JOURNAL OF VIROLOGY, Aug. 2006, p. 7844–7853 Vol. 80, No. 16
0022-538X/06/$08.00+0 doi:10.1128/JVI.00029-06

A Conserved Gly[436]-Trp-Leu-Ala-Gly-Leu-Phe-Tyr Motif in Hepatitis C Virus Glycoprotein E2 Is a Determinant of CD81 Binding and Viral Entry

Heidi E. Drummer,* Irene Boo, Anne L. Maerz, and Pantelis Poumbourios

The Macfarlane Burnet Institute for Medical Research and Public Health Ltd., 85 Commercial Road, Melbourne, Victoria, Australia 3004

Received 5 January 2006/Accepted 2 June 2006

The hepatitis C virus (HCV) glycoproteins E1 and E2 form a heterodimer that mediates CD81 receptor binding and viral entry. In this study, we used site-directed mutagenesis to examine the functional role of a conserved G[436]WLAGLFY motif of E2. The mutants could be placed into two groups based on the ability of mature virion-incorporated E1E2 to bind the large extracellular loop (LEL) of CD81 versus the ability to mediate cellular entry of pseudotyped retroviral particles. Group 1 comprised E2 mutants where LEL binding ability largely correlated with viral entry ability, with conservative and nonconservative substitutions (W437 L/A, L438A, L441V/F, and F442A) inhibiting both functions. These data suggest that Trp-437, Leu-438, Leu-441, and Phe-442 directly interact with the LEL. Group 2 comprised E2 glycoproteins with more conservative substitutions that lacked LEL binding but retained between 20% and 60% of wild-type viral entry competence. The viral entry competence displayed by group 2 mutants was explained by residual binding by the E2 receptor binding domain to cellular full-length CD81. A subset of mutants maintained LEL binding ability in the context of intracellular E1E2 forms, but this function was largely lost in virion-incorporated glycoproteins. These data suggest that the CD81 binding site undergoes a conformational transition during glycoprotein maturation through the secretory pathway. The G436P mutant was an outlier, retaining near-wild-type levels of CD81 binding but lacking significant viral entry ability. These findings indicate that the G[436]WLA GLFY motif of E2 functions in CD81 binding and in pre- or post-CD81-dependent stages of viral entry.

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family of small, enveloped plus-strand RNA viruses that has infected over 3% of the global human population, causing significant morbidity and mortality. Hepatitis C virus encodes two type I transmembrane glycoproteins, E1 and E2, which are cleaved from the viral polyprotein precursor by signal peptidases in the endoplasmic reticulum (ER). E1 and E2 form heterogeneous mixtures of covalently and noncovalently associated heterodimers that are largely retained in the ER via retention sequences located in their transmembrane domains (5, 6, 14). However, a small proportion of E1E2 heterodimer escapes the ER and matures through the secretory pathway (11). Retroviruses such as human immunodeficiency virus type 1 (HIV-1) can be pseudotyped with cell surface-expressed E1E2 (E1E2-pseudotyped particles [E1E2-pps]). E1E2-pps contain noncovalently associated E1E2 heterodimers and are capable of infecting primary human hepatocytes and various human liver cell lines including Huh7 cells (2, 11, 23). Viral entry of E1E2-pps and cell culture-grown HCV occurs via receptor-mediated endocytosis, the E1E2 glycoproteins mediating low-pH-dependent fusion (1, 2, 23, 24, 40).

The E2 glycoprotein mediates binding to cellular receptors, including the tetraspanin CD81 (34) and the high-density lipoprotein receptor scavenger receptor class B type 1 (SR-B1) (38). Glycoprotein E2-mediated viral entry is blocked by antibody to CD81 (3, 7, 23, 25, 44) and by short interfering RNA-mediated knockdown of CD81 expression (44). In addition, HepG2 cell lines that do not express CD81 can be made permissive for both E1E2-pps and cell culture-grown HCV after transfection with CD81 expression vectors (26, 28, 44). While antibody to SR-B1 can also block entry of E1E2-pps (3), its role in cell culture-grown HCV entry is yet to be verified. An examination of receptor expression profiles of entry-permissive and entry-nonpermissive cell lines indicates that the presence of both CD81 and SR-B1 correlates with viral entry. However, additional receptors or cofactors are likely to play a role in entry, as coexpression of both CD81 and SR-B1 in certain nonpermissive cell types does not allow infection with E1E2-pps (3).

In addition to its role in viral entry, the E2-CD81 interaction has been shown to cause inflammatory and immunomodulatory responses in certain cell types in vitro that are consistent with pathogenic processes observed in infected individuals. For example, E2-CD81 ligation lowers the threshold of T-cell activation, stimulating the production of inflammatory cytokines (42). In hepatic stellate cells, E2-CD81 interactions upregulate matrix metalloproteinase 2 expression, potentially contributing to liver inflammation and fibrosis (27). CD81-E2 ligation also leads to the suppression of NK cell activity, which may decrease the effectiveness of innate immune responses in clearing virus (8, 41). Thus, an understanding of the molecular basis of the E2-CD81 interaction is critical for the development of inhibitors of E2-CD81-mediated viral entry and immunomodulation.

The E2 binding site of CD81 is located within the large extracellular loop (LEL), which is bounded by transmembrane domains 3 and 4. The binding site comprises a solvent-exposed

* Corresponding author. Mailing address: The Macfarlane Burnet Institute for Medical Research and Public Health Ltd., GPO Box 2284, Melbourne, Victoria, Australia 3001. Phone: 613 9282 2179. Fax: 613 9282 2100. E-mail: hdrummer@burnet.edu.au.

hydrophobic ridge formed by the Ile^{181}-Ile^{182}-Leu^{185}-Phe^{186} cluster and an adjacent polar pocket formed by Asn^{184} and Thr^{166} (13). The affinity of interaction between E2 and the CD81 LEL is $\sim 10^{-8}$ M (13, 17, 22, 33, 34). The CD81 binding site is located within the E2 ectodomain residues 384 to 661 and is comprised of discontinuous sequences that form the binding surface (23, 31, 36, 43). Also located within the ectodomain of E2 are two hypervariable regions (HVR1, residues 384 to 410, and HVR2, residues 461 to 480) that appear to modulate the accessibility of the CD81 binding site without playing a direct role in CD81 receptor binding (36). For example, deletion of HVR1 (residues 384 to 410) from a truncated, soluble form of E2 increased LEL binding activity (36), whereas a larger deletion extending into the adjacent conserved sequence (residues 384 to 411) in E1E2-pps led to decreased LEL binding (4). Hypervariable region 1 is adjacent to the epitopes recognized by monoclonal antibodies (MAbs) 3/11 and AP33 (residues 412 to 423), which have been previously shown to inhibit CD81 binding and E1E2-pp entry (23, 31, 32). Thus, the hypervariable regions of E2 appear to be intimately associated with CD81 LEL binding determinants.

In this study, we examined the role of amino acids located in a conserved region of E2 located between HVR1 and HVR2, G^{436}WLAGLFY. Substitutions in the G^{436}WLAGLFY motif decreased or abrogated both the cellular entry ability of E1E2-pps and the CD81 binding function of virion-incorporated E1E2. An outlier mutant, G436P, was able to bind CD81 at near-wild-type levels but lacked significant viral entry function, consistent with an additional role for the motif in pre- or post-receptor-binding stages of viral entry.

MATERIALS AND METHODS

Cell lines and antibodies. HEK 293T, CHO-K1, and Huh7 cells were maintained in Dulbecco's minimal essential medium containing 10% fetal calf serum and 2 mM L-glutamine (DMF10). MAb H53 (9, 14) was a kind gift of Jean Dubuisson. Immunoglobulin G14 (IgG14) was purified from plasma obtained from an HIV-1-infected individual by using protein G Sepharose (Amersham Pharmacia Biotech). Monoclonal antibody 9E10, directed to the myc epitope tag, was purified from the cell line MYC 1-9E10.2 using protein G Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions and dialyzed into phosphate-buffered saline (PBS). The E2-specific MAb All was a kind gift from Harry Greenberg. IgG was purified from the serum of an HCV-infected individual with chronic infection by using protein G Sepharose (Amersham Pharmacia Biotech) according to the manufacturer's instructions and dialyzed into PBS.

Vectors. Construction of the pcDNA4HisMax (Invitrogen)-based vector, pE1E2H77c (wild type), which directs expression of E1E2 of the H77c strain from a cytomegalovirus promoter, has been described previously (11). The HIV-1 luciferase reporter vector pNL4-3.LUC.R$^-$E$^-$ was obtained from N. Landau through the NIH AIDS Research and Reference Reagent program (21). In vitro mutagenesis was carried out by standard overlap extension PCR techniques. Inserts were fully sequenced on an ABI automated sequencer. Primer sequences can be obtained from the corresponding author. The pcE2^{661}myc vector directs the expression of E2 residues 384 to 661 (H77c isolate), linked to an N-terminal tissue plasminogen activator leader sequence (13), and encodes a C-terminal myc epitope tag (E2^{661}myc). Mutated E2 384 to 661 fragments were amplified using the PCR primers 5′-CCA**GCTAGC**GAAACCCACGTCACCG GGGGAAATGC-3′ and 5′-CCG**TCTAGA**CTAATTCAGATCCTCTTCTGAGAT GAGTTTTTGTTCAGTACTCTCGGACCTGTCCCTGTC-3′ from the corresponding pE1E2H77c vectors. The E2^{661}myc PCR products were digested with NheI and XbaI and cloned directionally into the NheI-XbaI site of vector pcE2^{661}myc. The CD81 open reading frame was amplified by PCR from pcDM8-TAPA-1 (30) using the primers 5′-CCG**AAGCTT**CCACCATGGGAGTGGAGGGCTGC-3′ and 5′-GGC**TCTAGA**T TAGTACACGGAGCTGTTCCG-3′. The PCR product was cloned into pcDNA3 using HindIII and XbaI to generate the plasmid pcDNA3-CD81. The PCR-amplified DNA sequences were verified by automated DNA sequencing with Big Dye terminator chemistry (ABI). The vectors were transfected into HEK 293T or CHO-K1 cells using Fugene 6 (Roche) as described previously (11).

RIP. Radioimmunoprecipitations (RIPs) were performed as described previously (11). Intracellular forms of E1E2 were analyzed by radiolabeling E1E2-transfected HEK 293T cells with 150 μCi Tran^{35}S-label (ICN, Costa Mesa, CA) for 15 min followed by a 4-h chase in DMF10. Radiolabeling of E1E2–HIV-1 pseudotypes was performed using 293T cells that had been seeded at 350,000 cells/well in six-well culture dishes and transfected with 1 μg each of pNL4-3.LUC.R$^-$E$^-$ plus pE1E2H77c or empty pcDNA4HisMax vector. Twenty-four hours posttransfection, 75 μCi Tran^{35}S-label (ICN, Costa Mesa, CA) in methionine- and cysteine-deficient DMF10 was added, and labeling was performed for 18 h. The tissue culture fluid was clarified by centrifugation at 14,000 × *g* for 10 min, and radiolabeled viruses were pelleted by centrifugation at 14,000 × *g* for 2 h at 4°C. The E1E2-transfected cells or pelleted virions were lysed in RIP lysis buffer (0.6 M KCl, 0.05 M Tris, pH 7.4, 1 mM EDTA, 0.02% sodium azide, 1% Triton X-100) and immunoprecipitated with MAb H53, polyclonal IgG purified from the serum of an HCV-infected individual, or IgG from an HIV-1-infected individual (IgG14) prior to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and phosphorimage analysis.

E1E2-pseudotyped HIV-1 particle entry assay. Pseudotyped particle entry assays were performed as described previously (11). Briefly, HEK 293T cells were cotransfected with wild-type and mutated forms of pE1E2H77c or pcDNA4 empty vector plus pNL4-3.LUC.R$^-$E$^-$. At 72 h posttransfection, culture supernatants were filtered (0.45 μm) and applied to Huh7 cell monolayers. The p24 content of E1E2-pp was measured using a viral antigen detection kit (Vironostika; BioMerieux) according to the manufacturer's instructions. Luciferase was measured in Huh7 cell lysates 72 h later with a Polarstar plate reader (BMG Labtechnologies) fitted with luminescence optics using the Promega luciferase reagent system.

E2^{661}myc production. E2^{661}myc was secreted from HEK 293T cells transfected with pcE2^{661}myc using Fugene 6 (Boehringer Mannheim) as described previously (13). The tissue culture fluid was clarified through 0.22-μm-pore-size filters and then concentrated by approximately 10-fold in Centricon YM30 concentrators (Amersham). The relative amount of E2^{661}myc present in concentrates was determined by Western blotting. Protein samples were subjected to SDS-PAGE in 12% polyacrylamide gels under reducing conditions. The proteins were detected with MAb 9E10 and goat anti-mouse Alexa 680 (Invitrogen) antibody. The blots were scanned in a fluorescent scanner (Odyssey; LI-COR Biotechnology), and E2^{661}myc band intensity was quantitated using Odyssey software.

Solid-phase E1E2-CD81 binding assay. The expression and purification of a chimera composed of maltose-binding protein (MBP) linked to the CD81 LEL residues 113 to 201 (MBP-LEL$^{113–201}$) has been described previously (13). Ninety-six-well Maxisorb enzyme-linked immunosorbent assay plates (Nunc) were coated with 5 μg/ml of dimeric MBP-LEL$^{113–201}$ in PBS at room temperature overnight, followed by blocking of uncoated sites with PBS containing 10 mg/ml bovine serum albumin solution (BSA$_{10}$PBS) for 1 h at 37°C. The plates were washed four times with PBS containing 0.05% Tween 20. E1E2–HIV-1 pseudotypes were produced as described above, metabolically labeled with Tran^{35}S-label, and pelleted from the tissue culture medium by centrifugation at 14,000 × *g* for 2 h. The virions were lysed in RIP lysis buffer and applied at various dilutions to MBP-LEL$^{113–201}$-coated plates. Alternatively, pE1E2H77c-transfected 293T cells were lysed in RIP lysis buffer at 48 h posttransfection and serial dilutions of the clarified lysates were applied to MBP-LEL$^{113–201}$-coated plates. After incubation at room temperature for 2 h, the plates were washed and bound E2 was detected with MAb H53 and rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (DAKO). The plates were developed with tetramethylbenzidine substrate according to the manufacturer's recommendations (Sigma) and read at 450 nm, and the 620-nm background was subtracted. The data obtained using cell lysates were normalized for the amount of monomeric E2 expressed from each vector by subjecting proteins to SDS-PAGE analysis in 12% polyacrylamide gels under nonreducing conditions. Proteins were transferred to a nitrocellulose membrane, and E2 was detected with MAb All and goat anti-mouse Alexa 680 antibody. Membranes were scanned (Odyssey System; LI-COR Biotechnologies), and the amount of E2 was quantitated using Odyssey software (LI-COR Biotechnologies).

E2^{661}myc-CD81 cell surface binding assays. Cell surface binding assays were conducted as described previously (13). Briefly, CHO-K1 cells were seeded in 12-well culture plates at 1.25×10^5 cells/well and transfected with 2 μg pcDNA3-CD81 24 h later. At 48 h posttransfection the CD81-transfected CHO-K1 cells were chilled on ice and incubated with serial dilutions of wild-type or mutated E2^{661}myc in BSA$_{10}$PBS for 4 h on ice. The cells then were washed twice in

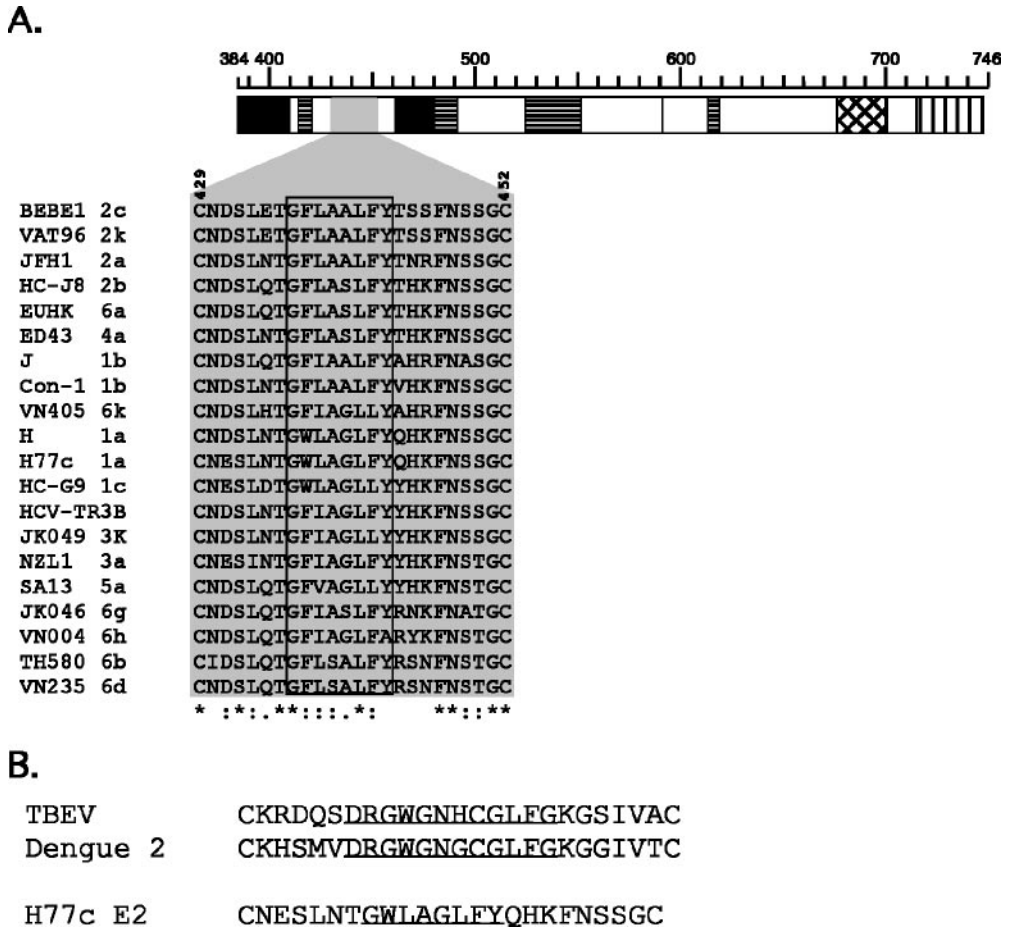

FIG. 1. (A) ClustalW alignment of prototype strains representative of each HCV genotype: 1a, H (GenBank accession no. M62631) and H77c (AF011751); 1b, J (D90208) and Con-1 (AJ238799); 1c, HC-G9 (D14853); 2a, JFH1 (AB047639); 2b, HC-J8 (D10988); 2c, BEBE1 (D50409); 2k, VAT96 (AB031663); 3a, NZL1 (D17763); 3b, HCV-TR (D49374); 3k, JK049 (D63821); 4a, ED43 (Y11604); 5a, SA13 (AF064490); 6a, EUHK2 (Y12083); 6b, TH580 (D84262); 6d, VN235 (D84263); 6g, JK046 (D63822); 6h, VN004 (D84265); and 6k, VN405 (D84264). Highlighted are the hypervariable regions located in E2 (black), the region under examination in this study (gray), the E2 stem region (crosshatched) (12), predicted locations of the transmembrane domains/signal peptide sequences (vertical stripes), and regions implicated in CD81 binding (horizontal stripes) (23, 31, 36, 43). Asterisk, identical; colon, highly conserved; period, partially conserved. (B) Fusion peptide sequences (underlined) of class II fusion proteins (tick-borne encephalitis virus Neudoerfl [U27495] and dengue virus type 2 [P12823]) are shown and compared to the region encompassing the G^{436}WLAGLFY sequence (underlined) in HCV E2.

BSA_{10}PBS prior to a 1-h incubation with ^{125}I-MAb 9E10 (10^6 cpm) that had been precleared with 10^7 CHO-K1 cells for 2 h on ice. After four further washes with BSA_{10}PBS, the cells were lysed in 1% SDS in PBS and counted in a Packard Auto-Gamma counter.

RESULTS

In this study, we used site-directed mutagenesis to examine the function of a conserved G^{436}WLAGLFY motif of E2. The motif is of interest as it is located between HVR1 and HVR2 (Fig. 1A), which modulate CD81 binding by E2 (36). Furthermore, the motif shares features in common with class II fusion peptides found in the phylogenetically related flaviviruses dengue virus and tick-borne encephalitis virus (Fig. 1B). The motif is bounded by two conserved cysteines (Cys^{429} and Cys^{452}), is rich in hydrophobic (29%) and glycine and alanine (16%) residues, and is predicted by the method of Garnier et al. (18) to form part of a β-strand/turn/α-helix/β-strand structure, believed to be a common feature of internal viral fusion peptides (10) (Fig. 1B).

Expression of E2 mutants. To examine the function of the conserved G^{436}WLAGLFY motif of E2, we determined how conservative and nonconservative substitutions in this region affected glycoprotein expression, maturation, CD81 receptor binding, and viral entry. The expression of mutated E1E2 complexes within transfected 293T cells was analyzed by pulse-chase metabolic labeling and immunoprecipitation with the conformation-dependent E2-specific MAb H53. In all cases, E1 and E2 were cleaved efficiently from the polyprotein, forming noncovalently associated heterodimers as assessed by nonreducing SDS-PAGE (Fig. 2A). We have shown previously that a small proportion of E1E2 is translocated to the cell surface, with E2 acquiring resistance to endoglycosidase H (11). These data suggest that E1E2 matures through the secretory pathway. The endoglycosidase H-resistant form of E2

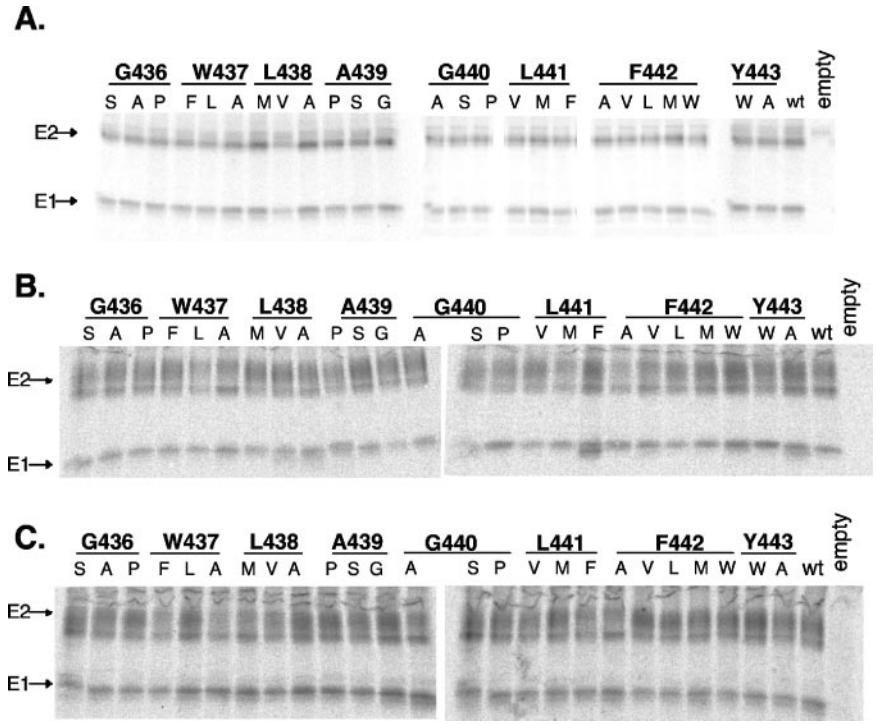

FIG. 2. Heterodimerization of E1E2 mutants in cell lysates and pseudotyped particles. (A) Metabolically labeled lysates of 293T cells, transfected with 2 μg wild type (wt), mutated pE1E2H77c, or empty pcDNA4 vector (no E1E2), were immunoprecipitated with the conformation-dependent anti-E2 MAb H53. The immunoprecipitants were examined by nonreducing SDS-PAGE on 10 to 15% polyacrylamide gradient gels. E1 and E2 were visualized by phosphorimage analysis. (B and C) Heterodimerization of E1E2 mutants incorporated into HIV-1 pseudotypes. Metabolically labeled viral particles were pelleted from the tissue culture fluid of pE1E2H77c plus pNL4-3.LUC.R$^-$E$^-$-transfected cells prior to lysis in RIP buffer. E1E2 heterodimers were immunoprecipitated with MAb H53 (B) or polyclonal IgG obtained from an HCV-infected individual (C) prior to nonreducing SDS-PAGE on 10 to 15% gradient gels and phosphorimage analysis.

can be incorporated into HIV-1 particles in association with E1 (11). Figure 2B indicates the coimmunoprecipitation of E1 with E2 mutants from ^{35}S-labeled E1E2-pp by MAb H53, consistent with formation of translocation-competent E1E2 heterodimers. In all cases, both E1 and E2 glycoproteins were efficiently immunoprecipitated from E1E2-pp using a human anti-HCV antibody (Fig. 2C) that possesses high-titer neutralizing antibodies (H. Drummer, unpublished observations),

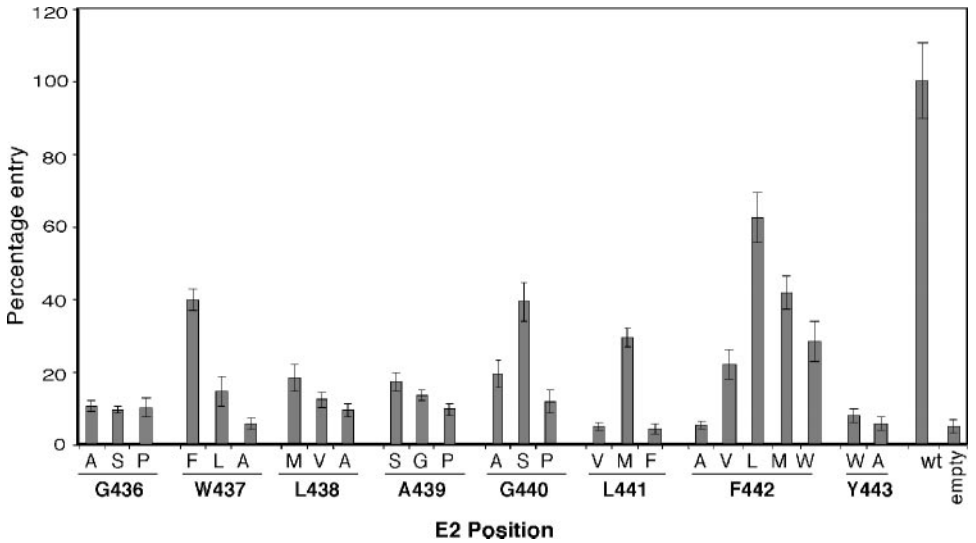

FIG. 3. Ability of E1E2-pseudotyped HIV-1 luciferase reporter viruses to enter Huh7 cells. Data represent the means ± standard deviations of an experiment performed in quadruplicate and are representative of five independent assays. wt, wild-type E1E2-pp; empty, HIV-1 particles lacking the E1E2 envelope glycoproteins.

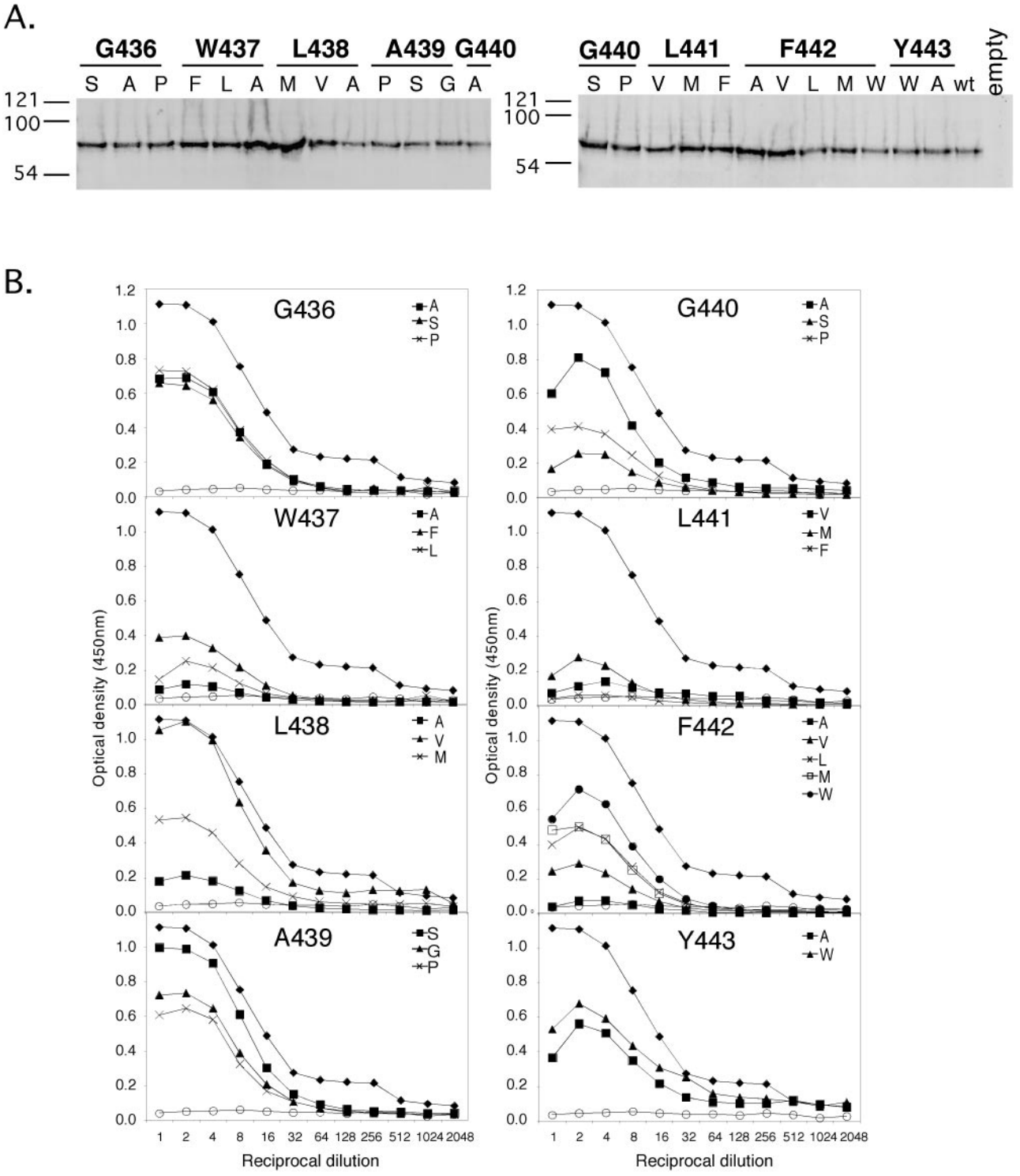

FIG. 4. Ability of E1E2 precursors in cell lysates containing mutations in the G^{436}WLAGLFY motif of E2 to bind CD81. (A) Detection of noncovalently associated E2 glycoprotein in cell lysates. Proteins were separated in 12% SDS-polyacrylamide gels under nonreducing conditions and then Western blotted with the E2-specific MAb All and goat anti-mouse Alexa 680 antibody. The amount of E2 in each lane was quantitated using Odyssey software and used to normalize the assay shown in panel B. Numbers at left of each panel are molecular masses in kilodaltons. (B) Ability of E2 in cell lysates to bind CD81. Dimeric MBP-$LEL^{113–201}$ was applied as a coating to enzyme immunoassay plates at 5 μg/ml. The cell lysates analyzed in panel A were titrated, and bound E2 was detected with MAb H53 and rabbit anti-mouse horseradish peroxidase in an enzyme immunoassay (13). The data were normalized against the amount of nonreduced E2 glycoproteins present and are representative of two independent experiments. Solid diamonds, wild-type pE1E2H77c-transfected cell lysates; open circles, empty vector control cell lysates.

TABLE 1. CD81 binding and viral entry abilities of G^{436}WLAGLFY mutants

Group and E2 mutant	Entry[a]	E1E2-LEL binding[b]	
		Virion incorporated	Cell lysate derived
No group			
WT[c]	100	+++	+++
Empty	5	0	0
1			
G436A	10	<	++
G436S	10	+	++
W437A	6	<	<
W437L	15	<	<
W437F	40	+	+
L438A	9	<	<
L438V	12	+	+++
L438M	18	++	+
A439G	14	++	++
A439S	17	+++	+++
A439P	9	++	+
G440P	12	+	+
L441V	5	<	<
L441F	4	<	<
F442A	5	<	<
F442M	42	+	+
Y443A	6	<	++
Y443W	8	<	++
2			
G440A	19	<	++
G440S	40	<	+
L441M	29	<	<
F442V	22	<	<
F442L	62	<	+
F442W	28	<	++
Outlier			
G436P	10	+++	++

[a] Percent entry data are derived from Fig. 3. Viral entry data are representative of five independent experiments.

[b] <, less than 25%; +, 25 to 50%; ++, 50 to 75%; +++, 75 to 100%. Binding was calculated as (optical density at 450 nm for mutant/optical density at 450 nm for wild type) × 100, calculated at a 1/4 dilution for E1E2-pp (Fig. 5A) and a 1/8 dilution for cell lysates (Fig. 4B) These dilutions correspond to approximately 75% of maximal binding for wild-type E1E2. Recombinant CD81 LEL binding data are representative of four independent experiments.

[c] WT, wild type.

suggesting that overall glycoprotein structure was preserved in the mutants. The immunoprecipitation of lysed virions with IgG14 from an HIV-1-infected individual indicated that the mutations had not affected the processing and virion incorporation of the HIV-1 structural protein $Pr55^{Gag}$, $p17^{MA}$, $p24^{CA}$, or $p66^{RT}$ (data not shown). These data suggest that the mutations did not significantly affect E1E2 maturation, recognition by neutralizing patient antiserum and a conformation-dependent antibody specific to E2, or incorporation into HIV-1 pseudotypes.

Effects of E2 mutations on viral entry. The ability of E1E2 mutants to mediate entry of E1E2-pps into Huh7 cells was next assessed (Fig. 3). Replacements of either Gly^{436} with Ser, Ala, or Pro or Gly^{440} with Ala or Pro led to >80% decreases in viral entry competence, indicating that both glycines are required for glycoprotein function, perhaps to confer backbone flexibility in the E2 motif. Consistent with this idea, the G440S mutant that would retain backbone flexibility (15) retained 40% of wild-type entry function. The aromatic residues at positions 437, 442, and 443 are well conserved across HCV genotypes and also represent determinants of viral entry competence because reductions in their side chain hydrophobicity resulted in severe entry defects. For example, entry was reduced by 60%, 85%, and 94% when Trp^{437} was mutated to Phe, Leu, or Ala, respectively. Replacement of Phe^{442} with Leu, Met, Val, and Ala reduced entry competence by 40%, 60%, 80%, and 95%, respectively, whereas replacement of the strictly conserved Tyr^{443} with Trp or Ala was not tolerated. Alterations to the hydrophobicity of the Leu^{438} and Leu^{441} side chains also caused severe entry defects with only L441M retaining viral entry competence (30% with respect to wild type). Thus, the G^{436}WLAGLFY motif of E2 is a key determinant of viral entry.

Effects of E2 mutations on CD81 binding. The conserved G^{436}WLAGLFY motif is positioned between HVR1 and HVR2, which have been shown to modulate the ability of E2 to recognize CD81 (36). We therefore examined whether the mutations affected the CD81 binding function of intracellular E1E2 precursors in a solid-phase assay employing MBP-$LEL^{113-201}$ (Fig. 4; Table 1). The intracellular forms of E1E2 were obtained by transfecting 293T cells with vectors encoding wild-type or mutant forms of E2. As it has been demonstrated that only non-disulfide-linked forms of E2 interact with CD81 (16), we determined the relative amounts of monomeric forms of wild-type and mutated E1E2 in cell lysates by nonreducing SDS-PAGE and Western blotting with MAb All (Fig. 4A). The ability of intracellular forms of E2 to interact with CD81 was then normalized for the relative amount of monomeric E2 expressed from each vector. The data show that in all but eight cases (W437A/L, L438A, L441V/M/F, and F442A/V) the mutated E1E2 intracellular precursors retained at least 25% of wild-type E2 LEL binding function (Fig. 4B; Table 1).

We next examined the ability of wild-type and mutated virion-derived E1E2 to interact with the CD81 LEL (Fig. 5A and B; Table 1). The amount of noncovalently associated E2 was assessed by immunoprecipitation of radiolabeled E1E2-pps using MAb H53. Proteins were separated in 10 to 15% SDS-polyacrylamide gels under nonreducing conditions. The amount of E2 and p24 incorporated into E1E2-pp was similar in all cases (Fig. 5B). The ability of E1E2-pp-derived E2 to interact with the LEL was determined in four independent experiments, and the mean and standard error are shown for a subset of mutants (Fig. 5A). Table 1 presents a summary of the data obtained for all E1E2-pps. The mutations caused more severe LEL binding defects in mature, virion-incorporated E1E2 than in intracellular forms (Table 1). For example, the intracellular forms of E1E2 bearing G436A/S, L438V, G440A, F442W, and Y443A/W mutations retained ≥50% LEL binding ability relative to wild type, whereas the corresponding virion-derived forms largely lacked this function. These data suggest that the CD81 binding site undergoes a conformational transition during E1E2 glycoprotein maturation in the biosynthetic pathway. For other E2 mutants, the LEL binding ability of the intracellular glycoprotein form generally corresponded to that of the virion-associated form. Thus, for both intracellular and virion-derived E1E2, G436P, W437F, L438M, A439S/G/P, G440P, and F442M retained >25% LEL binding, whereas W437A/L,

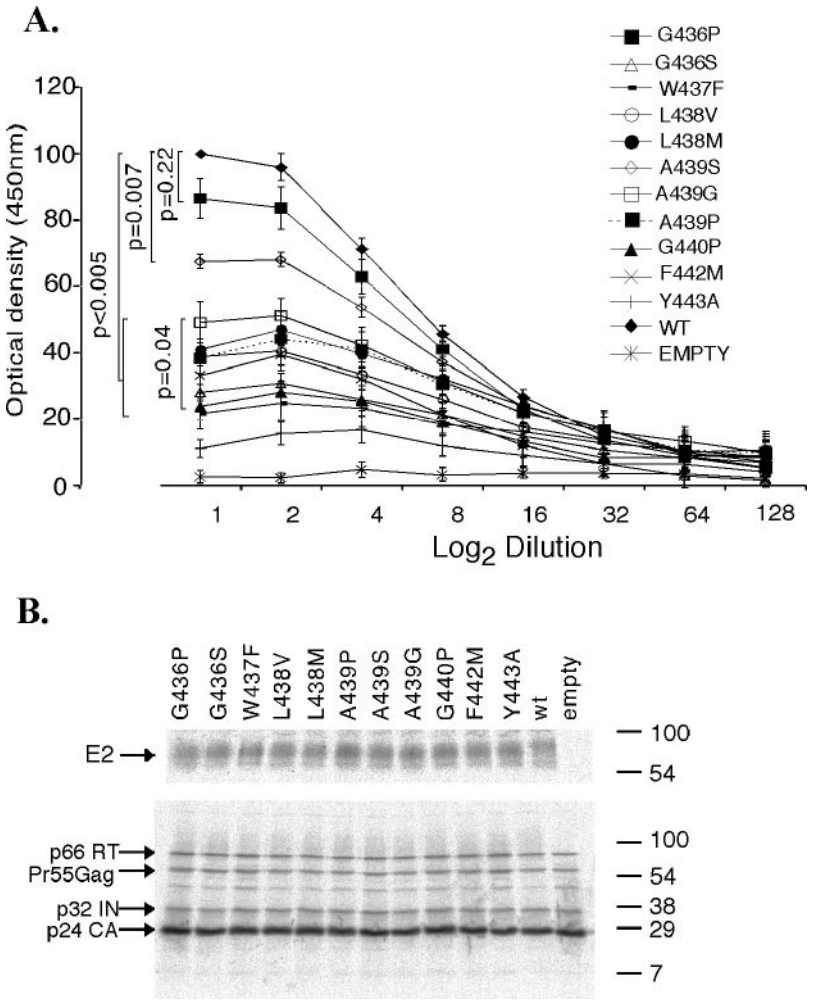

FIG. 5. Ability of E1E2 pseudotyped particles containing mutations in the E2 G^{436}WLAGLFY motif to bind CD81. (A) Dimeric MBP-$LEL^{113-201}$ was applied as a coating to enzyme immunoassay plates at 5 μg/ml. Wild-type (WT) and mutated E1E2-pp were titrated twofold, and bound E2 was detected with MAb H53 and rabbit anti-mouse horseradish peroxidase. Data were calculated as (optical density of each dilution of mutant E1E2-pp/optical density of undiluted wild-type E1E2-pp) × 100. Maximal binding values for the individual assays were 0.40, 0.334, 0.525, and 0.39 optical density units (450 nm). Data represent the means ± standard errors of four independent experiments. *P* values were determined for wild type versus G436P, wild type versus A439S, wild type versus A439G, wild type versus G440P, and A439G versus G440P using a two-sample Student *t* test assuming unequal variances at 75% of maximal binding by wild-type E2 (1/4 dilution). (B) Immunoprecipitation of E2 and HIV-1 core proteins. E1E2-pps were produced in 293T cells and metabolically labeled with Tran^{35}S-label for 18 h. E1E2 glycoproteins were immunoprecipitated with MAb H53 and protein G Sepharose, analyzed under nonreducing conditions in 10 to 15% SDS-polyacrylamide gels, and subjected to phosphorimage analysis (upper panel). Bands corresponding to E2 are shown. The HIV core proteins were immunoprecipitated with IgG from an HIV-positive individual, analyzed under reducing conditions in 7.5 to 15% SDS-polyacrylamide gels, and subjected to phosphorimage analysis (lower panel). RT, reverse transcriptase. Numbers at right are molecular masses in kilodaltons.

L438A, L441M/V/F, and F442A/V lacked LEL binding function. Because W437A/L, L438A, L441M/V/F, and F442A/V ablated LEL binding in both forms of E1E2, Trp^{437}, Leu^{438}, Leu^{441}, and Phe^{442} are likely to play a direct role in CD81 binding.

A comparison of the LEL binding ability of mature virion-incorporated E1E2 with entry competence allowed the placement of glycoprotein mutants into two groups (Table 1). Group 1 comprises mutants whose ability to bind CD81 correlated with viral entry competence. For example, viral entry was reduced by approximately 90% in E1E2-pp containing mutations at G436A/S, W437A, L438A, A439P, L441V/F, F442A, and Y443A/W with complete or almost-complete loss of LEL binding by virion-associated E1E2. In the case of group 2, where mutations were generally more conservative than in the group 1 glycoprotein counterparts, E1E2-pp retained 20 to 60% of entry competence in the absence of detectable LEL binding. The G436P mutant represents an outlier that retained wild-type levels of LEL binding but lacked viral entry competence. Therefore, the G^{436}WLAGLFY motif may also play a functional role in pre- or post-CD81-dependent stages of viral entry such as coreceptor binding or viral fusion.

Binding of E2 glycoproteins containing mutations in the G^{436}WLAGLFY motif to full-length CD81. The ability of group 2 mutants to mediate viral entry in the absence of detectable LEL binding function suggests either that the affinity for full-

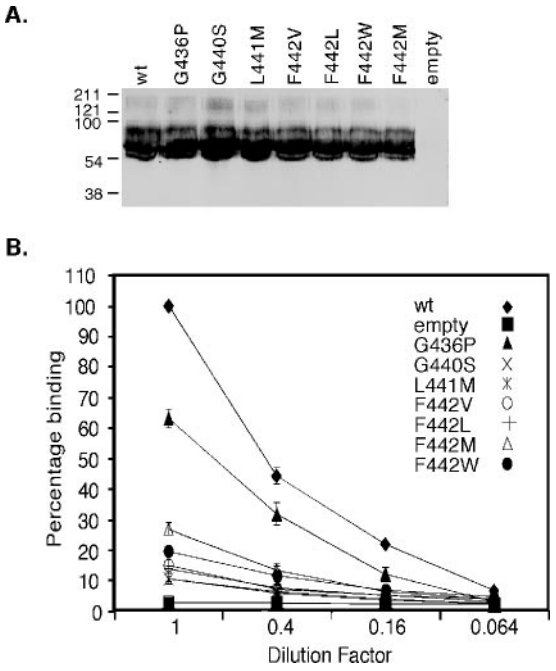

FIG. 6. Ability of E2 glycoproteins containing mutations in the G^{436}WLAGLFY motif to bind full-length CD81. (A) Recombinant forms of E2 were expressed from the pcE2^{661}myc vector in 293T cells. The secreted glycoproteins were concentrated from the tissue culture fluid of transfected cells and examined by SDS-PAGE and Western blotting with MAb 9E10 and goat anti-mouse Alexa 680 antibody. Blots were scanned in an Odyssey detector, and the amount of E2 in each lane was quantitated using Odyssey software. Numbers at left are molecular masses in kilodaltons. (B) Equivalent amounts of E2^{661}myc (derived from panel A) were added to CHO-K1 cells transfected with a vector encoding human CD81. Bound E2^{661}myc was detected using ^{125}I-MAb 9E10 and counted in a Packard gamma counter. Shown are the averages of two independent assays ± standard deviations. wt, wild type.

length CD81 is higher than for recombinant forms of CD81 or that an alternate pathway of entry may be utilized by HCV-pp. In order to determine whether residual CD81 binding could be detected for group 2 mutants, selected mutations were introduced into an E2 ectodomain fragment comprising residues 384 to 661, E2^{661}myc. This E2 fragment has been shown to retain CD81 binding function (17, 34, 37). A C-terminal c-*myc* epitope tag was incorporated to facilitate detection, and the native E2 leader sequence was replaced with the tissue plasminogen activator leader sequence to enhance expression levels (20). Figure 6A shows that E2^{661}myc was secreted into the tissue culture medium of transfected cells for all mutants as determined by SDS-PAGE and Western blotting with MAb 9E10. We next employed a CD81 binding assay to determine if E2^{661}myc containing mutations in the G^{436}WLAGLFY motif could bind full-length CD81 (13). CHO-K1 cells were transfected with a vector encoding human CD81. Forty-eight hours after transfection, the cells were placed on ice, and equivalent amounts of E2 were added to cells. Four hours later, bound E2 was detected with radioiodinated MAb 9E10, and the amount of bound E2 was quantitated. The results (Fig. 6B) show that the outlier mutant G436P had near-wild-type levels of CD81 binding, similar to that observed in LEL binding assays. The G440S, L441M, and F442V/L/W E2^{661}myc mutants all possessed 10 to 20% of CD81 binding ability while the group 1 mutant F442M retained 25% of CD81 binding, consistent with the binding activity of this mutant observed in the LEL assay. These data suggest that the ability of group 2 E1E2-pps to mediate viral entry may be explained by residual binding function for cell surface CD81.

DISCUSSION

The HCV glycoprotein E2 mediates attachment to the cellular receptor CD81, leading to the endocytosis of viral particles and low-pH-dependent membrane fusion. Previously we have shown that a hydrophobic heptad repeat (675 to 699), within the segment linking the E2 (384 to 661) receptor binding domain to the transmembrane domain, played a role in E1-E2 heterodimerization and viral entry (12). The function of this linking segment is analogous to that of the α-helical stem of the attachment/fusion glycoprotein E of flaviviruses, the prototype class II fusion glycoprotein. By analogy, HCV E2 may also act as a class II attachment/fusion glycoprotein; however, the location of the fusion peptide and the sequences involved in CD81 binding have not been identified. In this study, we characterized the role of the G^{436}WLAGLFY motif of E2, which resembles a class II fusion loop (Fig. 1B).

Mutations within the motif did not appear to affect glycoprotein maturation, as wild-type levels of noncovalently associated E1E2 heterodimers were immunoprecipitated by the conformation-dependent MAb H53 for both immature intracellular forms and mature virion-incorporated forms of the glycoproteins. However, defects in CD81 receptor binding were detected in a recombinant LEL binding assay (12). In the majority of cases, the LEL binding ability of virion-incorporated E1E2 bearing a given mutation predicted its ability to mediate viral entry (group 1, Table 1). The least conservative group 1 mutations, including G436A/S, W437A, L438A, L441V/F, F442A, and Y443A/W, reduced viral entry by 90% with complete or almost-complete loss of LEL binding by virion-incorporated E1E2. Furthermore, a subset of these mutations, W437A/L, L438A, L441M/V/F, and F442A/V, resulted in the complete loss of LEL binding function for both intracellular E1E2 precursors and mature, virion-incorporated glycoprotein. These data indicate that the G^{436}WLAGLFY motif is a CD81 binding determinant of mature E2 with Trp^{437}, Leu^{438}, Leu^{441}, and Phe^{442} directly contributing to the CD81 binding site. That the motif is intimately involved in CD81 binding is also indicated by the findings that MAbs 7/16b and 11/20, which are directed to epitopes encompassing the 436 to 447 sequence, block both E2-LEL interactions and E1E2-pp entry (23). The location of the G^{436}WLAGLFY motif between HVR1 and HVR2, previously shown to affect CD81 binding (36), is also of interest as it suggests that the hypervariable regions may modulate exposure of the proximal G^{436}WLA GLFY motif, thereby impacting on CD81 binding affinity.

Notably, the G^{436}WLAGLFY motif is comprised largely of hydrophobic and aromatic residues, the majority of which are important for CD81 binding. These residues may contribute to a hydrophobic surface that engages the Ile^{181}-Ile^{182}-Leu^{185}-Phe^{186} E2 binding cluster of the LEL (13). Roccasecca et al. (36) found that six simultaneous Ala substitutions for the C-

terminal Y^{613}RLWHY sequence of E2 also blocked LEL binding. The tertiary or quaternary fold of E2 may bring the discontinuous sequences G^{436}WLAGLFY and Y^{613}RLWHY together in a composite binding surface. Monoclonal antibody blocking studies have also implicated the amino acid sequences 412 to 423, 480 to 493, and 524 to 551 in CD81 binding; however, steric interference by antibody binding outside the CD81 interaction site of E2 may also account for the observed inhibition (23, 31, 43). A CD81 binding site composed of discontinuous E2 sequences contrasts the case with glycoprotein E of flaviviruses, where domain III is formed from a continuous C-terminal sequence and contains the receptor binding pocket (29, 35).

In the case of group 2 mutants, virion-incorporated E1E2 lacked detectable LEL binding function but E1E2-pp retained 20 to 60% of entry competence. Again, intracellular forms of E1E2 retained at least 25% to 50% of LEL binding in the majority of cases. The ability of group 2 mutants to enter cells is explained by the ability of truncated E2 glycoproteins bearing these mutations to bind full-length CD81 at levels corresponding approximately to 20% of that of wild-type E2. These data suggest that the affinity of E1E2 for full-length cellular CD81 may be higher than that for MBP-$LEL^{113–201}$, with residual CD81 binding leading to a low level of viral entry. That the residual entry activity observed for the group 2 mutants G440S, L441M, and F442W/L/V was further reduced by less conservative substitutions (G440A/P, L441V/F, and F442A) supports this scenario.

A number of mutations (G436A/S, L438V, G440A, F442W, and Y443A/W) affected the CD81 binding ability of the mature, virion-incorporated form of E1E2 more severely than that of the immature, intracellular glycoprotein form. These data suggest that the CD81 binding site undergoes a conformational transition during E1E2 maturation in the biosynthetic pathway with Gly^{436}, Leu^{438}, Gly^{440}, Phe^{442}, and Tyr^{443} contributing directly to the CD81 binding site in mature forms of E2. Alternatively, these residues may play an indirect role in the formation and/or maintenance of a functional CD81 binding site in mature E2. This may be the case if the G^{436}WLA GLFY motif plays a role in the formation and/or maintenance of the prefusion fold of E1E2. Such a role has been observed for the apparently homologous flavivirus E glycoprotein G^{100} WGNGCGLFG^{109} fusion loop motif, where Trp^{101} and Phe^{108} mediate stabilizing interactions with the receptor binding domain of a partner monomer (29, 35). However, unlike the flavivirus sequence, the HCV E2 G^{436}WLAGLFY sequence is unlikely to function as a fusion peptide unless disengagement from CD81 occurs prior to membrane fusion. The HCV fusion peptide sequence may reside elsewhere in E2 or perhaps in E1. For example, Garry and Dash proposed the highly conserved hydrophobic sequence C^{272}SALYVGDLC of E1 as the HCV fusion peptide with E1 functioning as a truncated class II fusion glycoprotein in concert with E2 (19).

The outlier mutant G436P exhibited near-wild-type CD81 binding activity, but viral entry function was significantly impaired (Table 1). The mutation is likely to lock the protein backbone in a *cis* or *trans* conformation (39), which would limit the rotational flexibility of this region. The minimal effect of this mutation on CD81 binding suggests that a pre- or post-CD81 binding stage of viral entry was blocked. Such an effect may be due to constraints imposed by proline on subsequent conformational changes that are required for coreceptor binding or low-pH-triggered membrane fusion activation of E1E2. Recently, Tscherne et al. showed that low pH was not sufficient to trigger fusion of cell culture-grown HCV with the plasma membrane and proposed that additional receptor binding or processing events must occur to trigger conformational changes in E1E2 in order for viral fusion to proceed (40). It is possible that the G^{436}WLAGLFY motif of E2 functions in such postreceptor conformational changes in addition to its direct role in CD81 binding.

ACKNOWLEDGMENTS

This work was supported by NHMRC grants 156713, 296200, and 345413.

We acknowledge the generosity of Jean Dubuisson, Charles Rice, and Harry Greenberg for providing reagents.

REFERENCES

1. **Bartosch, B., and F. L. Cosset.** 2006. Cell entry of hepatitis C virus. Virology **348:**1–12.
2. **Bartosch, B., J. Dubuisson, and F. L. Cosset.** 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J. Exp. Med. **197:**633–642.
3. **Bartosch, B., A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, and F. L. Cosset.** 2003. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. J. Biol. Chem. **278:**41624–41630.
4. **Callens, N., Y. Ciczora, B. Bartosch, N. Vu-Dac, F. L. Cosset, J. M. Pawlotsky, F. Penin, and J. Dubuisson.** 2005. Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. J. Virol. **79:**15331–15341.
5. **Cocquerel, L., S. Duvet, J. C. Meunier, A. Pillez, R. Cacan, C. Wychowski, and J. Dubuisson.** 1999. The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. J. Virol. **73:**2641–2649.
6. **Cocquerel, L., J. C. Meunier, A. Pillez, C. Wychowski, and J. Dubuisson.** 1998. A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. J. Virol. **72:**2183–2191.
7. **Cormier, E. G., F. Tsamis, F. Kajumo, R. J. Durso, J. P. Gardner, and T. Dragic.** 2004. CD81 is an entry coreceptor for hepatitis C virus. Proc. Natl. Acad. Sci. USA **101:**7270–7274.
8. **Crotta, S., A. Stilla, A. Wack, A. D'Andrea, S. Nuti, U. D'Oro, M. Mosca, F. Filliponi, R. M. Brunetto, F. Bonino, S. Abrignani, and N. M. Valiante.** 2002. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. J. Exp. Med. **195:**35–41.
9. **Deleersnyder, V., A. Pillez, C. Wychowski, K. Blight, J. Xu, Y. S. Hahn, C. M. Rice, and J. Dubuisson.** 1997. Formation of native hepatitis C virus glycoprotein complexes. J. Virol. **71:**697–704.
10. **Delos, S. E., J. M. Gilbert, and J. M. White.** 2000. The central proline of an internal viral fusion peptide serves two important roles. J. Virol. **74:**1686–1693.
11. **Drummer, H. E., A. Maerz, and P. Poumbourios.** 2003. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. FEBS Lett. **546:**385–390.
12. **Drummer, H. E., and P. Poumbourios.** 2004. Hepatitis C virus glycoprotein E2 contains a membrane-proximal heptad repeat sequence that is essential for E1E2 glycoprotein heterodimerization and viral entry. J. Biol. Chem. **279:**30066–30072.
13. **Drummer, H. E., K. A. Wilson, and P. Poumbourios.** 2002. Identification of the hepatitis C virus e2 glycoprotein binding site on the large extracellular loop of CD81. J. Virol. **76:**11143–11147.
14. **Dubuisson, J., H. H. Hsu, R. C. Cheung, H. B. Greenberg, D. G. Russell, and C. M. Rice.** 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. **68:**6147–6160.
15. **Eyal, E., R. Najmanovich, M. Edelman, and V. Sobolev.** 2003. Protein side-chain rearrangement in regions of point mutations. Proteins **50:**272–382.
16. **Flint, M., J. Dubuisson, C. Maidens, R. Harrop, G. R. Guile, P. Borrow, and J. A. McKeating.** 2000. Functional characterization of intracellular and secreted forms of a truncated hepatitis C virus E2 glycoprotein. J. Virol. **74:**702–709.
17. **Flint, M., C. Maidens, L. D. Loomis-Price, C. Shotton, J. Dubuisson, P. Monk, A. Higginbottom, S. Levy, and J. A. McKeating.** 1999. Characterization of hepatitis C virus E2 glycoprotein interaction with a putative cellular receptor, CD81. J. Virol. **73:**6235–6244.

18. **Garnier, J., D. J. Osguthorpe, and B. Robson.** 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J. Mol. Biol. **120:**97–120.
19. **Garry, R. F., and S. Dash.** 2003. Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. Virology **307:**255–265.
20. **Golden, A., D. A. Austen, M. R. van Schravendijk, B. J. Sullivan, E. S. Kawasaki, and M. S. Osburne.** 1998. Effect of promoters and signal sequences on the production of secreted HIV-1 gp120 protein in the baculovirus system. Protein Expr. Purif. **14:**8–12.
21. **He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan, and N. R. Landau.** 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G_2 phase of the cell cycle by inhibiting $p34^{cdc2}$ activity. J. Virol. **69:**6705–6711.
22. **Higginbottom, A., E. R. Quinn, C. C. Kuo, M. Flint, L. H. Wilson, E. Bianchi, A. Nicosia, P. N. Monk, J. A. McKeating, and S. Levy.** 2000. Identification of amino acid residues in CD81 critical for interaction with hepatitis C virus envelope glycoprotein E2. J. Virol. **74:**3642–3649.
23. **Hsu, M., J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C. M. Rice, and J. A. McKeating.** 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. USA **100:**7271–7276.
24. **Lavillette, D., B. Bartosch, D. Nourrisson, G. Verney, F. L. Cosset, F. Penin, and E. I. Pecheur.** 2006. Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes. J. Biol. Chem. **281:**3909–3917.
25. **Lavillette, D., A. W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A. H. Patel, J. Dubuisson, J. K. Ball, and F. L. Cosset.** 2005. Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus. Hepatology **41:**265–274.
26. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623–626.
27. **Mazzocca, A., S. C. Sciammetta, V. Carloni, L. Cosmi, F. Annunziato, T. Harada, S. Abrignani, and M. Pinzani.** 2005. Binding of hepatitis C virus envelope protein E2 to CD81 up-regulates matrix metalloproteinase-2 in human hepatic stellate cells. J. Biol. Chem. **280:**11329–11339.
28. **McKeating, J. A., L. Q. Zhang, C. Logvinoff, M. Flint, J. Zhang, J. Yu, D. Butera, D. D. Ho, L. B. Dustin, C. M. Rice, and P. Balfe.** 2004. Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner. J. Virol. **78:**8496–8505.
29. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. USA **100:**6986–6991.
30. **Oren, R., S. Takahashi, C. Doss, R. Levy, and S. Levy.** 1990. TAPA-1, the target of an antiproliferative antibody, defines a new family of transmembrane proteins. Mol. Cell. Biol. **10:**4007–4015.
31. **Owsianka, A., R. F. Clayton, L. D. Loomis-Price, J. A. McKeating, A. H. Patel, T. F. Baumert, S. Ito, D. T. Wong, and T. J. Liang.** 2001. Functional analysis of hepatitis C virus E2 glycoproteins and virus-like particles reveals structural dissimilarities between different forms of E2. J. Gen. Virol. **82:** 1877–1883.
32. **Owsianka, A., A. W. Tarr, V. S. Juttla, D. Lavillette, B. Bartosch, F. L. Cosset, J. K. Ball, and A. H. Patel.** 2005. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. J. Virol. **79:**11095–11104.
33. **Petracca, R., F. Falugi, G. Galli, N. Norais, D. Rosa, S. Campagnoli, V. Burgio, E. Di Stasio, B. Giardina, M. Houghton, S. Abrignani, and G. Grandi.** 2000. Structure-function analysis of hepatitis C virus envelope-CD81 binding. J. Virol. **74:**4824–4830.
34. **Pileri, P., Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, and S. Abrignani.** 1998. Binding of hepatitis C virus to CD81. Science **282:**938–941.
35. **Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. C. Harrison.** 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature **375:**291–298.
36. **Roccasecca, R., H. Ansuini, A. Vitelli, A. Meola, E. Scarselli, S. Acali, M. Pezzanera, B. B. Ercole, J. McKeating, A. Yagnik, A. Lahm, A. Tramontano, R. Cortese, and A. Nicosia.** 2003. Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. J. Virol. **77:**1856–1867.
37. **Rosa, D., S. Campagnoli, C. Moretto, E. Guenzi, L. Cousens, M. Chin, C. Dong, A. J. Weiner, J. Y. Lau, Q. L. Choo, D. Chien, P. Pileri, M. Houghton, and S. Abrignani.** 1996. A quantitative test to estimate neutralizing antibodies to the hepatitis C virus: cytofluorimetric assessment of envelope glycoprotein 2 binding to target cells. Proc. Natl. Acad. Sci. USA **93:**1759–1763.
38. **Scarselli, E., H. Ansuini, R. Cerino, R. M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, and A. Vitelli.** 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. **21:**5017–5025.
39. **Stites, W. E., and J. Pranata.** 1995. Empirical evaluation of the influence of side chains on the conformational entropy of the polypeptide backbone. Proteins **22:**132–140.
40. **Tscherne, D. M., C. T. Jones, M. J. Evans, B. D. Lindenbach, J. A. McKeating, and C. M. Rice.** 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J. Virol. **80:**1734–1741.
41. **Tseng, C. T., and G. R. Klimpel.** 2002. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. J. Exp. Med. **195:**43–49.
42. **Wack, A., E. Soldaini, C. Tseng, S. Nuti, G. Klimpel, S. Abrignani, C. Agrati, C. Nisii, A. Oliva, G. D'Offizi, C. Montesano, L. P. Pucillo, and F. Poccia.** 2001. Binding of the hepatitis C virus envelope protein E2 to CD81 provides a co-stimulatory signal for human T cells. Eur. J. Immunol. **31:**166–175.
43. **Yagnik, A. T., A. Lahm, A. Meola, R. M. Roccasecca, B. B. Ercole, A. Nicosia, and A. Tramontano.** 2000. A model for the hepatitis C virus envelope glycoprotein E2. Proteins **40:**355–366.
44. **Zhang, J., G. Randall, A. Higginbottom, P. Monk, C. M. Rice, and J. A. McKeating.** 2004. CD81 is required for hepatitis C virus glycoprotein-mediated viral infection. J. Virol. **78:**1448–1455.