An Optimized Hepatitis C Virus E2 Glycoprotein Core Adopts a Functional Homodimer That Efficiently Blocks Virus Entry

Kathleen McCaffrey,^{a,c,e} Irene Boo,^a Catherine M. Owczarek,^d Matthew P. Hardy,^d Matthew A. Perugini,^f Louis Fabri,^d Pierre Scotney,^d Pantelis Pountourios,^{a,b} Heidi E. Drummer^{a,b,c}

Centre for Biomedical Research, Burnet Institute, Melbourne, Australia^a; Department of Microbiology, Monash University, Clayton, Australia^b; Department of Microbiology and Immunology, The University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia^c; Research and Development, CSL Limited, Parkville, Victoria, Australia^d; Cellular Protein Chemistry, Utrecht University, Utrecht, The Netherlands^e; Department of Biochemistry and Genetics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Victoria, Australia^f

ABSTRACT The hepatitis C virus (HCV) envelope glycoprotein E2 is the major target of broadly neutralizing antibodies *in vivo* and is the focus of efforts in the rational design of a universal B cell vaccine against HCV. The E2 glycoprotein exhibits a high degree of amino acid variability which localizes to three discrete regions: hypervariable region 1 (HVR1), hypervariable region 2 (HVR2), and the intergenotypic variable region (igVR). All three variable regions contribute to immune evasion and/or isolate-specific structural variations, both important considerations for vaccine design. A high-resolution structural definition of the intact HCV envelope glycoprotein complex containing E1 and E2 remains to be elucidated, while crystallographic structures of a recombinant E2 ectodomain failed to resolve HVR1, HVR2, and a major neutralization determinant adjacent to HVR1. To obtain further information on E2, we characterized the role of all three variable regions in E2 ectodomain folding and function in the context of a recombinant ectodomain fragment (rE2). We report that removal of the variable regions accelerates binding to the major host cell receptor CD81 and that simultaneous deletion of HVR2 and the igVR is required to maintain wild-type CD81-binding characteristics. The removal of the variable regions also rescued the ability of rE2 to form a functional homodimer. We propose that the rE2 core provides novel insights into the role of the variable motifs in the higher-order assembly of the E2 ectodomain and may have implications for E1E2 structure on the virion surface.

IMPORTANCE Hepatitis C virus (HCV) infection affects ~2% of the population globally, and no vaccine is available. HCV is a highly variable virus, and understanding the presentation of key antigenic sites at the virion surface is important for the design of a universal vaccine. This study investigates the role of three surface-exposed variable regions in E2 glycoprotein folding and function in the context of a recombinant soluble ectodomain. Our data demonstrate the variable motifs modulate binding of the E2 ectodomain to the major host cell receptor CD81 and have an impact on the formation of an E2 homodimer with high-affinity binding to CD81.

KEYWORDS CD81 receptor, glycoproteins, hepatitis C virus

Hepatitis C virus (HCV) is one of the leading indicators of liver disease worldwide, and there is no vaccine available to prevent disease (1, 2). HCV is a positive-sense RNA virus and a member of the *Hepacivirus* genus of the *Flaviviridae* family that

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Address correspondence to Heidi E. Drummer, heidi.drummer@burnet.edu.au.

includes flaviviruses and pestiviruses. Due to the large degree of sequence variability of HCV, it is further categorized into seven genotypes (1 to 7) and various subtypes (a, b, c, etc.) that differ at the nucleotide level by approximately 30% and 20%, respectively (3). The HCV genome is translated as a single polyprotein that is cleaved to generate 10 viral proteins. The envelope proteins E1 (polyprotein residues 171 to 383; H77 genotype 1a numbering) and E2 (residues 384 to 746) are type I transmembrane (TM) proteins that assemble as a heterodimer during synthesis. The E1-E2 heterodimer mediates virus entry via a host cell receptor complex, including the tetraspanin CD81, scavenger receptor class B type I, and tight-junction membrane proteins claudin (claudin-1, -6, and -9) and occludin (4–8). Following clathrin-dependent endocytosis, HCV fusion is believed to occur at low pH within an endosomal compartment (9–11).

The E2 glycoprotein is a major target for broadly neutralizing antibodies and has therefore been the focus of efforts to design a prophylactic vaccine for HCV (12–15). Recombinant E2 and E1E2 have proven to be highly immunogenic; however, neutralizing antibody responses show limited breadth of neutralization (16–19). E2 exhibits the highest degree of amino acid variability encoded in the HCV genome, which localizes to three discrete variable regions. The majority of E2 variability is observed in the N-terminal-sequence hypervariable region 1 (HVR1; residues 384 to 409) (20). HVR1 is an immunodominant motif that acquires immune escape variants during the course of infection and is the major determinant of isolate-specific neutralizing antibody responses *in vivo* (21–24). Hypervariable region 2 (HVR2; residues 460 to 485) and the intergenotypic variable region (igVR; residues 570 to 580) display 20% and 0% amino acid identity between HCV genotypes, respectively, yet each retains a highly conserved N-linked glycosylation site (25). We have also recently reported that the igVR is under considerable *in vivo* immune selection pressure, by using a longitudinal analysis of glycoprotein sequence evolution in genotype 3a-infected individuals (26). In addition to amino acid variability, a high degree of structural flexibility recently reported within the variable regions as well as the conserved CD81-binding site has been predicted to account for a significant proportion of the nonneutralizing antibody response to E2 (27).

The structural arrangement of the HCV envelope complex at the virion surface and its fusion mechanism remain to be elucidated. This has proven to be a particularly challenging task due to the inherent heterogeneity of the virus, which has a nonuniform morphology and has been found to associate with (and within) lipoprotein complexes (28–30). Structural studies to date have therefore utilized a recombinant form of the E2 glycoprotein ectodomain (rE2; lacking transmembrane and membrane-proximal motifs) which can be independently expressed with the retention of CD81 and SR-B1 receptor binding function (4, 7, 31). Two high-resolution structures of the E2 ectodomain have recently reported a central immunoglobulin (Ig)-fold β -sandwich, flanked by front and back layers, that resembles domain III of flavivirus glycoprotein E but is otherwise an entirely novel structure (32, 33). These E2 “core” structures, however, were not fully glycosylated and required major modifications, including the truncation of the N-terminal HVR1 sequence as well as the substitution of a short linker for the large internal HVR2 sequence. Therefore, structural information about these key antigenic sites is still lacking.

We have previously reported the isolation of a minimal E2 “core” (Δ 123) protein via simultaneous replacement of the variable regions with short, flexible glycine-serine-serine-glycine (GSSG) linkers within the rE2 ectodomain (amino acid residues 384 to 661) which retains both native conformational and CD81 receptor binding characteristics. In this study, we undertook to further understand the significance of the E2 variable motifs in ectodomain folding and function. We confirm that the variable motifs can be removed from E2 ectodomains derived from diverse HCV isolates with the retention of wild-type CD81 binding function, suggesting that the E2 core is broadly conserved. Simultaneous replacement of HVR2 and the igVR was found to be necessary to retain wild-type CD81 binding, suggesting cooperation between these mutations in stabilizing the receptor-binding site and potentially neutralization-sensitive epitopes

FIG 1 Schematic and structural representations of the HCV glycoprotein E2 ectodomain. (A) The full-length E2 glycoprotein (polyprotein residues 384 to 746; H77 numbering) is depicted by an N-terminal ectodomain and C-terminal transmembrane domain (TMD) in dark gray. The locations of the variable regions HVR1, HVR2, and igVR are highlighted in black. The 18 conserved cysteine residues (C) and their disulfide arrangement as determined by Kong et al. is depicted by connecting lines (32). The locations of the 11 conserved N-linked glycan sites are indicated by asterisks, and known CD81-binding determinants are highlighted in pink (58, 63, 64). The color scheme of the E2 ectodomain backbone (light gray, blue, green, and orange) corresponds to structural motifs shown in panels B and C. The C-terminally truncated E2 ectodomain (rE2, residues 384 to 661) used in this study is depicted, including its six-histidine (6×HIS) epitope tag. The variable regions were deleted or replaced with short glycine-serine-serine-glycine linkers (GSSG) as previously described to isolate a minimal E2 core domain (Δ123) (25). (B) Ribbon diagram representing the three-dimensional structure of the E2 ectodomain (PDB 4MWF) lacking HVR1 and HVR2, as reported by Kong et al. (32). The central Ig-fold β-sandwich (blue) consists of two antiparallel β-sheets: an upper (dark blue) and a lower (light blue) sheet stabilized by two disulfides (yellow). An extended “CD81 binding loop” (pink) connecting upper and lower β-sheet motifs is a major CD81-binding determinant (63). Additional residues implicated in CD81 binding (pink) on the front layer of the E2 ectodomain (gray) and the back layer (green) are shown (58, 64). The predicted position of the deleted N-terminal HVR1 sequence (ΔHVR1) is shown as a black dashed line. The HVR2 sequence was replaced with a short linker (ΔHVR2 [GSSG]; black dashed line), and the igVR (black and black dashed line) is also labeled. (C) Surface representation of the E2 ectodomain structure as described for panel B.

therein. Detailed kinetic analysis further demonstrated that the removal of the variable regions accelerated E2 binding to CD81, suggesting a more accessible or optimal CD81-binding site configuration. Removal of the variable regions also unexpectedly reduced the propensity of the E2 ectodomain homodimer to misfold and aggregate and rescued high-affinity CD81 binding.

RESULTS

The functional core of the E2 ectodomain (Δ123) is conserved across diverse isolates of HCV and efficiently blocks virus entry into hepatocytes. We have previously reported that simultaneous replacements of HVR1 (residues 384 to 409), HVR2 (residues 460 to 485), and the igVR (residues 570 to 580) with short, flexible glycine-serine-serine-glycine (GSSG) linkers within the H77-derived E2 ectodomain (genotype 1a; polyprotein residues 384 to 661) does not alter native conformational characteristics or binding to the CD81 receptor (Fig. 1A, Δ123) (25). This strongly

suggests that these discrete variable regions form surface-exposed motifs on the surface of the ectodomain and are excluded from the core fold of the protein (Fig. 1C). To confirm the conservation of this $\Delta 123$ core domain across diverse isolates of HCV, we introduced the same deletions into E2 ectodomain sequences derived from Con1 (genotype 1b, residues 384 to 661) and JFH1 (genotype 2a, residues 384 to 665) isolates. Con1 and JFH1 share 78% and 65% amino acid identities with the H77-derived E2 ectodomain, respectively.

Expression, secretion, and folding of the Con1- and JFH1-derived ectodomains lacking individual (Δ HVR1, Δ HVR2, or Δ igVR [3]) or multiple ($\Delta 12$, $\Delta 13$, etc.) variable regions were characterized by transient transfection and metabolic labeling of cell monolayers prior to immunoprecipitation of the supernatant with a conformational sensitive monoclonal antibody (MAb), CBH-5 (Fig. 2A) (15, 34, 35). SDS-PAGE and radioisotope imaging showed a profile typical of the wild-type E2 ectodomain (E2wt) with a broad band between 50 and 80 kDa due to its extensive N-linked glycan modification. An SDS-resistant, yet dithiothreitol (DTT)-sensitive, dimer was also observed migrating between 100 and 120 kDa, as previously described (31). Mutants with deletion of individual or multiple variable regions migrated faster corresponding to the removal of these sequences and loss of an N-glycan within HVR2 or the igVR (3). No obvious defects in expression or assembly of the CBH-5 epitope were observed for any of the mutants, including the triple deletion construct ($\Delta 123$) representing the minimal E2 core domain, as previously observed for the H77 isolate (25).

E2 glycoprotein binding to the cell surface receptor CD81 via the large extracellular loop (LEL) is a critical determinant of HCV entry (36, 37). E2wt and $\Delta 123$ protein function was therefore determined by binding to a recombinant, dimeric form of the CD81-LEL that was previously demonstrated to interact with HCV E2 at a high affinity (38). As shown in Fig. 2B, protein supernatants representing E2wt and Δ HVR1 and $\Delta 123$ mutants from JFH1 (upper) and Con1 (lower) isolates were applied to immobilized CD81-LEL across a dilution series (right panel). As a loading control, the same lysates were applied to *Galanthus nivalis* agglutinin (GNA)-lectin-coated plates (Fig. 2B, left panel). As previously described for the H77 isolate, the Con1-derived $\Delta 123$ mutant retained wild-type levels of binding to CD81-LEL. Notably, the JFH1-derived $\Delta 123$ ectodomain exhibited an approximately 4-fold enhancement in CD81-LEL binding, as determined by a 50% effective concentration (EC_{50}) shift from a dilution factor of 0.022 (E2wt) to 0.006 ($\Delta 123$; $P < 0.0001$). Enhanced binding to CD81 has been previously reported upon deletion of HVR1 (39, 40); however, we did not observe this across any of the isolates tested, suggesting that alternative determinants confer this improved binding efficiency in this isolate (Fig. 2B, Δ HVR1).

JFH1 is the prototype isolate for the HCV infectious cell culture system (HCVcc), and therefore we validated its phenotype in the context of the full-length CD81 receptor as present on the cell surface (41–43). To do this, we undertook large-scale protein expression and nickel affinity purification of JFH1-derived E2wt and $\Delta 123$ proteins. The purified proteins were then preincubated with Huh7.5 cell monolayers prior to infection with a genotype 2a-derived (J6/JFH1) virus containing a luciferase reporter (41). In Fig. 2C, measurement of relative luciferase activity (RLU) demonstrated that, indeed, JFH1 $\Delta 123$ could efficiently inhibit virus entry with a 50% inhibitory concentration (IC_{50}) of 0.24 μ M versus an IC_{50} of 1.37 μ M for E2wt. This is in agreement with the *in vitro* CD81-LEL binding assays described above, although the IC_{50} shift was not statistically significant ($P = 0.360$).

Together these data indicate that the $\Delta 123$ protein represents a highly conserved E2 core that maintains both conformational features and CD81 binding properties of the wild-type ectodomain.

The $\Delta 123$ protein causes allosteric changes that disrupt conformational epitopes in immunogenic domain A. To further assess potential sites of conformational change caused by deletion of the variable regions, we immunoprecipitated metabolically labeled E2wt and $\Delta 123$ supernatants with a well-characterized panel of human conformation-sensitive antibodies (Fig. 3) (35). These antibodies have

FIG 2 The functional Δ 123 E2 core is conserved across diverse isolates of HCV and efficiently blocks virus entry. (A) Expression, secretion, and folding of radiolabeled Con1 (genotype 1b; residues 384 to 661) and JFH1 (genotype 2a; residues 384 to 665) E2 ectodomain constructs lacking individual (Δ HVR1, Δ HVR2, Δ gVR(3)) or multiple (Δ 12, Δ 13, Δ 23, Δ 123) variable regions as detected by immunoprecipitation with a conformation-dependent domain B-specific antibody, CBH-5. Eluted proteins were analyzed by SDS-PAGE under nonreducing (-DTT) and reducing (+DTT) conditions and by phosphorimaging. (B) Solid-phase binding assays of JFH1-derived (top) and Con1-derived (bottom) E2wt (black), Δ HVR1 (blue), and Δ 123 (red) proteins binding to recombinant, dimeric CD81-LEL (right panels) or GNA-lectin loading control (left panels). Data are representative of the mean results of three-independent experiments $\pm$ standard errors. EC_{50} s were calculated using nonlinear regression analysis, represented by a solid line, and P values for ascribing statistical significant differences to E2wt were derived using the sum-of-squares F test in GraphPad Prism v6.0. (C) Inhibition of a chimeric, genotype 2a-derived virus containing a luciferase reporter was determined by preincubation of Huh7.5 cells with increasing concentrations of JFH1-derived E2wt (black circles) or Δ 123 (red squares). (Continued on next page)

FIG 3 Isolate-specific changes in domain A epitopes are observed in the $\Delta 123$ mutant. Global antigenic profiles of Con1-derived (left panel) and JFH1-derived (right panel) E2wt (upper) and $\Delta 123$ (lower) proteins as determined by immunoprecipitation using a panel of conformation-sensitive MAbs (CBH MAbs indicated at the top of each lane) with epitopes representing three nonoverlapping domains within the E2 glycoprotein (A, B, and C), as described by Keck et al. (34, 44). CBH-17 is not conformation sensitive and immunoprecipitates only denatured E2. Eluted proteins were analyzed by SDS-PAGE under reducing conditions (+DTT) and phosphorimaging. The relative density of $\Delta 123$ protein-specific signal was calculated as a ratio to E2wt immunoprecipitated under the same conditions ($\Delta 123/\text{E2wt}$ protein) using ImageJ software.

been used to describe three conserved, nonoverlapping antigenic domains within E2, namely, A, B, and C. Domain B and C antibodies are neutralizing, and their epitopes overlap known CD81-binding determinants, whereas domain A antibodies are nonneutralizing and cannot inhibit E2-CD81 interactions (34, 44).

SDS-PAGE and radioisotope imaging demonstrated largely similar binding profiles for both domain B and C antibodies in Con1 (left)- and JFH1 (right)-derived E2wt and $\Delta 123$ ectodomains, although JFH1 $\Delta 123$ did exhibit less efficient binding to CBH-11 (Fig. 3, compare upper and lower panels). This suggests that the $\Delta 123$ mutant can assemble the CD81-binding site, as reported above, and conserved neutralization determinants therein. Binding to the domain A antibody CBH-4G was also similar for both E2wt and the $\Delta 123$ mutant; however, both Con1 and JFH1 $\Delta 123$ displayed a marked loss of binding to the CBH-4B and -4D antibodies, and this was more pronounced in the JFH1 isolate (compare upper and lower panels within the dashed rectangle), suggesting that nonneutralizing antibody binding sites may be displayed differently in the core domains of different HCV genotypes.

Together these data suggest that localized, isolate-specific conformational changes occur in domain A upon replacement of HVR2 and the igVR with GSSG linkers but that the overall fold of E2 and key neutralizing antibody epitopes are retained.

Simultaneous replacement of HVR2 and the igVR within the E2 ectodomain is required for wild-type CD81-LEL binding. A major challenge to the analysis of the role of the variable regions in E2 folding and function was the extreme heterogeneity of the secreted ectodomain. In Fig. 4A, size exclusion chromatography (SEC) profiles of affinity-purified H77-derived ectodomains lacking one or multiple variable regions

FIG 2 Legend (Continued)

squares) protein. Luciferase activity was measured as a function of virus entry and calculated as a percentage of maximum values obtained in the absence of E2-derived proteins. Data represent the mean results of three independent experiments $\pm$ standard errors. IC_{50} s were calculated using nonlinear regression analysis, represented by a solid line, and *P* values for ascribing statistical significant differences to E2wt were derived using the sum-of-squares *F* test in GraphPad Prism v6.0.

FIG 4 Simultaneous deletion of HVR2 and the igVR is necessary for wild-type CD81-LEL binding function. (A) Preparative size exclusion chromatography profiles of affinity-purified H77-derived E2wt and the individual (Δ HVR1, Δ HVR2, Δ igVR) or multiple (Δ 12, Δ 13, Δ 23, Δ 123)-variable-region deletion proteins (dashed lines). These profiles are overlaid with analytical SEC profiles of the monomer fractions isolated from the same preparations (solid lines). (B) Solid-phase binding assays of monomeric E2 lacking individual (Δ HVR1, Δ HVR2, Δ igVR) or multiple (Δ 12, Δ 13, Δ 23, Δ 123) variable regions in the upper and lower panels, respectively. Binding to CD81-LEL and GNA-lectin (loading control) are shown in the right and left panels, respectively. Data are representative results of two independent experiments $\pm$ standard deviations. EC_{50} s were calculated using nonlinear regression analysis, represented by a solid line, and P values for ascribing statistical significant differences to E2wt were derived using the sum-of-squares F test in GraphPad Prism v6.0.

display two major peaks corresponding to heavily glycosylated monomer and dimer forms as well as a high-molecular-weight shoulder species (dashed line). It has been previously reported that only the monomer form is functional (45). However, it is difficult to estimate the proportion of monomeric species of E2 within different preparations, as this varies significantly between the deletion mutants and between preparations. To conduct a higher-resolution analysis of these mutants, we further purified the monomer form to $\sim 90\%$ purity (Fig. 4A, solid line) and repeated our

analysis of recombinant CD81-LEL binding using the solid-phase binding assay as described above (Fig. 4B).

In Fig. 4B, top panel, individual deletion of HVR1, HVR2, or the igVR(3) within the monomeric E2 ectodomain demonstrated that the HVR1 deletion (Δ HVR1) does not significantly alter CD81-LEL binding compared to that of the wild-type ectodomain (E2wt), with EC_{50} s of 2.14 μ M versus 1.39 μ M, respectively ($P = 0.147$). In contrast, monomeric Δ HVR2 and Δ igVR(3) exhibited a statistically significant loss of CD81-LEL binding affinity, with EC_{50} s of 20.83 μ M and 75.62 μ M ($P < 0.0001$), suggesting impaired assembly or stability of the CD81-LEL binding site. However, in Fig. 4B, lower panel, comparison of monomeric multiple-variable-region deletions indicated that the triple deletion mutant representing the E2 core (Δ 123) exhibited wild-type levels of CD81-LEL binding, with an EC_{50} of 2.57 μ M ($P = 0.033$). Deletion of HVR1 in combination with HVR2 or the igVR (Δ 12 and Δ 13) did not appear to be able to fully account for this rescue of function, as these mutants still displayed significantly impaired binding compared to that of E2wt, at 6.70 μ M and 10.68 μ M ($P < 0.0001$), respectively. However, simultaneous deletion of HVR2 and the igVR resulted in wild-type levels of CD81-LEL binding (Δ 23, 3.18 μ M; $P = 0.0002$), and this was marginally improved upon by deletion of HVR1 (Δ 123, 2.57 μ M; $P = 0.033$).

This suggests that simultaneous replacement of HVR2 and the igVR with GSSG linkers cooperatively stabilizes the CD81-binding site within E2 despite the individual mutations resulting in reduced CD81-LEL binding function.

Monomeric Δ 123 protein exhibits altered CD81-LEL binding kinetics. Our analysis above strongly suggests that removal of the variable regions introduces subtle changes at the surface of E2 while maintaining the structural integrity of the core CD81 receptor-binding domain. To further investigate this, we decided to perform a kinetic analysis of CD81-LEL binding to the monomeric forms of E2wt and Δ 123 ectodomains representing H77, Con1, and JFH1 isolates as described above using surface plasmon resonance (SPR) techniques. Isolation of the monomer using nickel affinity chromatography and preparative SEC (Fig. 5A, top panel) was confirmed by using both analytical SEC (bottom panel) and SDS-PAGE (Fig. 5B). The purity and molecular mass were determined by both mass spectrometry and sedimentation velocity analysis (Table 1).

Monomeric E2wt and Δ 123 proteins were applied to CD81-LEL immobilized to the SPR chip across a broad concentration range. The E2 binding-incompetent CD81-LEL (F186S) mutant was immobilized to the proceeding flow cell to subtract for nonspecific interactions. Representative association curves (association rate [k_a], 250 s) and dissociation curves (dissociation rate [k_d], 600 s) are shown in Fig. 5C (upper panel). The raw data (solid lines) were fitted using a 1:1 Langmuir interaction model (dashed line) to derive affinity constants (K_D) and the kinetic parameters summarized in Table 2. As predicted from the steady-state experiments, the Δ 123 protein exhibited either equivalent (H77) or improved (Con1 and JFH1) affinity for CD81-LEL compared to that of E2wt, with K_D values of 0.94 μ M versus 0.71 μ M (H77), 0.45 μ M versus 0.75 μ M (Con1), and 0.08 μ M versus 0.29 μ M (JFH1), respectively. Notably, the genotype 2a (JFH-1) monomeric protein displayed a higher intrinsic affinity for CD81-LEL than the genotype 1a (H77) isolate.

However, closer analysis revealed that the Δ 123 protein had significantly higher association rates (k_a) across all isolates coupled with significantly higher dissociation rates (k_d) (Table 2, compare k_a and k_d values of the Δ 123 mutant with those of E2wt). The specific affinities (K_D) obtained for the Δ 123 protein therefore reflect a balance between apparently contradictory forces: an improvement in CD81-LEL binding efficiency and a reduction in the stability of this interaction. For example, the H77-derived Δ 123 mutant did not exhibit a large increase in association rate compared to that of E2wt (k_a of 4.6 versus 3 units, $P = 0.008$) and consequently equivalent K_D values are reported. In contrast, JFH1 Δ 123 displayed a 5-fold increase in association rates compared to that of E2wt (k_a values of 65 versus 13 units, $P = 0.014$) and, therefore,

FIG 5 $\Delta 123$ protein displays altered CD81-LEL binding kinetics and adopts a functional homodimer. (A) Preparative size exclusion chromatography (SEC) profiles of affinity-purified H77-, Con1-, and JFH1-derived E2wt (black) and $\Delta 123$ (gray) proteins are shown in the upper panels. Analytical SEC profiles of the peak monomer (solid lines) and dimer (dashed lines) fractions isolated from the preparative SEC profiles are shown in the lower panels. (B) SDS-PAGE analysis and Coomassie staining of individual fractions isolated from the preparative SEC in panel A representing H77-derived E2wt (left) and $\Delta 123$ (right) proteins. Proteins were separated under nonreducing (–DTT) and reducing (+DTT) conditions. The fractions corresponding to monomers, dimers, and putative high-molecular-weight (HMW) forms of E2 are indicated above each gel. Fractions corresponding to the monomer and dimer forms used for further analysis are marked by

(Continued on next page)

TABLE 1 Molecular masses of H77-derived E2wt and $\Delta 123$ fractions determined by MS and SV analyses^a

Analysis	Molecular mass (kDa) of indicated fraction			
	Monomer		Dimer	
	E2wt	$\Delta 123$	E2wt	$\Delta 123$
MS	56.1	45.3	107.7	87.8
SV ^b	53	45	104	82

^aMS, mass spectrometry; SV, sedimentation velocity.^bPartial specific volumes were experimentally determined to be 0.69 ml/g.

despite all isolates having increased dissociation rates, has an overall higher affinity for CD81-LEL than its E2wt counterpart.

Together these findings suggest that the removal of the variable regions results in a more accessible or optimal CD81-binding site configuration within the E2 ectodomain. It also reveals, however, that the $\Delta 123$ mutant generally adopts a less stable E2-CD81 complex after binding and dissociates more readily than the E2wt, potentially due to a failure to adopt a receptor-induced conformational change.

The $\Delta 123$ mutant adopts a functional homodimer with high-affinity CD81 binding. There are reports that the CD81 receptor adopts a dimeric structure at the cell surface (46, 47), and solved structures of recombinant CD81-LEL also describe a homodimer (48). However, a recently reported structure of full-length CD81 depicts a monomer in which the LEL dimerization interface is within 3.5 Å of the TM1/TM2 bundle, suggesting that the LEL homodimer is the result of lattice packing effects in the absence of the TM domains (49). However, the stoichiometry of binding between E2 and CD81 is unknown. To further investigate this, the homodimer fractions of E2wt and the $\Delta 123$ mutant were also tested for CD81-LEL binding using the SPR method described above (Fig. 5D).

In agreement with previous reports of misfolding and aggregation of the E2 ectodomain homodimer (45), the E2wt dimer displayed significantly less CD81-LEL binding than the E2wt monomer despite the application of equivalent concentrations ($\mu\text{g/ml}$) to immobilized CD81-LEL (Fig. 5C and D, compare relative response units [RUs]). In contrast, the $\Delta 123$ homodimer displayed efficient binding to CD81-LEL across all isolates (Fig. 5D, compare relative RUs for black and gray lines). This suggests that the $\Delta 123$ homodimer is less likely to form nonfunctional disulfide-linked aggregates, and indeed, we observed that E2wt exhibited more dithiothreitol (DTT)-resistant material in its dimer and high-molecular-weight (HMW) fractions than the $\Delta 123$ mutant, as analyzed by reducing SDS-PAGE (Fig. 5B, compare +DTT panels).

Assuming that the E2wt and $\Delta 123$ homodimers represent bivalent analytes, we could not correctly derive affinity constants (K_D) using SPR for direct comparison with their monomeric counterparts due to the concentration dependency of bivalent interaction kinetics. As the CD81-LEL is also a dimeric protein, we also could not obtain a value by simply inverting the experimental setup (47). However, a comparison of concentration-independent dissociation rates (k_d) (Table 3) did reveal significantly lower CD81-LEL dissociation rates for the E2 homodimer than for the monomeric protein typical of a bivalent analyte: for example, the JFH1 E2wt monomer dissociation rate (k_d) is 3.7 units (Table 2), whereas the homodimer dissociation rate is almost a magnitude lower, at 0.43 units (Table 3). Notably, the low response units obtained in

FIG 5 Legend (Continued)

an asterisk. (C) Representative surface plasmon resonance (SPR) association (k_a , 250 s) and dissociation (k_d , 600 s) profiles obtained for H77-, Con1-, and JFH1-derived E2wt (black) and $\Delta 123$ (gray) monomers binding to dimeric CD81-LEL. Raw data and fitting using a 1:1 Langmuir association model are shown by solid and dashed lines, respectively. Mean affinity constants (K_D) obtained from global fitting of the results of at least three independent assays are indicated. Statistically significant differences between E2wt and $\Delta 123$ K_D values under comparable conditions, as determined by Student's *t* test ($P < 0.05$), are shown. (D) Representative SPR association (k_a , 250 s) and dissociation (k_d , 600 s) profiles obtained for H77-, Con1-, and JFH1-derived E2wt (black) and $\Delta 123$ (gray) dimers binding to dimeric CD81-LEL. The representative curves displayed are at 100 $\mu\text{g/ml}$ (H77 and Con1) or 25 $\mu\text{g/ml}$ (JFH1).

TABLE 2 Kinetic parameters and affinity constants (K_D) of diverse E2wt and $\Delta 123$ monomer proteins binding to CD81-LEL determined using surface plasmon resonance

Parameter	Value ^a for:					
	H77c (genotype 1a)		Con1 (genotype 1b)		JFH1 (genotype 2a)	
	E2wt	$\Delta 123$ protein	E2wt	$\Delta 123$ protein	E2wt	$\Delta 123$ protein
k_{on} [$1/(M \times s)$] $\times 10^3$	3.0	4.6*	4.8	17*	13	65*
k_{off} (1/s) $\times 10^{-3}$	2.1	4.2*	3.4	7.6*	3.7	4.7*
K_D (k_{off}/k_{on}) (μM)	0.71	0.94	0.75	0.45*	0.29	0.08*
χ^2 statistic	2.7	4.2	6.9	14.8	9.8	19

^aAll values are the mean result of at least three independent assays, and asterisks indicate values that are significantly different from that for E2wt at a P value of <0.05 (Student's t test). See the text and Fig. 5C for exact P values.

this concentration range for the Con1 E2wt dimer did not allow global fitting and derivation of k_d values for this isolate.

In order to determine the stoichiometry and relative binding affinities in the absence of valency effects, we therefore performed in solution sedimentation equilibrium (SE) analysis of E2 binding to CD81-LEL. The H77-derived monomer and dimer fractions were tested in isolation or in association with dimeric CD81-LEL at two different molar ratios (Fig. 6A). Multispeed data were globally fitted to either a single-site heterogeneous association model (1-site) or a two-site heterogeneous association model (2-site) using SEDPHAT software (50, 51). The model with the global best fit and residuals and corresponding thermodynamic parameters for each of the E2 proteins are listed in Table 4.

Monomeric E2wt and $\Delta 123$ proteins both displayed a preference for the 2-site model of binding to CD81-LEL with similar primary affinity constants (K_{D1}), 6.0 μM and 8.5 μM , respectively. The 2-site model has two affinity constants but assumes equivalent secondary binding sites with a ratio of 1:4 to derive the K_{D2} values listed in Table 4. This indicates that dimeric CD81-LEL encompasses two functional E2-binding sites and exhibits the same stoichiometry of binding to both the E2wt- and $\Delta 123$ -derived monomers. In contrast, the E2wt and $\Delta 123$ homodimers favored a 1-site binding model, with K_D values of 12.4 μM and 0.4 μM , respectively. Attempts to fit a 2-site model in which the CD81-LEL dimer binds two E2 dimers or, inversely, that the E2 dimer binds two CD81-LEL dimers resulted in very large K_{D2} values that essentially reverted to 1-site binding model with similar K_{D1} values. This indicates that the CD81-LEL dimer has a 1:1 stoichiometry with the E2 homodimer, although the affinity of this interaction is significantly less for the E2wt, presumably due to a proportion of this sample being nonfunctional, as suggested by the SPR analysis above.

Our *in vitro* analysis above suggested that the $\Delta 123$ homodimer exhibits an affinity for dimeric CD81-LEL that is almost an order of magnitude higher than that of the monomeric proteins. This may reflect the engagement of two CD81-LEL determinants and therefore high avidity binding to the CD81 receptor. To validate this finding, the same fractions were tested for competitive inhibition of HCVcc entry into hepatocytes *in vivo* as described above (Fig. 6B). In Fig. 6B, top panel, the monomeric $\Delta 123$ fraction has an inhibition profile very similar to that of E2wt, as fitted by nonlinear regression analysis, with relative IC_{50} s of 0.17 μM and 1.36 μM ($P = 0.301$), respectively. In Fig. 6B,

TABLE 3 Dissociation rates (k_d) of diverse E2wt and $\Delta 123$ dimer protein binding to CD81-LEL determined using surface plasmon resonance

Parameter	Value ^a for:					
	H77c (genotype 1a)		Con1 (genotype 1b)		JFH1 (genotype 2a)	
	E2wt	$\Delta 123$ protein	E2wt	$\Delta 123$ protein	E2wt	$\Delta 123$ protein
k_d (1/s) $\times 10^{-3}$	0.8	1.1	NA	2.08	0.43	0.54
χ^2 statistic	2.5	2.5	NA	1.03	1.1	6.7

^aAll values are the mean result of at least three independent assays. NA, not applicable.

FIG 6 The $\Delta 123$ homodimer has a high affinity for CD81-LEL and can efficiently inhibit HCV entry into hepatocytes. (A) Sedimentation equilibrium (SE) profiles of E2 binding to the recombinant CD81-LEL dimer. Representative SE profiles are shown at 14,000 rpm for the E2wt monomer alone (dashed line) or binding to recombinant, dimeric CD81-LEL in solution at two different molar ratios, 1:1 (diamonds) or 3:1 (triangles). Global model fitting in SEDPHAT using a two-site heterogeneous association model is represented by a solid line (upper panel). The fitting residuals are shown in the lower panel. (B) Inhibition of a chimeric, genotype 2a-derived virus containing a luciferase reporter was determined by preincubation of Huh7.5 cells with increasing concentrations of H77-derived monomers (top panel) or dimers (bottom panel) of E2wt (black circles) or $\Delta 123$ (red squares) protein. Luciferase activity was measured as a function of virus entry and calculated as a percentage of maximum values obtained in the absence of E2-derived proteins. Data represent results of two independent experiments $\pm$ standard deviations. IC_{50} s and 95% confidence intervals were calculated using nonlinear regression analysis, represented by a solid line, and P values for ascribing statistical significant differences to E2wt were derived using the sum-of-squares F test in GraphPad Prism v6.0.

bottom panel, nonlinear regression analysis derived an ambiguous fit for the E2wt dimer and could not derive an IC_{50} within this concentration range that was consistent with its lower-affinity constant for CD81-LEL *in vitro*. In contrast, the $\Delta 123$ homodimer efficiently inhibited virus entry within this concentration range, with a relative IC_{50} of $0.27 \mu M$ and a 95% confidence interval of 0.04 to $1.67 \mu M$.

Together these data indicate that the $\Delta 123$ mutant adopts a stable homodimer that has a high affinity and avidity for CD81-LEL in solution that is not observed for the E2wt

TABLE 4 Thermodynamic parameters of H77 E2wt and $\Delta 123$ protein binding to CD81-LEL determined using sedimentation equilibrium analysis

Parameter	Value ^a for:			
	Monomers (2-site model)		Dimers (1-site model)	
	E2wt	$\Delta 123$ protein	E2wt	$\Delta 123$ protein
K_D1 (μM)	6.0	8.5	12.4	0.4
K_D2 (μM)	24.0	34.2		
Reduced χ^2	1.1	1.1	0.9	1.7
MC (95% CI) ^b	5.8–6.3	8.2–9	11.6–13.9	0.31–0.51

^aA two-site model of best fit was used for the monomers, and a one-site model of best fit was used for the dimers. The two-site model assumes an equivalent second binding site or a ratio of 1:4 to derive K_D2 .
^bMonte Carlo (MC) statistical analysis with a 95% confidence interval (CI) of global fitting using a single-site heterogeneous association model (1-site) or a two-site heterogeneous association model (2-site) as listed in SEDPHAT software.

homodimer. The $\Delta 123$ mutant also blocked virus entry with an efficiency similar to that of the monomeric ectodomain, either E2wt or the $\Delta 123$ mutant, suggesting a high affinity but not necessarily a high avidity for the CD81 receptor at the cell surface.

DISCUSSION

In agreement with our initial study, our analysis confirms that HVR1, HVR2, and the igVR can be simultaneously deleted from E2 ectodomains representing diverse isolates of HCV to yield a broadly conserved functional E2 core domain ($\Delta 123$) that efficiently blocks virus entry (25). A detailed kinetic characterization of highly purified, monomeric $\Delta 123$ protein further showed that removal of the variable regions accelerates E2 binding to CD81-LEL, suggesting that the receptor-binding site adopts either a more accessible or optimal configuration within the $\Delta 123$ protein. In the context of the virion-incorporated envelope, HVR1 has been reported to be a key determinant in the accessibility of the CD81-binding site and occlusion of these neutralization-sensitive epitopes from immune recognition (39, 40), although we failed to observe a significant increase in CD81-LEL binding upon deletion of HVR1 in the context of the recombinant E2 ectodomain. Our analysis clearly demonstrated that simultaneous replacement of HVR2 and the igVR cooperatively stabilized the CD81-binding site within E2, despite these individual mutations resulting in reduced CD81-LEL binding. We have recently reported that the ability of both a neutralizing antibody and an apparently nonneutralizing antibody to inhibit E2-CD81 binding was significantly improved when HVR2 and the igVR were deleted, with or without HVR1, suggesting that these variable regions can modulate the CD81-LEL binding site and epitopes therein (52).

Our kinetic analysis of the $\Delta 123$ protein, however, demonstrated increased dissociation rates from CD81-LEL compared to that of the wild-type E2 ectodomain across all isolates tested, indicating a seemingly contradictory loss of affinity for the receptor. Since the $\Delta 123$ protein exhibits an optimal CD81-binding site, this rather suggests that it fails to adopt a conformational change induced upon receptor ligation which helps stabilize wild-type E2-CD81 interactions. A receptor-induced conformational change within the E2 ectodomain is in agreement with reports that HCV virions require pretreatment with CD81-LEL, in addition to exposure to low pH, to initiate virus fusion and entry into target cells (53). We also observed that Con1- and JFH1-derived $\Delta 123$ ectodomains had decreased binding to two conformation-sensitive antibodies within the nonneutralizing antigenic domain A. The amino acid sequences of the deleted variable regions do not directly contribute to domain A epitopes, which locate to the back layer of E2 (residues 630 to 635) (Steven K. H. Fong, personal communication), and rather suggest that the substitution of GSSG linkers for HVR2 and/or the igVR introduces localized conformational constraints on the nonneutralizing face of E2. The more severe loss of these epitopes observed for JFH1 $\Delta 123$ may therefore be due to an additional two amino acids within both HVR2 and the igVR that are necessary for epitope assembly in this isolate. Notably, domain A antibodies display increased binding to the HCV envelope after low-pH treatment, suggesting that their epitopes are exposed during a post-receptor binding step in virus entry (54). The limited ability of the $\Delta 123$ ectodomain to adopt domain A epitopes may therefore also reflect a reduced flexibility and inability to transition to post-CD81 receptor binding states.

Unexpectedly, we observed that removal of the variable regions rescued the CD81-binding function of the E2 ectodomain dimer. The stoichiometry of the E2-CD81 interaction *in vivo* is unknown, although there is evidence that CD81 forms a dimer at the cell surface (46, 47). However, a recent structure of full-length CD81 reported a monomer and suggests that the observed dimeric structure of the isolated CD81-LEL may be nonphysiological (48, 49). Our *in vitro* analysis indicated that the E2 homodimer has a 1:1 binding stoichiometry with a recombinant, dimeric form of CD81-LEL, compared with a 2:1 ratio for the monomer, suggesting that the $\Delta 123$ homodimer has two CD81-LEL competent binding sites. It also displayed an approximately 10-fold-higher affinity for CD81-LEL than the monomeric proteins (both wild-type and $\Delta 123$ proteins),

which is likely to reflect additional high-avidity interactions with the CD81-LEL *in vitro*. High-avidity interactions at the cell surface is predicted to be beneficial for HCV entry; however, the $\Delta 123$ homodimer did not exhibit an improvement in its ability to inhibit virus entry that was equivalent to that of the $\Delta 123$ monomer (or E2wt monomer), suggesting that such bivalent interactions may not occur at the cell surface. As there is currently no high-resolution structure of the intact HCV envelope, the relevance of an E2 ectodomain homodimer *in vivo* remains unclear.

In conclusion, we propose that the $\Delta 123$ protein represents an intriguing HCV vaccine candidate, as it lacks both the HVR1 and igVR sequences which are subject to immune selection pressure during infection (23, 26) yet retains key neutralization epitopes within the CD81-binding site when coupled with deletion of HVR2. The broad conservation of the $\Delta 123$ protein across diverse isolates of HCV as well as its more accessible or optimal CD81-LEL binding site configuration in the absence of the variable loops is predicted to favor a protective response against infection. The reduced conformational flexibility of the $\Delta 123$ ectodomain proposed here may further limit the presentation of nonneutralizing epitopes, as a remarkable degree of structural flexibility recently reported within the CD81-binding site has been proposed to account for nonneutralizing antibody responses to this otherwise conserved, immunogenic face of E2 (27). The $\Delta 123$ protein may also limit the presentation of nonneutralizing domain A epitopes which are exposed at post-CD81 binding stages in virus entry and represents an antigenic cluster accounting for a substantial portion of isolate-specific antibody responses to E2 (13). We recently reported that higher-molecular-weight forms of $\Delta 123$ protein also generate more potent neutralizing antibodies *in vivo* due at least in part to the burial of nonneutralizing domain A epitopes at intermolecular interfaces (19). The $\Delta 123$ protein therefore represents a unique tool to further investigate how such immunogenic sites are presented within E2 and to evaluate the potential of recombinant E2 as a vaccine candidate.

MATERIALS AND METHODS

Plasmid constructs, cell lines, and antibodies. Recombinant E2 proteins corresponding to the truncated ectodomain, HCV polyprotein residues 384 to 661 (H77 and Con1) or 384 to 665 (JFH1), were derived from H77 genotype 1a (GenBank accession number [AF011751](#)), Con1 genotype 1b (GenBank accession number [AJ238799](#)), and JFH1 genotype 2a (GenBank accession number [AB237837](#)) isolates and introduced into pcDNA3.1 expression vector (Life Technologies) with a C-terminal six-histidine epitope tag (6 $\times$ His). To generate the $\Delta 123$ protein, HVR1 (residues 387 to 409) was deleted and HVR2 (residues 460 to 485 or 487) or igVR (residues 570 or 572 to 580 or 584) sequences were replaced with glycine-serine-serine-glycine (GSSG) linkers by overlapping extension PCR. For radioimmunoprecipitation experiments, HVR1 was replaced with a GSSG linker as previously described (25).

HEK 293T cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine. FreeStyle 293-F cells were maintained in FreeStyle 293-F expression medium (Life Technologies). The infectious HCV clone (genotype 2a chimera, J6/JFH1) and Huh7.5 cells were kind gifts from Charles Rice (41). Huh7.5 cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 0.1 mM nonessential amino acids. Human conformation-dependent monoclonal antibodies (CBH panel) were a kind gift from Steven Fong (34, 35). Mouse conformation-dependent monoclonal antibody H53 was a kind gift from Jean Dubuisson and Harry Greenberg (55, 56). The rabbit polyclonal antibody against the polyhistidine epitope tag (anti-6 $\times$ His) is commercially available from Rockland Immunochemicals, Inc.

Radioimmunoprecipitation. Plasmids encoding the truncated E2 ectodomain lacking individual or multiple variable regions were transiently transfected into HEK 293T cells using FuGENE 6 (Roche) according to the manufacturer's instructions. At 24 h posttransfection, cells were cultured in cysteine- and methionine-deficient medium (MP Biomedicals) prior to labeling with Trans³⁵S-label (ICN Pharmaceuticals) for 1 h. Cells were then transferred into complete medium for 6 h before harvesting and clarification of the cell culture medium containing secreted protein. Proteins were immunoprecipitated using the relevant antibodies and protein-G-Sepharose beads and eluted in sample buffer prior to analysis by SDS-PAGE and radioisotope imaging.

Steady-state CD81-LEL interaction assays. The expression and purification of a dimeric chimera composed of maltose-binding protein (MBP) linked to the CD81 large extracellular loop (LEL) between residues 113 and 201 have been previously described and extensively used to characterize E2-CD81 and CD81-claudin interactions (38, 46, 57, 58). For steady-state binding assays, 5 μ g/ml of dimeric CD81-LEL was immobilized onto 96-well immunosorbent plates (Nunc Maxisorp; Denmark) and E2 was applied to the plates at serial dilutions. Bound E2 was detected using anti-6 $\times$ His antibody and anti-rabbit IgG conjugated to horseradish peroxidase (HRP) antibody (Dako, Denmark) and developed with tetramethylbenzidine substrate (TMB; Sigma) according to the manufacturer's instructions. For direct binding

assays, 96-well plates were coated at 5 $\mu\text{g/ml}$ with *Galanthus nivalis* agglutinin (GNA lectin; Sigma), followed by E2 proteins. Nonlinear regression analysis was performed using Prism v6.0 software (GraphPad Software, Inc.).

Inhibition of virus entry. HCV RNA was *in vitro* transcribed from plasmid DNA carrying the infectious J6/JFH1 genotype 2a chimeric genome containing a *Gaussia* luciferase gene as previously described (41). RNA was transfected into Huh7.5 cell monolayers using DMRIE-C (Invitrogen) according to the manufacturer's instructions. HCV-containing supernatants were harvested after 72 h, and infectious titers were determined by a limiting dilution assay. Huh7.5 monolayers seeded in 48-well tissue culture dishes were preincubated with different concentrations of purified E2-derived proteins on ice for 15 min. HCV was then applied to cells, incubated at 37°C for 4 h, and then replaced with cell culture medium for an additional 72 h. Virus containing a mutation in HCV polymerase NS5B was included as a negative control. Clarified supernatants were then measured for luciferase activity using a Steady-Glo luciferase kit (Promega) and a FLUOstar microplate reader (BMG Labtech) fitted with luminescence optics according to the manufacturer's instructions. Nonlinear regression analysis was performed using Prism v6.0 software (GraphPad Software, Inc.).

Protein purification and size exclusion chromatography. Transient transfections of expression plasmids using FreeStyle 293-F cells were performed using 293fectin transfection reagent (Life Technologies) according to the manufacturer's instructions. The cell culture supernatants were harvested by centrifugation at 2,500 rpm and were passed through a 0.45- μm filter (Nalgene) prior to purification by nickel affinity chromatography (Ni-NTA; Qiagen). The protein was then further purified using ion-exchange chromatography and dialyzed into phosphate-buffered saline (PBS; 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , 137 mM NaCl, 2.7 mM KCl, pH 7.4). To isolate distinct oligomers of E2wt or $\Delta 123$ protein, the purified protein was subject to high-resolution size exclusion chromatography using a preparative-scale 21.5- by 600-mm TSKgel 3000SW column (Tosoh Bioscience) and an AKTA Explorer fast protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences) to collect 2-ml fractions. Analytical size exclusion chromatography was performed using a 4.8- by 300-mm TSKgel 3000SWXL column (Tosoh Bioscience) using an Alliance high-performance liquid chromatograph (HPLC; Waters Corporation). Protein concentrations were determined using a MicroBCA protein quantification kit (ThermoScientific) according to the manufacturer's instructions and analyzed by SDS-PAGE and Coomassie staining techniques. The monomeric and dimeric E2wt or $\Delta 123$ proteins were further purified using ZipTip (Millipore) for native molecular mass determination using a matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometer (Bruker). The stabilities of the monomer and dimer fractions were tested at room temperature across 72 h to confirm that they were highly stable, as no dissociation or assembly/aggregation was observed for either (data not shown).

SPR. Surface plasmon resonance (SPR) experiments were performed using a Biacore 2000 (GE Healthcare) system. Purified, dimeric CD81-LEL (described above) was buffer exchanged into 10 mM sodium acetate, pH 4.2, for immobilization onto a CM5 chip via amine coupling. For all experiments, the E2 binding-incompetent CD81-LEL F186S was immobilized to the proceeding flow cell to allow background subtraction (38). E2-derived analytes were injected at a broad range of concentrations for 250 s to obtain an association rate (k_a) and allowed to dissociate for 600 s to obtain a dissociation rate (k_d). Kinetic experiments were performed at a flow rate of 10 $\mu\text{l/min}$ in HEPES buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.05% Tween 20, pH 7.4), and the surface was regenerated by two 15- μl pulses of 100 mM phosphoric acid at a flow rate of 100 $\mu\text{l/min}$. The SPR data were subjected to global fitting using BIAevaluation software (GE Healthcare) with a 1:1 Langmuir association model. Experiments were performed at least three times to obtain average kinetic parameters, and statistical significance between comparative conditions (P value < 0.05) was calculated using Student's t test.

Analytical ultracentrifugation. Experiments were performed with a Beckman Coulter XL-I analytical ultracentrifuge using the absorbance optics and standard two-sector quartz window cells. The partial specific volume of the glycoproteins was experimentally determined using sedimentation equilibrium (SE) experiments and the "single species of interacting system" model in SEDPHAT to be 0.69 ml/g and was in agreement with theoretical formulas for glycoprotein density using mass spectrometry (59–61). Sedimentation velocity (SV) experiments were performed at 40,000 rpm at 20°C. Intensity data were collected in a continuous mode at 235 nm at a step size of 0.003 cm without averaging, converted to pseudoabsorbance data, and modeled using two-dimensional spectrum analysis (2DSA) by employing Ultrascan software (62). SE experiments were performed at molar ratios of 1:1 or 3:1 of recombinant, dimeric CD81-LEL (described above) to the relevant E2 proteins. Absorbance data were collected in step mode at 4-h intervals at a wavelength of 260 nm using a step size of 0.001 cm and 10 averages until sedimentation equilibrium was attained ($t = 24$ to 28 h). Following data collection at 8,000 rpm and 14,000 rpm, baseline offsets were determined by high-speed (50,000-rpm) depletion. Data were then globally fitted to either a "single-site heterogeneous association model" (1-site) or a "two-site heterogeneous association model" (2-site) using SEDPHAT software to obtain thermodynamic parameters of the E2-CD81 interaction (50, 51). The models representing the global best fit as determined by the reduced chi-square (χ^2) value were each subject to Monte Carlo (MC) statistical evaluation with a 95% confidence interval (CI) using SEDPHAT software.

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