 96 h postelectroporation, supernatants were collected, pooled, and stored immediately at −80°C. Virus-containing medium was added to target cells plated at 1.5 × 10^6 cells/cm^2 and incubated for 1 h. Unbound virus was removed replaced with 3% FBS-DMEM, and the cells were incubated at 37°C. After 72 h, infected cells were visualized by staining methanol-fixed cells for NS5A expression using the anti-NS5A 9E10 MAb and Alexa Fluor conjugated anti-mouse IgG (Invitrogen, CA).

Generation of AgGFP- and DsRed-tagged CD81, CLDN1, and CLDN4. To generate pTRIP lentiviral vectors expressing Aequorea coelestes GFP (AgGFP)-CD81 (g.CD81_H11022) and DsRed-tagged CD81-CD81 (r.CD81_H11022) and DsRed-CLDN1 (r.CLDN1_H11022), the AgGFP and DsRed open reading frames were cloned into preexisting constructs encod-
ing CD81 and CLDN1 (27). A C-terminal deletion mutant of CLDN1 (r.CLDN1ΔC), was generated using the method described by Evans and colleagues and expressed in pTRIP (25). pBABE vectors encoding CLDN1 and CLDN4 were modified in a similar manner to generate g.CLDN1BABE, r.CLDN1ΔC, and r.CLDN4BABE (82).

**SDS-PAGE and Western blotting.** Huh-7.5 and 293T-CLDN1TRIP cells were plated at 1.5 x 10^6 cells/cm² and the following day lysed in 1% TriSep 10, 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM CaCl₂, and 1 mM MgCl₂, containing protease inhibitors (Complete medium; Roche). Lysates were clarified by centrifugation (20,000 x g, 30 min), and protein concentrations were determined by using protein assay reagent (Pierce) according to the manufacturer’s instructions. Defined concentrations of cell lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes for incubation with an anti-CLDN1 (JAY.8) and Fd (12, 17, 56, 72). Images of cells expressing donor or acceptor fluorescent proteins were collected with all laser permutations to determine the degree of residual spectral bleed-through (see Fig. S1 in the supplemental material). Fluorophore lifetimes in cells expressing AcGFP- or DsRed-tagged proteins were measured by photobleaching at low and high laser powers. As expected, both fluorophores showed an exponential decay over long time periods at a high laser power, whereas a low laser power resulted in a linear decay over the relatively short time periods used in cell imaging (see Fig. S2 in the supplemental material).

**Measurement of fluorophore lifetime in all regions of protein colocalization allowed us to account for the relative abundances of the donor and the acceptor and to correct for photobleaching in a site-specific manner (72).**

Energy transfer from a donor fluorophore to an acceptor can be used to infer their separation. However, several parameters, including cross talk between fluorophores due to spectral overlap, the relative contribution of FRET and non-FRET energies to the measured fluorescence intensity, and the impact of relative donor and acceptor concentrations on overall FRET efficiency, must be considered (reviewed in reference 9). AcGFP and DsRed proteins fluoresce as monomers, display a 30% overlap in their excitation and emission spectra, and are ideally suited for FRET studies (65). To minimize spectral bleed-through, we utilized the meta-head function of the microscope at the following wavelengths (λ): for AcGFP, excitation λ 488 nm and emission λ 520 nm, and for DsRed, excitation λ 561 nm and emission λ 600 nm (see Fig. S1 and S3 in the supplemental material). Analysis of donor and acceptor photobleaching in several cell lines demonstrated similar levels of cross talk for AcGFP and DsRed. Providing these two parameters are constant, FRET values can be inferred over a wide range of fluorophore concentrations (donor/acceptor ratios of 1:10 to 10:1) (9, 17). Quantification of CD81 and CLDN1/CLDN4 in transfected cells showed an approximately threefold range in expression levels.

**Cell treatments.** Huh-7.5 cells expressing g.CD81/r.CD81 or g.CD81/r.CD81 were grown on 22-mm² round coverslips for FRET analysis or in 48-well tissue culture plates for HCVpp or HCVcc infection. Cells were treated at 37°C for 1 h with increasing concentrations of HCV-1 E2v1 or E1-E2 in PBS and anti-CD81 M38 or control antibody. Cells were fixed immediately in ice-cold methanol prior to confocal imaging and FRET analysis. Replica wells were stained with anti-E2 MAb 1/39 to visualize cell-bound antigens. In parallel experiments, cells were treated with the agents for 1 h at 37°C and infected with HCVpp, MLVpp, Env pp, or JFH-1 for 1 h at 37°C. Unbound virus and agents were removed by washing, and the cells were cultured for 72 h and assessed for viral infection.

**Statistics.** Differences between FRET distances were assessed using a non-parametric Kruskal-Wallis test and Dunn’s multiple-comparison test. The relationship between fluorophore fluorescence intensity and distance was assessed by linear regression. Differences in %FRET observed between samples were compared by Fisher’s exact test. Corrections for multiple sampling (Bonferroni method) were used when appropriate. Statistical analyses were carried out with the ImageJ program or using the Prism 4 package (GraphPad Software, CA), with probabilities as described in the figure legends and table footnotes.

**RESULTS**

**CD81 and CLDN1 cellular localization.** The cellular expression of CD81 and CLDN1 was studied by LSCM. Huh-7.5 cells support efficient HCV entry and express CD81 and CLDN1 at the PM, with some punctate intracellular CD81 observed (Fig. 1A). CD81 and CLDN1 colocalize in Huh-7.5 cells (Fig. 1A). Comparable patterns of CD81-CLDN1 colocalization were observed in permissive hepatoma (HepG2.CD81) and epithelial (293T-CLDN1) cell lines and a nonpermissive colorectal carcinoma T84 cell line (Fig. 1B to D), suggesting that receptor colocalization per se does not predict cellular permissiveness for HCV entry.

**Characterization of AcGFP- and DsRed-tagged CD81 and CLDN1 proteins.** CD81 and CLDN1 fusion proteins with where FRET occurs (%FRET) is an indicator of the frequency of protein-protein association. To determine the FRET efficiency (E), we used an approach based on the photobleaching FRET methods described by Zal and Gascoigne (79). Currently available confocal microscopes offer tunable lasers which allow the simultaneous photobleaching of donor and acceptor molecules. E values were determined using the donor fluorophore showed an exponential decay over long time periods at a high laser power, whereas a low laser power resulted in a linear decay over the relatively short time periods used in cell imaging (see Fig. S2 in the supplemental material). Fluorophore lifetimes in cells expressing AcGFP- or DsRed-tagged proteins were measured by photobleaching at low and high laser powers. As expected, both fluorophores showed an exponential decay over long time periods at a high laser power, whereas a low laser power resulted in a linear decay over the relatively short time periods used in cell imaging (see Fig. S2 in the supplemental material). Fluorophore lifetime in all regions of protein colocalization allowed us to account for the relative abundances of the donor and the acceptor and to correct for photobleaching in a site-specific manner (72). Energy transfer from a donor fluorophore to an acceptor can be used to infer their separation. However, several parameters, including cross talk between fluorophores due to spectral overlap, the relative contribution of FRET and non-FRET energies to the measured fluorescence intensity, and the impact of relative donor and acceptor concentrations on overall FRET energy, must be considered (reviewed in reference 9). AcGFP and DsRed proteins fluoresce as monomers, display a 30% overlap in their excitation and emission spectra, and are ideally suited for FRET studies (65). To minimize spectral bleed-through, we utilized the meta-head function of the microscope at the following wavelengths (λ): for AcGFP, excitation λ 488 nm and emission λ 520 nm, and for DsRed, excitation λ 561 nm and emission λ 600 nm (see Fig. S1 and S3 in the supplemental material). Analysis of donor and acceptor photobleaching in several cell lines demonstrated similar levels of cross talk for AcGFP and DsRed. Providing these two parameters are constant, FRET values can be inferred over a wide range of fluorophore concentrations (donor/acceptor ratios of 1:10 to 10:1) (9, 17). Quantification of CD81 and CLDN1/CLDN4 in transfected cells showed an approximately threefold range in expression levels.

**Cell treatments.** Huh-7.5 cells expressing g.CD81/r.CD81 or g.CD81/r.CD81 were grown on 22-mm² round coverslips for FRET analysis or in 48-well tissue culture plates for HCVpp or HCVcc infection. Cells were treated at 37°C for 1 h with increasing concentrations of HCV-1 E2v1 or E1-E2 in PBS and anti-CD81 M38 or control antibody. Cells were fixed immediately in ice-cold methanol prior to confocal imaging and FRET analysis. Replica wells were stained with anti-E2 MAb 1/39 to visualize cell-bound antigens. In parallel experiments, cells were treated with the agents for 1 h at 37°C and infected with HCVpp, MLVpp, Env pp, or JFH-1 for 1 h at 37°C. Unbound virus and agents were removed by washing, and the cells were cultured for 72 h and assessed for viral infection.

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AcGFP and DsRed at their N termini were constructed in pTRIP and pBABE retroviral vectors for FRET studies. To ascertain if AcGFP or DsRed fluorophores modulate protein activity, HepG2 (CD81 negative) and 293T (CLDN1 negative) cells were transduced to express the parental and tagged proteins and screened for viral receptor activity and protein localization with specific antibodies. Parental and tagged versions of CD81 and CLDN1 permitted comparable levels of HCVpp entry into cells (Fig. 2A). 293T cells transduced with pBABE-CLDN1 were approximately fivefold less permissive for HCVpp entry than were cells transduced with pTRIP-CLDN1, which most likely reflects the reduced levels of CLDN expression observed in pBABE-transduced cells (Fig. 3D). MLVpp infected all cells with similar efficiencies independent of CD81 or CLDN expression (data not shown). As a control, 293T cells were transduced with the related CLDN family member CLDN4, and both parental and r.CLDN4 failed to support HCV entry (Fig. 2A), despite levels of PM CLDN4 expression comparable to that observed for CLDN1 (Fig. 3A).

Quantification of r.CD81 and r.CLDN1 expression by direct enumeration of the fluorescent tag or indirectly via receptor specific antibodies identified intracellular forms of r.CLDN1 that were not recognized by anti-CLDN1 antibodies which may represent unfolded or immature forms of tagged CLDN1 (Fig. 2C). CD81 and CLDN1 demonstrated a preferential localization at CJs and tagged proteins expressed at the PM were

![Image](http://jvi.asm.org/)

**FIG. 1.** CD81 and CLDN1 colocalization. Huh-7.5 (A), HepG2.CD81 (B), 293T.CLDN1 (C), and T84 cells (D) were grown on poly-L-lysine-treated glass coverslips and stained with normal mouse IgG (mlG) or antibodies specific for CD81 (M38) and CLDN1 (1C5-D9). Bound antibodies were visualized using the Alexa Fluor 488 anti-mouse IgG1 (M38; green) and Alexa Fluor 633 anti-mouse IgG2a (1C5-D9; red). LSCM images were obtained using a 63× 1.2-NA objective (the scale bar represents 10 μm). Areas of CD81-CLDN1 colocalization at CJs are labeled with an arrow.

HCV-specific infectivity levels (expressed in relative light units [RLU]) were as follows: for Huh-7.5 cells, 139 × 10⁴ RLU; for HepG2.CD81 cells, 9 × 10⁴ RLU; for 293T.CLDN1 cells, 14 × 10⁴ RLU; and for T84 cells, 0.2 × 10⁴ RLU.
recognized by receptor-specific antibodies (Fig. 2B and C). Consequently, FRET experiments will focus on PM-expressed forms of the coreceptors.

**Validation and FRET measurement of CD81 and CLDN1 protein interactions.** To study CD81 and CLDN1 interaction(s), we utilized a cell-based FRET method to investigate protein association(s). FRET is a process in which energy is transferred from an excited donor to an acceptor molecule and can occur only when the donor and acceptor are less than 10 nm apart (28). We utilized a gradual acceptor photobleaching FRET methodology in which donor and acceptor fluorescence intensities are monitored throughout the period of photobleaching (9, 17, 32, 72, 79). This method provides photobleaching coefficients for both fluorophores and allows us to determine the frequency of colocalized molecules where FRET occurs and the amount of energy transferred ($E_{\text{FRET}}$). $E_{\text{FRET}}$ values allow the distance(s) between donor and acceptor proteins to be estimated. Although the relative orientations of AcGFP and DsRed in the fusion proteins are unknown, if we assume that proteins expressed in the same cell background are under the same constraints, it is possible to estimate distances between tagged molecules.

CD81 is reported to form homodimers in the formation of higher-order oligomeric structures characteristic of the tetrarasin web (41). We therefore measured FRET between AcGFP (donor)- and DsRed (acceptor)-tagged CD81 proteins.

FIG. 2. Characterization of fluorescently N terminus-tagged CD81 and CLDN1. (A) HepG2 and 293T cells were transduced with retroviral vector pTRIP or pBABE expressing CD81, r.CD81, g.CD81, CLDN1, r.CLDN1, g.CLDN1, CLDN4, or r.CLDN4 and infected with HCVpp-H77, MLVpp, or Env-pp. Data are expressed as levels of specific infectivity and represent the mean luciferase levels (relative light units [RLU]) determined from replicate infections, with the Env-pp value subtracted (270 RLU for HepG2 cells and 360 RLU for 293T cells). HepG2 cells expressing r.CD81 (B) or 293T cells expressing r.CLDN1 (C) were stained with antibodies specific for CD81 (anti-CD81) or CLDN1 (anti-CLDN1), respectively. Linear profiling of the fluorescence signal emitted by the tagged protein (black line) and the indirect fluorescence signal from antibody staining (gray line) is shown. The mean fluorescence intensities from fluorescently tagged proteins (r.CD81 and r.CLDN1) and from antibody-stained (anti-CD81 and anti-CLDN1) receptors were obtained by profiling 50 cells. Regions were defined as the nonjunctional PM (black bar), intracellular junctions (white bar), and CJs (gray bar). All cells were imaged under the same conditions, and the data are expressed as arbitrary fluorescence units (F). The data from a single experiment are presented and are representative of two further experiments.
expressed in Huh-7.5, 293T, and T84 cells. g.CD81 and r.CD81 colocalized (Fig. 3A), and FRET was detected with varying efficiencies across cell types (42% to 84%), with estimated distances in the range of 4.2 to 6.9 nm (Table 1). The variable frequencies of FRET may reflect the presence of untagged endogenous receptors which may compete with tagged receptors for protein association (69). Due to the low frequency of transduction of HepG2 cells, we were unable to ascertain g.CD81-r.CD81 FRET values in a cellular background lacking CD81 expression.

CLDN1 is reported to form dimers and higher-order oligomeric structures (43, 60), and we therefore investigated g.CD81-r.CLDN1 FRET in CLDN-null 293T cells. CLDN1 localized predominantly to CJs and FRET occurred between 52% of colocalized g.CD81 and r.CLDN1 molecules (Fig. 3A and Table 1). We measured FRET between g.CD81-r.CLDN1 in 293T cells in the presence and absence of untagged competitor CLDN1. pTRIP lentivirus efficiently transduced >95% of 293T cells to express CLDN1 (Fig. 3B), and the level of expression was comparable to the endogenous levels observed in Huh-7.5 cells (Fig. 3C). FRET occurred between 59% of colocalted g.CD81 and r.CLDN1 in naïve 293T cells, and this percentage was reduced to 15% in the presence of untagged CLDN1, with an increase in the estimated distance(s) between associating molecules (Table 1). In contrast, CLDN1 expression had no significant effect on %FRET between g.CD81 and r.CD81 (Table 1). Exchanging the donor and acceptor fluorophores maintained comparable levels of FRET between colocalized CD81 and CLDN1 (Table 1). We noted that CLDN1 expression levels in 293T pTRIP-CLDN1-transduced cells were significantly higher than the levels for those transduced with pBABE-CLDN1 (Fig. 3D), allowing us to study the effect(s) of CLDN1 expression on FRET association with CD81. Comparable frequencies of FRET were observed between g.CD81 and r.CLDN1.
and r.CLDN1 expressed from pTRIP or pBABE (Table 1). To assess the specificity of FRET between CD81 and CDLN1, we investigated FRET between CD81 and the inactive HCV receptor CLDN4 in 293T cells, which lack endogenous CLDN1/CLDN4 expression. CD81 colocalized with CLDN4 and showed a low level of FRET (Fig. 3A and Table 1), comparable to that seen between g.CD81 and r.CLDN1 in 293T cells expressing untagged CLDN1, suggesting a baseline of 15% FRET for nonspecific protein interactions.

To investigate whether differences in FRET between g.CD81 and r.CLDN1 exist between permissive (Huh-7.5 and 293T) and nonpermissive (T84) targets, the cells were transduced to express the tagged proteins, and FRET analyses were completed. Since CD81 and CLDN1 preferentially localize at CJs in contrast to nonopposing regions of the PM, we quantified protein localization and compared FRETs at these cellular locations (Fig. 4). FRET occurred between g.CD81 and r.CLDN1 in all three cell lines, with comparable frequencies

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<sup>a</sup> Areas of fluorescent protein colocalization were selected and imaged by confocal microscopy. All tests were performed using the Mann-Whitney test (*, P < 0.05; ***, P < 0.001).

<sup>b</sup> Huh-7.5, 293T, 293T-CLDN1<sub>TRIP</sub> and T84 cells were transduced with pTRIP vectors expressing g.CD81 and r.CD81.

<sup>c</sup> 293T cells were transduced with pBABE vectors expressing g.CD81 and r.CLDN1.

<sup>d</sup> 293T or 293T-CLDN1<sub>TRIP</sub> cells were transduced with pTRIP and pBABE vectors expressing g.CD81, r.CD81, g.CLDN1, r.CLDN1, or r.CLDN4 as indicated.

<sup>e</sup> N<sub>col</sub>, the number of colocalized regions analyzed.

FIG. 4. Localization and FRET between fluorescently N terminus-tagged CD81 and CLDN1. The localizations of g.CD81, r.CLDN1, and r.CLDN1<sub>AC</sub> in Huh-7.5 (A), 293T (B), and T84 (C) cells were assessed by quantifying the fluorescence intensity at the nonjunctional PM (black bar), intracellular junctions (IC; white bar), and CJs (gray bar) of 50 transduced cells. Data are presented as the relative localization of the tagged protein in each cell line. A number of regions (N<sub>col</sub>) where CD81 and CLDN1 colocalize at the PM or CJs were selected for FRET analysis. The %FRET and the estimated distance between fluorophores (nm) are shown. The data presented are from a single experiment and is representative of two further experiments. *, P < 0.05.
and distance estimates at both PM and CJs (Fig. 4). These data suggest that CD81 associates with CLDN1 in permissive and nonpermissive cell types.

The C terminus of CLDN1 has been reported to mediate interactions with junction adhesion molecule and zonula occludens-1 in the formation of TJ protein complexes (36, 71). To investigate if the C-terminal region of CLDN1 is important for associating with CD81, we generated an r.CLDN1 lacking the C-terminal region (r.CLDN1AC) and investigated its localization, association with CD81, and viral receptor activity. r.CLDN1AC colocalized with g.CD81 in Huh-7.5 and 293T at the PM and CJs. A greater quantity of intracellular protein was noted in 293T cells compared to full-length r.CLDN1, suggesting that the C-terminal region may have some localizing properties in 293T that are not apparent in Huh-7.5 cells (Fig. 4A and B). The %FRET between g.CD81 and r.CLDN1AC in Huh-7.5 and 293T cells was comparable to that observed for g.CD81 and r.CLDN1; however, the distance estimate(s) between CD81 and CLDN1AC at the PM was reduced compared to those at the CJs (Fig. 4A and B). Deletion of the C-terminal region reduced viral receptor activity by approximately 50%, with HCVpp infecting 293T cells expressing r.CLDN1 and r.CLDN1AC having specific infectivities of 26 × 10^4 and 13 × 10^4 RLU, respectively. In contrast, 293T cells expressing both forms of tagged CLDN1 were equally susceptible to infection by MLVpp (data not shown). These data suggest that the C-terminal region of CLDN1 is not critical for an association with CD81 in nonpolarized Huh-7.5 and 293T cells; however, this motif may regulate the distance(s) between CD81-CLDN1 complexes at the PM and may contribute to the reduced viral receptor activity.

Measurement of indirect FRET between CD81-CLDN1 in healthy and HCV-infected liver tissues. To investigate if our observations of FRET between CD81 and CLDN1 in cell lines are consistent with protein association in hepatocytes within liver tissue, we labeled liver biopsy specimens with CD81 and CLDN1 receptor-specific antibodies bearing fluorophores Alexa Fluor 488 and TRITC and assessed whether FRET occurred. For a control for this indirect method of FRET measurement, we stained Huh-7.5 cells with the same antibodies and measured FRET. The autofluorescence of liver tissue was subtracted from the specific antibody-dependent fluorescence. CD81 and CLDN1 showed some level of colocalization in hepatocytes within healthy (Fig. 5A) and HCV-infected (Fig. 5B) liver tissues. FRET was detected between receptor-specific antibodies bound to healthy (NL1 and NL7) and HCV-infected (HCV4 and HCV5) liver tissues, confirming CD81-CLDN1 association(s) (Fig. 5C).

Hepatocytes are polarized cells with their PMs separated by TJs into apical (canalicular) and basolateral (sinusoidal) domains (76). As previously reported, CLDN1 expression was increased at the basolateral surface of hepatocytes in HCV-infected samples (Fig. 5B) (63). In healthy liver tissue, CD81 colocalized with CLDN1 preferentially at apical regions (Fig. 5A); however, this pattern was not apparent in the infected tissue, with proteins colocalizing at both cellular domains (Fig. 5B). %FRET values for anti-CD81 and anti-CLDN1 were comparable at apical and basolateral domains (Fig. 5C). In summary, these data suggest that CD81 and CLDN1 colocalize at both apical and basolateral domains of hepatocytes and that FRET occurs between specific antibodies at both sites, supporting an association between CD81 and CLDN1 in primary liver tissue.

Effect of HCV gps on CD81-CLDN1 association. HCV encodes two gps, E1 and E2, which are critical for viral entry into cells (reviewed in reference 19). To investigate whether HCV gps modulate CD81-CLDN1 association(s), we utilized recombinant preparations of truncated E2715 and E1-E2 as a model for studying virus interaction with cell-surface-expressed coreceptors. E2715 and E1-E2 bound to Huh-7.5 cells, demonstrating a punctate staining pattern at the cell surface, with no detectable internalization (Fig. 6A). Huh-7.5 cells expressing...
g.CD81/r.CLDN1 were incubated with increasing concentrations of E2715 or E1-E2 (0.03 to 1 μM) for 1 h at 37°C and fixed in ice-cold methanol, and areas of colocalization were selected for FRET analysis. %FRET was unchanged by E2715 or E1-E2 treatments (data not shown); however, E1-E2 reduced the estimated distance between g.CD81 and r.CLDN1 (**, P < 0.05 [Dunn’s test]). (C) Huh-7.5 cells expressing g.CD81/r.CD81 were incubated with a saturating concentration (1.0 μM) of E2715 or E1-E2 for 1 h at 37°C and fixed in ice-cold methanol, and areas of colocalization at the nonjunctional PM (black bars) and CJs (gray bars) were selected for FRET analysis. E2715 or E1-E2 treatment(s) had no detectable effect on %FRET and distance(s) between g.CD81 and r.CD81 in comparison to control values (data not shown). (D) Control or E2715- or E1-E2-treated cells were infected for 1 h with HCVcc JFH-1, HCVpp-H77, or MLVpp. Unbound virus was removed by washing, and cells were incubated for 72 h. For HCVcc JFH-1, results are the means from three replicate infections and expressed as relative infectivities compared to the infection of control cells; for HCVpp and MLVpp, specific infectivities are shown and represent the mean luciferase levels (relative light units [RLU]) determined from three replicate infections, with the average Envpp value subtracted (420 RLU). Incubation with E1-E2 significantly reduced HCVcc and HCVpp relative infectivities (*, P < 0.05; **, P < 0.01 [Dunn’s test]). The data presented are from a single experiment and are representative of two independent experiments.

FIG. 6. Effect of HCV gps on FRET between fluorescently N terminus-tagged CD81 and CLDN1 and viral infectivity. (A) Huh-7.5 cells were incubated with mock or E1-E2 gps at 37°C for 1 h, and bound protein was visualized with anti-E2 1/39 and anti-rat TRITC (red). (B) Huh-7.5 cells expressing g.CD81/r.CLDN1 were incubated with increasing concentrations of E2715 or E1-E2 (0.03 to 1 μM) for 1 h at 37°C and fixed in ice-cold methanol, and areas of colocalization were selected for FRET analysis. FRET-inferred distances between CD81 and CLDN1 at the nonjunctional PM and CJs were determined. %FRET was unchanged by E2715 or E1-E2 treatments (data not shown); however, E1-E2 reduced the estimated distance between g.CD81 and r.CLDN1 (**, P < 0.05 [Dunn’s test]). Huh-7.5 cells expressing g.CD81/r.CD81 were incubated with a saturating concentration (1.0 μM) of E2715 or E1-E2 for 1 h at 37°C and fixed in ice-cold methanol, and areas of colocalization at the nonjunctional PM (black bars) and CJs (gray bars) were selected for FRET analysis. E2715 or E1-E2 treatment(s) had no detectable effect on %FRET and distance(s) between g.CD81 and r.CD81 in comparison to control values (data not shown). (D) Control or E2715- or E1-E2-treated cells were infected for 1 h with HCVcc JFH-1, HCVpp-H77, or MLVpp. Unbound virus was removed by washing, and cells were incubated for 72 h. For HCVcc JFH-1, results are the means from three replicate infections and expressed as relative infectivities compared to the infection of control cells; for HCVpp and MLVpp, specific infectivities are shown and represent the mean luciferase levels (relative light units [RLU]) determined from three replicate infections, with the average Envpp value subtracted (420 RLU). Incubation with E1-E2 significantly reduced HCVcc and HCVpp relative infectivities (*, P < 0.05; **, P < 0.01 [Dunn’s test]). The data presented are from a single experiment and are representative of two independent experiments.

Effect of anti-CD81 treatment on CD81-CD81 and CD81-CLDN1 association. Several laboratories have reported that treatment of hepatoma cells with anti-CD81 MAb inhibits HCV entry; however, the underlying mechanism of action is...
unknown. To assess whether the neutralizing CD81-specific MAb M38 modulates CD81 protein association(s), we studied the effect(s) of M38 on FRET between g.CD81-r.CD81 and g.CD81-r.CLDN1 in Huh-7.5 cells. Treatment of Huh-7.5 cells with MAb M38 inhibited HCVcc strain JFH-1 or HCVpp for 1 h, unbound virus was removed by washing, and cells were incubated at 37°C for 72 h. M38 significantly reduced HCVcc and HCVpp infectivity (*, P < 0.05 [Dunn’s test]). Data are expressed as relative infectivities compared to control untreated cells and represent the means from three replicate infections. (B) Huh-7.5 cells expressing g.CD81/r.CD81 or g.CD81/r.CLDN1 were incubated with anti-CD81 M38 (2 μM) or isotype-matched control MAb for 1 h at 37°C at a concentration shown to saturate cell-surface-expressed CD81 by flow cytometry. Cells were fixed, and regions of colocalization at the nonjunctional PM and CJs were imaged by confocal microscopy. The numbers of colocalized regions analyzed (N_{tot}), the %FRET values, and the estimated distances between fluorescent proteins are shown. M38 significantly reduced the %FRET and estimated distances between CD81-CD81 (*, P < 0.05; **, P < 0.01; ***, P < 0.001 [Fisher’s exact test and Mann Whitney test]).

**FIG. 7.** Effect of anti-CD81 on FRET between fluorescently N terminus-tagged CD81 and CLDN1 and viral infectivity. (A) Huh-7.5 cells were incubated with increasing concentrations of anti-CD81 MAb (M38) for 1 h at 37°C. Control and M38-treated cells were infected with HCVcc strain JFH-1 or HCVpp for 1 h, unbound virus was removed by washing, and cells were incubated at 37°C for 72 h. M38 significantly reduced HCVcc and HCVpp infectivity (*, P < 0.05 [Dunn’s test]). Data are expressed as relative infectivities compared to control untreated cells and represent the means from three replicate infections. (B) Huh-7.5 cells expressing g.CD81/r.CD81 or g.CD81/r.CLDN1 were incubated with anti-CD81 M38 (2 μM) or isotype-matched control MAb for 1 h at 37°C at a concentration shown to saturate cell-surface-expressed CD81 by flow cytometry. Cells were fixed, and regions of colocalization at the nonjunctional PM and CJs were imaged by confocal microscopy. The numbers of colocalized regions analyzed (N_{tot}), the %FRET values, and the estimated distances between fluorescent proteins are shown. M38 significantly reduced the %FRET and estimated distances between CD81-CD81 (*, P < 0.05; **, P < 0.01; ***, P < 0.001 [Fisher’s exact test and Mann Whitney test]).

Effect of HCV infection on CD81-CD81 and CD81-CLDN1 FRET. To assess whether HCV infection alters coreceptor protein association(s), Huh-7.5 cells expressing g.CD81/r.CD81 or g.CD81/r.CLDN1 were infected with HCVcc JFH-1, and FRET was measured. HCV-infected cells were identified by expression of the nonstructural viral protein NS5A, and regions of CD81-CD81 and CD81-CLDN1 colocalization were selected for FRET analysis. HCV infection had no effect on the expression levels of the tagged coreceptors. Infection significantly reduced %FRET between CD81-CD81 at the PM and CJs and reduced the estimated distance between the molecules specifically at the PM (Table 2). In contrast, the %FRET and estimated distances between CD81-CLDN1 were unaltered following HCV infection.

**DISCUSSION**

Confocal imaging and FRET analysis between fluorescence-tagged CD81 and CLDN1 molecules expressed in various cell types demonstrate protein association, consistent with the formation of coreceptor complexes. FRET between cell-surface-expressed g.CD81 (donor) and r.CLDN1 (acceptor) occurred in Huh-7.5 cells, 293T cells transduced to express CLDN1, and T84 colorectal carcinoma cells, suggesting that coreceptor localization and association is not unique to cells which support HCV entry. This is in contrast to a recent report by Yang and
CLDN1 association, we used recombinant soluble E2715 and sive data. Future experiments will need to address the role of have been technically challenging and have provided inconclu-

ciased in polarized Caco-2 cells demonstrated that HCVpp and HCVcc can infect the colorectal ad-

gestated that CD81-CLDN1 FRET association in polarized Caco-2 cells nature of the cells, suggesting that CLDN coreceptor activity
class cells (77), who demonstrated minimal cell surface CLDN1 expression in nonpermissive HeLa cells, leading the authors to conclude that CLDN1 and CD81 localization predicts cellular permissiveness to HCV entry. In our experience, transduction of some cell types (HeLa and WIF-B) to express exogenous CLDN1 leads to an accumulation of the protein within cells and no detectable cell surface expression, consistent with an inability to support HCVpp entry (data not shown). However, interpreting data from cells in which CLDN1 demonstrates incorrect localization is fraught with difficulties. In contrast, the identification of nonpermissive cells with native patterns of CLDN1 and CD81 localization supports the conclusion that coreceptor localization and FRET-defined association does not predict cellular permissiveness to HCV infection.
The C-terminal region of CLDN1 is not critical for its association with CD81 in Huh-7.5 and 293T cells (Table 1), suggesting that the form of CLDN1 interacting with CD81 is not complexed with other TJ protein constituents. However, Huh-7.5 and 293T cells do not polarize or form TJs in cell culture (C. J. Mee, unpublished observations) and are not suitable for the study of the effects of polarization on CD81 and CLDN1 association and receptor activity. Recent experiments demonstrated that HCVpp and HCVcc can infect the colorectal adenocarcinoma Caco-2 cell line independent of the polarized nature of the cells, suggesting that CLDN coreceptor activity may be independent of its TJ function (55). Attempts to study CD81-CLDN1 FRET association in polarized Caco-2 cells have been technically challenging and have provided inconclusive data. Future experiments will need to address the role of cell polarization on CD81-CLDN1 association(s) and their sensitivity to inhibitors of various signaling pathways.
To investigate whether HCV gps promote CD81 and CLDN1 association, we used recombinant soluble E2715 and E1-E2 to model virus-receptor interactions. Both gp preparations bound to Huh-7.5 cells according to confocal imaging (Fig. 6A) and flow cytometry (data not shown). Neither antigen increased the %FRET between CD81 and CLDN1, sug-

ting that receptor complexes preexist within cells and that their formation or stability is not dependent upon or promoted by interaction(s) with the viral gps. This is in contrast to reports demonstrating that association between the HIV receptors CD4 and CCR-5 is dependent on the viral envelope gp (1, 31, 69, 78). The interaction of E1-E2, but not E2715, with Huh-7.5 cells reduced the estimated distance between CD81 and CLDN1 and inhibited HCV infection (Fig. 6). In contrast, saturating concentrations of E2715 and E1-E2 had no effect on %FRET or estimated distances between CD81-CD81, suggest-
ging that the viral gps modulate CD81 association with CLDN1. We are unable to ascertain if this effect is regulated at the level of E1-E2 binding, since we cannot discriminate between E1-E2 binding to CD81 in association with CD81 and with CLDN1.

The exact role of each gp in mediating receptor-dependent attachment and fusion of viral and cell membranes is unknown. A recent report demonstrating the presence of potential fusion peptides in both E1 and E2 (48) lends further support to the model that both gps are required for productive viral entry. There are several interpretations of the inhibitory effect(s) of E1-E2 on HCV infection. First, the gp-dependent reduction in CD81-CLDN1 FRET distances may reflect an induced change in the stoichiometry or distance between coreceptor molecules that is necessary for efficient HCV entry. Second, gp occupation of the CD81-CLDN1 complex may sterically block HCV interaction. Third, gp binding may lead to an internalization of receptor complexes. At the present, it is difficult to discriminate between these alternatives; however, current data do not support a gp-dependent internalization of the E1-E2–receptor complex (Fig. 6A).

Several publications have suggested that SR-BI may be the primary receptor defining HCV attachment to target cells (reviewed in reference 74). Our attempts to generate and express AcGFP- or DsRed-tagged SR-BI for FRET studies resulted in a series of molecules that accumulated within the cytoplasm and fail to express at the PM (Joe Grove, unpublished data). More-recent experiments with SR-BI fusion proteins with eGFP and mCherry added to the N terminus demonstrate PM expression; however, these fluorophores are not suitable for FRET studies, as they require dimerization to fluoresce. Future experiments will seek to investigate the localization and association of SR-BI with CD81 and CLDN1 in polarized and nonpolarized cell culture systems (55).

The principal site of HCV replication is believed to be hepato
tocytes within the liver. Hepatocytes are polarized, with TJs separating their PM into apical and basolateral domains (76). FRET occurred between hepatocyte-bound antibodies specific for CD81 and CLDN1 in healthy and HCV-infected liver tissues, suggesting that CD81 associates with CLDN1 at apical and basolateral surfaces (Fig. 5). However, the heterogeneity of coreceptor expression in hepatocytes and the limitations of indirect FRET methodology make comparison of CD81-CLDN1 FRET efficiencies between liver samples difficult to interpret (63).

Tetraspanins are four-transmembrane proteins that typically reside at the cell surface and assemble with themselves and other proteins to form tetraspanin-enriched microdomains (reviewed in reference 50). Multiple regions within the extracellular and transmembrane domains of CD81 have been reported to be important for oligomerization (15, 16, 41).

**TABLE 2. Effect of HCV infection on FRET between fluorescently N-terminus-tagged CD81 and CLDN1**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Colocalized region</th>
<th>N&lt;sub&gt;col&lt;/sub&gt;</th>
<th>%FRET</th>
<th>Distance (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>g.CD81/r.CD81</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>PM</td>
<td>49</td>
<td>80</td>
<td>6.11 ± 2.04</td>
</tr>
<tr>
<td>NS5A-positive</td>
<td>CI</td>
<td>44</td>
<td>84</td>
<td>6.19 ± 2.64</td>
</tr>
<tr>
<td></td>
<td>PM</td>
<td>54</td>
<td>46</td>
<td>1.54 ± 2.70</td>
</tr>
<tr>
<td></td>
<td>CI</td>
<td>156</td>
<td>64</td>
<td>5.10 ± 3.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.CD81/r.CLDN1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>PM</td>
<td>51</td>
<td>57</td>
<td>6.72 ± 2.49</td>
</tr>
<tr>
<td>NS5A-positive</td>
<td>PM</td>
<td>65</td>
<td>52</td>
<td>6.24 ± 3.44</td>
</tr>
<tr>
<td></td>
<td>CI</td>
<td>121</td>
<td>58</td>
<td>5.58 ± 2.76</td>
</tr>
</tbody>
</table>

<sup>a</sup> Huh-7.5 cells expressing g.CD81/r.CD81 or g.CD81/r.CLDN1 were infected with HCVcc JFH-1. At 72 h postinfection, naive and infected cells were fixed and stained for NS5A, and regions of fluorescent protein colocalization at the PM and CJs were imaged by confocal microscopy for FRET analysis.

<sup>b</sup> N<sub>col</sub>, the number of colocalized regions analyzed.

<sup>c</sup> HCV infection significantly decreased the estimated distance between g.CD81 and r.CD81 at the PM (P < 0.001 [Mann-Whitney test]).
Partner proteins for tetraspanins include integrins, Ig superfamily proteins, G protein-coupled receptors, and signaling enzymes (7, 37, 68). CLDN proteins similarly contain four transmembrane domains; however, the sequences and functions of the CLDN and tetraspanin families are quite distinct. CLDNs have been reported to form homophilic and heterophilic interactions at TJs between apposing cells (23, 61). The estimated distances between CD81-CLDN1 and CLDN1-CLDN1 of 5.5 to 7.0 nm at CJs are likely to reflect intracellular protein associations, as both donor and acceptor fluorophores are located intracellularly, and the average thickness of two opposing PMs is within the order of 10 to 12 nm (40).

Approaches to investigating tetraspanin-protein interactions have generally utilized mass spectroscopic analysis of immunoprecipitates from detergent-lysed cell preparations (49, 52, 66, 67). However, several reports suggest that microdomains isolated from detergent-lysed cells may not reflect the organization of protein-protein complexes in intact cells (reviewed in reference 46). Kovalenko et al. recently developed a cysteine cross-linking method to identify tetraspanin-protein associations and reported an interaction between CD9 and CD81 with CLDN1 (42). Given the reported interaction between CD81 and CD9 (reviewed in reference 37), we cannot discriminate whether the FRET-determined association(s) between CD81 and CLDN1 is direct or mediated via CD9. However, Huh-7.5 cells express minimal levels of CD9, transduction to overexpress CD9 has no effect on HCV entry, and treatment with anti-CD9 antibodies has no effect on HCV infection, suggesting that CD9 has no role in the HCV entry process (33, 81; A. Jennings, unpublished data). Our experiments to coprecipitate CD81 and CLDN1 from Huh-7.5 and 293T cells have been inconclusive and may reflect the specific association of the coreceptors at the PM, where intracellular protein interactions may mask the detection of protein associations at the cell surface. It is important to note that techniques to cross-link cell surface proteins involve the chipping of cells on ice and that recent experiments demonstrate that this low-temperature treatment reduces coreceptor expression at the PM, making these experiments difficult to interpret (M. Farquhar, unpublished data).

Treatment of Huh-7.5 cells with the neutralizing anti-CD81 MAb M38 reduced the %FRET between CD81-CD81 complexes and had a negligible effect on the %FRET between CD81-CD81 complexes and had a negligible effect on the %FRET between CD81 and CLDN1 (Fig. 7), suggesting that M38 can discriminate between CD81 in association with CD81 and that in association with CLDN1. Further investigation will require the generation of fluorescently labeled M38 to study its interaction with CD81 in discrete protein complexes. Current data suggest that anti-CD81 antibodies may inhibit HCV infection through mechanisms more complex than simple blocking of receptor-virus interactions, involving the reorganization of CD81 in the PM.

In summary, we have demonstrated that FRET occurs between CD81- and CLDN1-tagged molecules in cultured cells, consistent with protein complex association. The colocalization of CD81 and CLDN1 in liver tissue (76) and indirect FRET between receptor-specific antibodies presented here lend further support for the presence of receptor complexes in polarized cells that localize beyond the apically positioned TJs. Recent experiments studying coreceptor localization in polarized Caco-2 cells demonstrate CLDN1 expression beyond the lateral apical cell junctions, in agreement with our observations with human liver tissue (55, 63). These data support a model in which HCV may utilize forms of CLDN1 that are not associated with TJs. Perturbation of %FRET and estimated distances between CD81-CD81 and CD81-CLDN1 with HCV E1-E2 gpsi, anti-CD81 MAb M38, and HCV infection suggest that these complexes may have distinct role(s) in the viral entry process and offer new targets for antiviral intervention.

ACKNOWLEDGMENTS

We thank Takaji Wakita for JFH-1, Charles Rice for Huh-7.5 cells and anti-NS5A 9E10 MAb, Adrian Thrasher for CSGW, Paul Bieniasz for HIV gag-pol plasmid, and Fedor Berditchevski for anti-CD81 MAb M38. We thank Joe Grove and Adam Jennings for permission to cite their unpublished work.

This work was supported by PHS grant AI50798, the MRC, and the Wellcome Trust.

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