Structure-Based Design of Hepatitis C Virus E2 Glycoprotein Improves Serum Binding and Cross-Neutralization

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ABSTRACT An effective vaccine for hepatitis C virus (HCV) is a major unmet need, and it requires an antigen that elicits immune responses to key conserved epitopes. Based on structures of antibodies targeting HCV envelope glycoprotein E2, we designed immunogens to modulate the structure and dynamics of E2 and favor induction of broadly neutralizing antibodies (bNAbs) in the context of a vaccine. These designs include a point mutation in a key conserved antigenic site to stabilize its conformation, as well as redesigns of an immunogenic region to add a new N-glycosylation site and mask it from antibody binding. Designs were experimentally characterized for binding to a panel of human monoclonal antibodies (HMAbs) and the coreceptor CD81 to confirm preservation of epitope structure and preferred antigenicity profile. Selected E2 designs were tested for immunogenicity in mice, with and without hypervariable region 1, which is an immunogenic region associated with viral escape. One of these designs showed improvement in polyclonal immune serum binding to HCV pseudoparticles and neutralization of isolates associated with antibody resistance. These results indicate that antigen optimization through structure-based design of the envelope glycoproteins is a promising route to an effective vaccine for HCV.

IMPORTANCE Hepatitis C virus infects approximately 1% of the world’s population, and no vaccine is currently available. Due to the high variability of HCV and its ability to actively escape the immune response, a goal of HCV vaccine design is to induce neutralizing antibodies that target conserved epitopes. Here, we performed structure-based design of several epitopes of the HCV E2 envelope glycoprotein to engineer its antigenic properties. Designs were tested in vitro and in vivo, demonstrating alteration of the E2 antigenic profile in several cases, and one design led to improvement of cross-neutralization of heterologous viruses. This represents a proof of concept that rational engineering of HCV envelope glycoproteins can be used to modulate E2 antigenicity and optimize a vaccine for this challenging viral target.

KEYWORDS hepatitis C virus, structure-based design, antigenicity, human monoclonal antibodies, immunogenicity, vaccine

Hepatitis C virus (HCV) infection is a major global disease burden, with 71 million individuals, or approximately 1% of the global population, chronically infected worldwide, and 1.75 million new infections per year (1). Chronic HCV infection can lead to cirrhosis and hepatocellular carcinoma, the leading cause of liver cancer, and in the
United States HCV was found to surpass HIV and 59 other infectious conditions as a cause of death (2). While the development of direct-acting antivirals has improved treatment options considerably, several factors impede the effective use of antiviral treatment, such as the high cost of antivirals, viral resistance, occurrence of reinfections after treatment cessation, and lack of awareness of infection in many individuals since HCV infection is considered a silent epidemic. Therefore, development of an effective preventative vaccine for HCV is necessary to reduce the burden of infection and transmission and to achieve global elimination of HCV (3).

Despite decades of research resulting in several HCV vaccine candidates tested in vivo and in clinical trials (4, 5), no approved HCV vaccine is available. There are a number of barriers to the development of an effective HCV vaccine, including the high mutation rate of the virus, which leads to viral quasispecies in individuals and permits active evasion of T cell and B cell responses (6). Escape from the antibody response by HCV includes mutations in the envelope glycoproteins, as observed in vivo in humanized mice (7), studies in chimpanzee models (8), and through analysis of viral isolates from human chronic infections (9). This was also clearly demonstrated during clinical trials of a monoclonal antibody, HCV1, which, in spite of its targeting a conserved epitope on the viral envelope, failed to eliminate the virus as viral variants with epitope mutations emerged under immune pressure and dominated the rebounding viral populations in all treated individuals (10, 11).

There have been a number of successful structure-based vaccine designs for variable viruses such as influenza virus (12, 13), HIV (14, 15), and respiratory syncytial virus (RSV) (16, 17) using rationally designed immunogens that optimize presentation of key conserved epitopes, mask sites using N-glycans, or stabilize conformations or assembly of the envelope glycoproteins. Recent studies have reported use of several of these strategies in the context of HCV glycoproteins, including removal or modification of N-glycans to improve epitope accessibility (18, 19), removal of hypervariable regions (HVRs) (18, 20, 21), or presentation of key conserved epitopes on scaffolds (22, 23). However, such studies have been relatively limited compared with those with other viruses in terms of design strategies employed and number of designs tested, and immunogenicity studies have not shown convincing improvement of glycoprotein designs over native glycoproteins in terms of neutralization potency or breadth (18, 21), with the possible exception of an HVR-deleted high-molecular-weight form of the E2 glycoprotein that was tested in guinea pigs (20).

Here, we report the generation, characterization, and in vivo immunogenicity of novel structure-based designs of the HCV E2 glycoprotein, which is the primary target of the antibody response to HCV and a major vaccine target. Designs were focused on antigenic domain D, which is a key region of E2 targeted by broadly neutralizing antibodies (bNAb) that are resistant to viral escape (24), as well as antigenic domain A, which is targeted by nonneutralizing antibodies (25, 26). Based on the intrinsic flexibility of the neutralizing face of E2 (27), which includes antigenic domain D, and on the locations of bNAb epitopes to this domain (24), we identified a structure-based design substitution to reduce the mobility of that region and preferentially form a bNAb-bound conformation. We also tested several substitutions to hyperglycosylate and mask antigenic domain A located in a unique region on the back layer of E2, as determined by fine epitope mapping (28), which represents an approach that has been applied to mask epitopes in influenza virus (29) and HIV (30) glycoproteins. Designs were tested for antigenicity using a panel of monoclonal antibodies (MAbs), and selected designs were tested individually and in combinations for in vivo immunogenicity. Assessment of immune serum revealed that certain E2 designs yielded improvements in serum binding to recombinant HCV particles, as well as viral cross-neutralization, while maintaining serum binding to soluble E2 glycoprotein and key epitopes. This provides a proof of concept that rational design of HCV glycoproteins can lead to improvements in immunogenicity and neutralization breadth.
RESULTS

Structure-based design of E2. We utilized two approaches to design variants of the E2 glycoprotein to improve its antigenicity and immunogenicity (Fig. 1). For one approach, we used the previously reported structure of the affinity-matured bNAb HC84.26.5D bound to its epitope from E2 antigenic domain D (31) (PDB code 4Z0X), which shows the same epitope conformation observed in the context of other domain D human monoclonal antibodies (HMAbs) targeting this site (32). Analysis of this epitope structure for potential proline residue substitutions to stabilize its HMAb-bound conformation identified several candidate sites (Fig. 1A and Table 1). We selected one of these substitutions, H445P, which is adjacent to core contact residues for domain D located at amino acids (aa) 442 to 443 (32), for subsequent experimental characterization due to its position in a region with no secondary structure and its location between residues Y443 and K446, both of which make key antibody contacts in domain D antibody complex structures (31, 32). This also represents a region of the epitope that is distinct from a substitution that we previously described and tested (A439P) (28).

Another design approach, hyperglycosylation, was utilized to mask antigenic domain A, which is an immunogenic region on the back layer of E2 associated with nonneutralizing antibodies (25, 26, 28). Other antibodies with some binding determi-
nants mapped to this region, including HMAbs AR1A and HEPC46, exhibit limited or weak neutralization. NXS (asparagine-X-serine) and NXT (asparagine-X-threonine) N-glycan sequon substitutions were modeled in Rosetta at solvent-exposed E2 positions in antigenic domain A (Fig. 1B and Table 2), followed by visual inspection of the modeled E2 mutant structures to confirm exposure of the mutant asparagine residues. This analysis suggested that designs with N-glycans at residues 627 (F627N to V629T), 628 (K628N to R630S), 630 (R630N to Y632T), and 632 (Y632N to G634S) warranted further investigation for effects on antigenicity.

**Initial screening of mutant antigenicity using ELISA.** We first screened the structure-based designs described above to assess their effects on E2 glycoprotein antigenicity, to confirm that designs preserved the structure of key E2 epitopes, and to disrupt nonneutralizing antigenic domain A HMAb binding in the case of the N-glycan designs. These designs were cloned in E1E2 and assessed using enzyme-linked immunosorbent assays (ELISA) with a panel of representative HMAbs to antigenic domains A (Fig. 2). Only two HMAb concentrations were tested in this assay in order to detect major disruptions to HMAb binding, or lack thereof, rather than quantitative measurements. The results indicate that mutant H445P maintained approximately wild-type levels of binding to antibodies, while truncations of HVR1 had various effects. Binding of domain E HMAb HC33.4 and, to a lesser extent, HC33.1 was negatively affected by truncation of all of HVR1 (residues 384 to 410 removed; referred to here as ΔHVR1411), whereas a more limited HVR1 truncation (residues 384 to 407 removed; referred to here as ΔHVR1) largely restored binding of these bNAbs. The design of ΔHVR1 was based on the observation that residue 408 located within HVR1 affected the binding of HC33.4 but not HC33.1 (34). Likewise, designed N-glycan substitutions showed various effects on antigenicity, with pronounced reduction of binding for several bNAbs for F627NT.

**TABLE 1** Backbone structure and proline mutant analysis of antigenic domain D residues

| Residue no. | Wild-type residue | Backbone angle (°) | Backbone analysis | Rosetta proline ΔΔG^b |
|-------------|-------------------|--------------------|-------------------|-----------------------|
|             |                   | Φ                  | Ψ                 | Pre-Pro               |                       |
| 437         | Trp               | −73.66             | −18.28            | Yes                   | No                    | 0.5                   |
| 438         | Leu               | −59.18             | −59.67            | Yes                   | No                    | 0.3                   |
| 439         | Ala                | −59.14             | −37.12            | Yes                   | Yes                   | 0                     |
| 440         | Gly                | −56.62             | −26.31            | Yes                   | Yes                   | 0.1                   |
| 441         | Leu               | −68.3              | −40.87            | Yes                   | Yes                   | 2.1                   |
| 442         | Phe                | −80.55             | −40.18            | Yes                   | Yes                   | 2.7                   |
| 443         | Tyr                | −159.11            | 143.78            | No                    | Yes                   | 2.5                   |
| 444         | Gln                | −110.46            | 128.63            | No                    | Yes                   | 2.2                   |
| 445         | His                | −58.15             | 153.22            | Yes                   | Yes                   | 0                     |

^aValues and proline backbone analysis were obtained from the Ramachandran plot analysis web server (https://zlab.umassmed.edu/bu/rama/) (54). Pre-Pro assessments correspond to preproline Ramachandran plot conformation for the backbone of the preceding residue. Unfavorable Pro or preproline conformations are noted in boldface.

^bPredicted binding energy change for proline epitope mutant to the HC84.26.5D HMAb, based on the X-ray structure of the complex (PDB code 4Z0X) and computational mutagenesis in Rosetta (55). Predicted destabilizing values (>0.5 in Rosetta energy units) are indicated in boldface.

**TABLE 2** Calculated surface accessibility of E2 residues in antigenic domain A

| Residue no. | Amino acid | Side chain ASA^a |
|-------------|------------|------------------|
| 627         | Phe        | 62.7             |
| 628         | Lys        | 89.4             |
| 629         | Val        | 38.4             |
| 630         | Arg        | 60.4             |
| 631         | Met        | 14               |
| 632         | Tyr        | 62.3             |
| 633         | Val        | 1.5              |

^aAccessible surface areas (ASA) were calculated using the X-ray structure of H77 E2 core (PDB code 4MWF) and the NACCESS program (57) with default parameters. Values reflect relative side chain surface accessibility (normalized to 100).
(F627N and V629T) and R630NT (R630N and Y632T), while K628NS (K628N and R630S) did not exhibit ablation of domain A antibody binding. In contrast, Y632NS (Y632N and G634S) disrupted binding for both tested domain A HMAbs, with limited loss of binding for other HMAbs. Based on this antigenic characterization, designs H445P, ΔHVR1, and Y632NS were selected for further testing.

**Biophysical and antigenic characterization of E2 designs.** The two candidate structure-based E2 designs H445P and Y632NS, as well as ΔHVR1, were expressed and purified as monomeric soluble E2 (sE2) glycoproteins and tested for thermostability and binding affinity to a panel of HMAbs, as well as the CD81 receptor (Table 3). Pairwise combinations of these designs and a design with all three modifications, here termed "triple," were also expressed and tested. As noted previously by others (27), wild-type sE2 was found to exhibit high thermostability (melting temperature \( T_m \) of 84.5°C) (Table 3). All designs likewise showed high thermostability, with only minor reductions in \( T_m \), with the exception of the combined triple design, which had the lowest measured thermostability among the tested E2 mutants \( (T_m = 76.5°C) \).

**TABLE 3** Antigenic and biophysical characterization of E2 designs

| sE2 constructa | \( T_m \) (°C)b | Antibody/receptor binding \( K_D \) (nM)c |
|----------------|----------------|------------------------------------------|
|                | Domain A       | Domain B       | Domain D       | Domain E       | Receptor     |
| sE2 wild-type  | 84.5           | 8.4            | 15             | 1.8            | 5.1          | 4.3          | 30           | 42           | 9.5          | 22          |
| H445P          | 83.1           | 14             | 15             | 1.1            | 4.6          | 0.4          | 31           | 70           | 8.0          | 23          |
| Y632NS         | 81.4           | 37             | 180            | 1.2            | 4.6          | 4.6          | 83           | 26           | 6.5          | 31          |
| ΔHVR1          | 84.5           | 5.8            | 20             | 1.5            | 7.0          | 3.1          | 60           | 42           | 7.9          | 15          |
| ΔHVR1-H445P    | 82.9           | 11             | 15             | 1.5            | 2.3          | 2.4          | 51           | 46           | 7.6          | 10          |
| ΔHVR1-Y632NS   | 80.0           | 7.7            | 140            | 1.8            | 9.0          | 2.2          | 110          | 50           | 6.7          | 140         |
| Triple         | 76.5           | 22             | 110            | 1.4            | 4.6          | 2.8          | 58           | 27           | 9.3          | 40          |

a sE2 wild-type corresponds to residues 384 to 661 of H77C E2, and listed designs represent point mutants or truncations of that sequence. Y632NS is an abbreviation for the double mutant Y632N to G634S, and ΔHVR1 denotes deletion of most of the HVR1 sequence at the N terminus of E2, with the resultant construct containing residues 408 to 661. Triple, combination of the ΔHVR1, H445P, and Y632NS designs.

b \( T_m \) values were measured by differential scanning calorimetry.

c \( K_D \) values were measured by Octet biolayer interferometry. Antibodies are classified by their mapping to antigenic domains A, B, D, and E on E2 (28). Values in bold denote \( K_D \) changes of more than 5-fold versus the wild-type E2 level for that antibody.

**FIG 2** Antigenic characterization of E2 designs using ELISA. Designs were cloned and expressed in the context of E1E2 as previously described (28) and tested for binding to a panel of HMAbs that target E2 antigenic domain A (CBH-4G and CBH-4B), B (HC-1), C (CBH-7), D (HC84.20, HC84.24, and HC84.26), and E (HC33.1 and HC33.4), at concentrations of 1 µg/ml and 5 µg/ml. Binding was tested to wild-type H77C E1E2 and compared with levels for the designs ΔHVR1 (E2 residues 384 to 407 deleted), ΔHVR1-411 (E2 residues 384 to 410 deleted), H445P, F627NT (F627N and V629T), R630NT (R630N and Y632T), K628NS (K628N and R630S), and Y632NS (Y632N and G634S). Asterisks denote designs that were tested in the context of ΔHVR1-411 (E2 residues 384 to 410 deleted) rather than full-length E1E2.
To assess antigenicity of glycoprotein designs, solution binding affinity measurements were performed with Octet using HMAbs that target E2 antigenic domains A, B, D, and E, with two antibodies per domain, as well as the receptor CD81 (Table 3). These antibodies have been previously characterized using multiple global alanine scanning studies (28, 35) (CBH-4G, CBH-4D, HC33.1, AR3A, and HC33.1) and X-ray structural characterization studies (AR3A, HEPC74, HC84.1, HC33.1, and HCV1) (32, 36–39). The HC84.26.WH.5DL antibody is an affinity-matured clone of the parental HC84.26 antibody with improved affinity and neutralization breadth over that of the parental antibody (31). The binding site of CD81 has been mapped to E2 residues in antigenic domains B, D, and E (35); thus, CD81 binding provides additional assessment of antigenicity of that E2 supersite (6). Binding experiments with this panel showed nanomolar binding affinities to wild-type sE2 which were largely maintained for sE2 designs. A 10-fold increase in binding affinity of sE2 design H445P for the domain D HMAb HC84.26.WH.5DL was observed, showing that this design, located within antigenic domain D, not only maintained affinity but also improved engagement in that case; a steady-state binding fit for that interaction is shown in Fig. 3A. However, this effect was not observed for combinations of designs including H445P, suggesting possible interplay between designed sites. As expected, domain A hyperglycosylation designs Y632NS, ΔHVR1-Y632NS, and triple (ΔHVR1-H445P-Y632NS) showed loss of binding (>5-fold for each) to the antigenic domain A HMAb CBH-4G (Y632N-CBH-4G binding measurement is shown in Fig. 3B) though we did not observe disruption of binding to CBH-4D. Additionally, design ΔHVR1-Y632NS showed moderate (6-fold) loss of CD81 binding, which was not the case for other designs. As domain A HMAbs have distinct, albeit similar, binding determinants on E2 (28), differential effects on domain A antibody binding by Y632NS variants reflect likely differences in HMAb docking footprints on E2. Measurements of glycan occupancy at residue 632 using mass spectroscopy showed partial levels of glycosylation at that site for Y632NS and combinations (Table 4), which may be responsible for incomplete binding ablation to the tested antigenic domain A HMAbs. As alanine substitution at Y632 was previously found to disrupt binding of domain A antibodies (28), it is

### TABLE 4 Percentage of occupancy for engineered N-glycan at position 632, determined by mass spectrometry

| Design        | % Aglycosylation | Endo F efficiency (%) |
|---------------|------------------|------------------------|
| Y632NS        | 51               | 96                     |
| ΔHVR1-Y632NS  | 44               | 97                     |
| Triple<sup>a</sup> | 22               | 100                    |

<sup>a</sup>Combination of ΔHVR1, H445P, and Y632NS.
possible that the Y632N amino acid substitution in the Y632NS mutant may be responsible, in addition to partial N-glycosylation, for effects on domain A antibody binding. Regardless, these results suggest at least partial binding disruption and N-glycan masking of this region, supporting testing of these designs as immunogens in vivo.

**In vivo immunogenicity of E2 designs.** Following confirmation of antigenicity, E2 designs were tested in vivo for immunogenicity to assess elicitation of antibodies that demonstrate potency and neutralization breadth. CD1 mice (6 per group) were immunized with H77C sE2 and designs, with priming at day 0 followed by three biweekly boosts. Serum samples were obtained at day 56 after initial injection (2 weeks after the final boost) and tested for binding to H77C sE2 and key conserved epitopes (AS412/domain E and AS434/domain D) (Fig. 4). Peptide epitopes were confirmed for expected monoclonal antibody specificity using ELISAs (Fig. 4B). Endpoint titers demonstrated that sera from mice immunized with E2 designs maintained recognition of sE2 and tested epitopes. Intragroup variability resulted in a lack of statistically significant differences in serum binding levels between immunized groups; however, mean titers from the ΔHVR1 group were moderately lower than those of the wild-type sE2 group, and other mutants yielded moderately higher levels of serum binding to the tested epitopes. Notably, design H445P elicited antibodies that robustly cross-reacted with the wild-type AS434/domain D epitope. To assess differential binding to conformational epitopes on E2, serum binding competition with selected HMAbs was performed (Fig. 5). The observation of competition in the majority of antisera suggests that the antibodies elicited to domain D are to native conformational epitopes although there were no major differences between immunized groups. Likewise, no substantial differences in serum competition for binding to antigenic domains A or B were detected among immunized groups.

**Serum binding to HCV E1E2 and HCVpp.** For further analysis of immune serum binding, we tested binding to purified recombinant H77C E1E2 and HCV pseudoparticles (HCVpp) from H77C and two heterologous genotypes (Fig. 6 and Table 5). While
binding to H77 E1E2 resembled binding to H77 sE2, with no apparent difference between immunized groups, we observed notable differences in binding to HCVpp representing H77C, UKNP1.18.1, and J6 for H445P-immunized mice versus that in mice immunized with wild-type sE2. The difference between J6 HCVpp binding from H445P-immunized mice versus that from sE2-immunized mice was highly significant (P < 0.0001, Kruskal-Wallis test). To confirm this difference in HCVpp binding between sE2-and H445P-immunized groups, given the relatively low levels of overall titers, H77C HCVpp were concentrated and tested in ELISAs for binding to pooled sera from sE2- and H445P-immunized mice. This confirmed differences between immunized groups for sera from day 56, as well as day 42, which corresponds to three, rather than four, immunizations (Fig. 7). To demonstrate native-like E2 and E1E2 assembly of the HCVpp in the context of the ELISA, concentrated HCVpp showed binding to monoclonal antibodies that target linear and conformational epitopes on E2 (HCV1, HC84.26.WH.5DL, and AR3A) and conformational epitopes on E1E2 (AR4A and AR5A) and did not interact with a negative-control antibody (CA45) (Fig. 8). The molecular basis for the differential serum reactivity when HCVpp were used instead of purified recombinant E1E2 and E1E2 in ELISAs is unclear, particularly given that sE2 was used as an immunogen, yet these results collectively provide evidence that H445P may improve targeting of conserved glycoprotein epitopes on the intact HCV virion.

Homologous and heterologous serum neutralization. To assess effects of antibody neutralization potency and breadth from the E2 designs, we tested serum

**FIG 5** Serum binding competition with monoclonal antibodies. Serum inhibition of binding by biotinylated monoclonal antibodies at a concentration of 1 μg/ml was tested at the serum dilutions shown, using ELISAs. The monoclonal antibodies tested for serum competition target E2 antigenic domains A (CBH-4G), B (HC-1), and D (HC84.26).
neutralization of HCVpp representing homologous H77C and six heterologous isolates (Fig. 9). The heterologous isolates collectively diverge substantially in sequence from H77C and represent neutralization phenotypes ranging from moderately to highly resistant (Table 5), with the latter group represented by three of the most resistant tested HCVpp from a previous study that performed characterization with a panel of neutralizing monoclonal antibodies (40) (UKNP2.4.1, UKNP4.1.1, and UKNP1.18.1). As we
found previously with immunization of H77C-based sE2 (19), there was relatively large intragroup variability in neutralization of H77C, and no statistically significant differences between groups were observed. However, 50% inhibitory dilution (ID$_{50}$) values for individual mice varied less within immunized groups for heterologous isolates. Comparison between groups immunized with sE2 designs and wild-type sE2 showed significantly higher neutralization in some cases. Notably, two resistant isolates had increased neutralization for H445P immune sera versus wild-type sE2 immune sera (UKNP1.18.1, J6).

### Analysis of correlates of immunogenicity and antigenicity

Based on our *in vitro* and *in vivo* measurements, we assessed correlations between serum neutralization of different genotypes, serum antigen binding, and antigenicity (Fig. 10). First, we performed correlations between immunogenicity measurements for individual murine serum samples, corresponding to 42 points per data set. Measurements of HCVpp serum binding were not included in this analysis due to low and unquantifiable binding measurements for multiple mice for those assays (Fig. 6B to D). Top correlations between immunogenicity measurements (Fig. 10A) include serum binding values (50% effective concentration [EC$_{50}$]) to sE2 versus that to E1E2 ($r$ = 0.84), J6 neutralization (ID$_{50}$) versus UKNP1.18.1 neutralization ($r$ = 0.66), and UKNP2.4.1 neutralization versus UKNP1.18.1 neutralization ($r$ = 0.51), all of which were highly significant ($P$ $<$ 0.001). The last two correlations highlight shared patterns of neutralization of HCVpp with resistant phenotypes; a plot of UKNP2.4.1 HCVpp ID$_{50}$ values versus those for UKNP1.18.1 HCVpp ID$_{50}$ values is shown in Fig. 10B.

To assess possible associations between antigenicity and immunogenicity, we calculated correlations between measured binding affinity values for HMAbs and group immunogenicity measurements (endpoint titer or HCVpp ID$_{50}$). Such analysis has been

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**TABLE 5 Panel of viral isolates used in neutralization assays**

| Isolate | Genotype | Neutralization phenotype$^a$ | Neutralization resistance rank$^a$ | % identity with:$^b$
|---------|----------|------------------------------|-------------------------------|------------------|
| H77     | 1a       | Moderate                     | 22                            | 100 100          |
| UKNP1.18.1 | 1b   | Resistant                    | 3                             | 79 82            |
| UKNP1.20.3 | 1b   | Moderate                     | 32                            | 79 82            |
| UKNP2.4.1 | 2a   | Resistant                    | 1                             | 72 74            |
| UKNP2.1.2 | 2i   | Moderate                     | 23                            | 70 73            |
| UKNP4.1.1 | 4a   | Resistant                    | 2                             | 71 75            |
| J6      | 2a       | Resistant                    | 11                            | 71 74            |

$^a$Neutralization phenotype and neutralization resistance rank were based on assessment of 78 HCVpp with a panel of monoclonal antibodies by Urbanowicz et al. (40).

$^b$Percent amino acid sequence identity with H77C sE2 (aa 384 to 661) and ΔHVR1 (aa 408 to 661).
used by other investigators with other viral antigen designs to examine antigenic properties associated with immunogenicity (16). Top correlations based on significance ($P$ value) are shown in Fig. 10C. As with the individual mouse correlation analysis noted above, HCVpp endpoint titers were excluded from this analysis due to insignificant

![HCVpp binding graph](image)

**Fig. 8** Binding of concentrated HCV pseudoparticles (HCVpp), pseudotyped with H77C E1E2, to monoclonal antibodies. Binding measurements were performed using ELISAs with antibodies targeting E2 (HCV1, HC84.26.WH.5DL, and AR3A), E1E2 (AR4A and AR5A), and a negative-control antibody (CA45).

![Serum neutralization graph](image)

**Fig. 9** Serum neutralization of homologous (H77C) and heterologous HCVpp. Immune murine serum neutralization was tested using HCV pseudoparticles (HCVpp) representing H77C as well as six heterologous isolates. Neutralization for four HCVpp representing isolates with resistant phenotypes is shown on the right, as indicated. Neutralization titers are represented as serum dilution levels required to reach 50% virus neutralization (ID$_{50}$), calculated by curve fitting in GraphPad Prism software. Serum dilutions were performed as 2-fold dilutions starting at 1:64, and minimum dilution levels (corresponding to 1:64) are indicated as dotted lines for reference. Murine sera with low (calculated ID$_{50}$ of $<10$) or incalculable ID$_{50}$ values due to low or background levels of neutralization (observed only for some mice for J6 HCVpp neutralization) have the ID$_{50}$ shown as 10. Due to insufficient sera, J6 neutralization measurements did not include two mice from group 1 (sE2) and one mouse from group 4 ($\Delta$HVR1). $P$ values for between-group ID$_{50}$ values were calculated using Kruskal-Wallis analysis of variance with Dunn’s multiple-comparison test, and significant $P$ values for comparisons of results between the sE2 control and sE2 design groups are shown (*, $P \leq 0.05$; **, $P \leq 0.01$).
binding values in several groups. As expected due to the limited number of data points and limited overall variability in binding affinity measurements (Table 3), few correlations between antigenic and immunogenic parameters were highly significant though binding of the domain D HMAb HC84.26.WH.5DL was highly correlated with neutral-
ization of J6 HCVpp ($r = 0.97, P = 0.0003$), as well as neutralization of UKNP1.18.1 HCVpp ($r = 0.88, P = 0.008$), while anticorrelations were detected for other antibody binding measurements (HEPC74 and HCV1) and HCVpp group neutralization values at lower significance levels. The high correlations involving HMAb HC84.26.WH.5DL are not unexpected, based on the higher HMAb binding affinity to the H445P sE2 antigen and higher NAb responses induced by H445P; Fig. 10D shows a comparison of UKNP1.18.1 neutralization with HC84.26.WH.5DL binding, in which the point corresponding to H445P is in the upper right.

**DISCUSSION**

In this study, we applied a variety of rational design approaches to engineer the HCV E2 glycoprotein to improve its antigenicity and immunogenicity. One of these approaches, removal of HVR1 (ΔHVR1), has been tested in several recent immunogenicity studies in the context of E2 (18, 20, 23) and E1E2 (21). In this study, we tested the E2 ΔHVR1 mutant in which residues 384 to 407 have been removed and residues 408 to 661 of E2 are retained; this is a more conservative truncation than that of previously tested ΔHVR1 mutants in order to retain residue 408 which is a binding determinant for the HC33.4 HMAb and other HMAbs (28, 34). Here, we found that this mutant was not advantageous from an immunogenicity standpoint, which is in agreement with findings of most other previous immunogenicity studies testing ΔHVR1 mutants (18, 21, 23). Although HVR1 is an immunogenic epitope, its removal from recombinant E2 glycoprotein does not appear to increase homologous or heterologous NAb titers, with the latter suggesting that the level of antibodies targeting conserved NAb epitopes did not increase upon HVR1 removal. Based largely on studies of engineered viruses in cell culture, as summarized in a recent review (41), removal of HVR1 is associated with increased NAb sensitivity and CD81 receptor binding, while a recent study has indicated that HVR1 may modulate viral dynamics and open and closed conformations during envelope breathing (42). Despite the importance of HVR1 in the context of the virion and its dynamics, its removal appears to have a neutral or minimal effect on the immunogenicity of recombinant envelope glycoproteins.

Another design strategy tested in this study was hyperglycosylation through structure-based addition of N-glycan sequons to mask antigenic domain A, which is associated with nonneutralizing antibodies (25, 26, 28, 43). The concept of downmodulating immunity to this region was based on the observation that this region is highly immunogenic and may divert antibody responses to bNAb epitopes of lower immunogenicity. Through the efforts of isolating bNAbs to distinct regions on E2 from multiple HCV-infected individuals, nonneutralizing antibodies to domain A are consistently identified (S. Foung, personal communication). This strategy has been successfully employed for other glycoprotein immunogens, including for HIV Env SOSIP trimers, in which the immunogenic V3 loop was masked with designed N-glycans (30). Surprisingly, some of the designs in this study exhibited an impact on recognition by antibodies targeting antigenic domain D on the front layer of E2, suggesting a possible interplay between the front and back layers of E2, as proposed previously based on global alanine scanning mutagenesis (35). As observed by Ringe et al. in the context of HIV Env (30), the designed E2 N-glycan variant tested for immunogenicity in this study (Y632NS) did not show improvements in NAb elicitation. However, its combination with ΔHVR1 did lead to modest improvement in NAb titers against one resistant isolate (UKNP2.4.1; $P < 0.05$) compared to those of wild-type sE2. Previously, we used insect cell expression to alter the N-glycan profile of sE2 versus that of mammalian cell-expressed sE2 (19), and other investigators have recently tested immunogenicity for glycan-deleted E2 and E1E2 variants (18); in neither case was a significant improvement in homologous and heterologous NAb responses observed for immunogens with altered glycans. Collectively, these results suggest that glycoengineering of E2 or E1E2 represents a more challenging, and possibly less beneficial, avenue for HCV immunogen design; however, a report of success by other investigators through insect cell-expressed sE2 indicates that altered glycosylation may help in some instances (44).
The designed substitution H445P, which was generated to preferentially adopt the bNAb-bound form in a portion of E2 antigenic domain D that exhibits structural variability (31), showed the greatest level of success, both with regard to improvements in serum binding to homologous and heterologous HCVpp and HCVpp neutralization of heterologous HCVpp. This design lies within a supersite of E2 associated with many broadly neutralizing antibodies (5, 6, 45, 46), and through biophysical characterization and molecular dynamics simulation experiments, other investigators have found that this region is likely quite flexible (27, 47), providing a rationale for stabilizing key residues to engage and elicit bNabs. Interestingly, a residue adjacent to the site of this design appears to be functionally important, with the Q444R substitution restoring viral infectivity in the context of an HCVpp with a domain E glycan shift substitution, N417S (8). The design strategy of utilizing proline residue substitutions to stabilize conformations of viral glycoproteins has been successful for HIV Env (48), respiratory syncytial virus (RSV) F (49), Middle East respiratory syndrome (MERS) coronavirus spike (50), and, recently, the novel coronavirus (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) spike (51). The data from this study suggest that this approach is also useful in the context of HCV E2 and possibly E1E2.

This study provides a proof of concept for computational structure-based design of the HCV E2 glycoprotein to modulate its antigenicity and immunogenicity. Future studies with the H445P design include testing of its antigenicity and immunogenicity in the context of HCV E1E2 and testing of its immunogenicity in other animal models, as well as confirmation of its impact on E2 structure through high-resolution X-ray structural characterization and additional biophysical characterization. Confirmation of improved elicitation of neutralizing antibodies with a cell culture-based HCV (HCVcc) assay versus the pseudoparticle-based (HCVpp) assay used in this study can provide further insight into the impact of these and other HCV envelope glycoprotein variants. However, the employment of HCVpp does permit a greater ease in testing against clinical isolates. Furthermore, additional designed proline substitutions in this flexible E2 “neutralizing face” supersite may confer greater improvements in homologous and heterologous NAb elicitation; these can be generated using structure-based design or with a semirational library-based approach, as was used to scan a large set of proline substitutions for HIV Env (52). This study provides a promising design candidate for follow-up studies, underscoring the value of the set of previously determined, though somewhat limited, E2-bNAb complex structures. Prospective elucidation of the structure of E2 in complex with additional bNabs, as well as characterization of the E1E2 complex structure, will facilitate future structure-based design studies to engineer and optimize immunogens for an effective HCV vaccine.

MATERIALS AND METHODS

Computational modeling and design. Proline substitution designs to stabilize epitopes were modeled as previously described for design of T cell receptor binding loops (53), using a Ramachandran plot server to assess epitope residue backbone conformations for proline and preproline conformational similarities (https://zlab.umassmed.edu/bu/rama/) (54), as well as explicit modeling of energetic effects of proline substitutions using the point mutagenesis mode of Rosetta, version 2.3 (55). N-glycan sequon substitutions (NKS and NXT) were modeled using Rosetta (55), followed by modeling of the N-glycan structure using the Glyprot web server (56). Assessment of residue side chain accessible surface areas was performed using NACCESS (57) with default parameters.

Protein and antibody expression and purification. Expression and purification of recombinant soluble HCV E2 (sE2) and designs were performed as previously described (19). Briefly, the sequence from isolate H77C (GenBank accession number AF011751; residues 384 to 661) was cloned into the pSecTag2 vector (Invitrogen), transfected with 293Fectin into FreeStyle HEK293-F cells (Invitrogen), and purified from culture supernatants by sequential HisTrap Ni²⁺-NTA and Superdex 200 columns (GE Healthcare). For recombinant HCV E1E2 expression, the H77C E1E2 glycoprotein coding region (GenBank accession number AF011751) was synthesized with a modified tissue plasminogen activator (tPA) signal peptide (58) at the N terminus and cloned into the vector pcDNA3.1+ at the cloning sites of KpnI/NotI (GenScript). Expi293 cells (Thermo Fisher) were used to express the E1E2 glycoprotein complex. In brief, the Expi293 cells were grown in Expi293 medium (ThermoFisher) at 37°C at 125 rpm in 8% CO₂ and 80% humidity in Erlenmeyer sterile polycarbonate flasks (VWR). The day before the transfection, 2.0 × 10⁶ viable cells/ml was seeded in a flask, and the manufacturer’s protocol (expression kit no. A14524; ThermoFisher) was followed for transfection performance. After 72 h posttransfection, the cell pellets
were harvested by centrifuging cells at 3,000 g for 5 min, and the cell pellets were then stored at −80°C for further processing. Recombinant E1E2 was extracted from cell membranes using 1% NP-9 and purified via sequential Fractogel EMD TMAE (Millipore), Fractogel EMD SO3− (Millipore), HC84.26 immunoaffinity, and Galanthus nivalis lectin (GNL; Vector Laboratories) affinity chromatography. Monoclonal antibody HC1V was provided by Yang Wang (MassBiologics, University of Massachusetts Medical School), and monoclonal antibodies AR3A, AR4A, and ARSA were provided by Mansun Law (Scripps Research Institute). All other monoclonal antibodies used in ELISA and binding studies were produced as previously described (24, 25, 59). A clone for mammalian expression of the CD81 large extracellular loop (LEL), containing N-terminal TPA signal sequence and a C-terminal twin Strep tag, was provided by Joe Grove (University College London). CD81-LEL was expressed through transient transfection in Expi293F cells (ThermoFisher) and purified from supernatant with a Gravity Flow Strep-Tactin Superflow high-capacity column (IBA Lifesciences). Purified CD81-LEL was polished by size exclusion chromatography (SEC) with a Superdex 75 10/300 GL column (GE Healthcare) on an Akta fast-performance liquid chromatograph (FPLC; GE Healthcare).

**ELISA antigenic characterization and competition assays.** Cloning and characterization of E2 mutant antigenicity using ELISA were performed as described previously (28). Mutants were constructed in plasmids carrying the 1a H77C E1E2 coding sequence (GenBank accession number AF009606), as described previously (60). All of the mutations were confirmed by DNA sequence analysis (EliL Biopharmaceuticals, Inc., Hayward, CA) for the desired mutations and for absence of unexpected residue changes in the full-length E1E2-encoding sequence. The resulting plasmids were transfected into HEK 293T cells for transient protein expression using the calcium-phosphate method. Individual E2 protein expression was normalized by binding of CBH-17, an HCV E2 HMAb to a linear epitope (61). Serum samples at specified dilutions were tested for their ability to block the binding of selected HCV HMABs conjugated with biotin or G. nivalis agglutinin (GNA)-captured E1E2 glycoprotein ELISA, as described previously (24). Data are shown as mean values of two experiments performed in triplicate.

**Biolayer interferometry.** The interaction of recombinant sE2 glycoproteins with CD81 and HMABs was measured using an Octet RED96 instrument and Ni2+-NTA biosensors (Pall ForteBio). The biosensors were loaded with 5 μg/ml of purified His6-tagged wild-type or mutant sE2 for 600 s. Association for 300 s followed by dissociation for 300 s against a 2-fold concentration dilution series of each antibody was performed. Data analysis was performed using Octet Data Analysis, version 10.0, software and utilized reference subtraction at 0 nM antibody concentration, alignment to the baseline, interstep correction to the dissociation step, and Savitzky-Golay fitting. Curves were globally fitted to obtain Kd (dissociation constant) values.

**Differential scanning calorimetry.** Thermal melting curves for monomeric E2 proteins were acquired using a MicroCal PEAC differential scanning calorimetry (DSC) automated system (Malvern Panalytical). Purified monomeric E2 proteins were dialyzed into phosphate-buffered saline (PBS) prior to analysis, and the dialysis buffer was used as the reference in the experiments. Samples were diluted to 10 μM in PBS prior to analysis. Thermal melting was probed at a scan rate of 90°C · h−1 over a temperature range of 25 to 115°C. All data analyses, including estimation of the melting temperature, were performed using standard protocols that are included with the PEAC-DSC software.

**MS.** Digestion was performed on 40 μg each of HCK293-derived sE2 glycan sequon substitutions by denaturing using 6 M guanidine HCl and 1 mM EDTA in 0.1 M Tris, pH 7.8, reduced with a final concentration of 20 mM dithiothreitol (DTT) (65°C for 90 min) and alkylated at a final concentration of 50 mM iodoacetamide (room temperature for 30 min). Samples were then buffer exchanged into 1 M urea in 0.1 M Tris, pH 7.8, for digestion. Sequential digestion was performed using trypsin (1/50 enzyme/protein, wt/wt) for 18 h at 37°C, followed by chymotrypsin (1/20 enzyme/protein, wt/wt) overnight at room temperature. Samples were then absorbed onto Sep-Pak tC18 columns to remove proteolytic digestion buffer, eluted with 50% acetonitrile–0.1% trifluoroacetic acid (TFA) buffer, and concentrated to dryness in a centrifugal vacuum concentrator. The samples were then resuspended in 50 mM sodium acetate, pH 4.5, and incubated with endoglycosidase (endo) F1, endo F2, and endo F3 (Q Bio) at 37°C for 72 h to remove complex glycans. Liquid chromatography–UV-mass spectrometry (LC–UV-MS) analyses were performed using an UltiMate 3000 LC system coupled to an LTQ Orbitrap Discovery equipped with a heated electrospray ionization (HESI) source and operated in a top–five dynamic exclusion mode. A volume of 25 μl (representing 10 μg of digested protein) of sample was loaded via the autosampler onto a C8 peptide column (AdvanceBio Peptide column, 2.7-μm particle size, 2.1 by 150 mm [catalog no. 653750-902; Agilent]) enclosed in a thermostatted column oven set to 50°C. Samples were held at 4°C while queued for injection. The chromatographic gradient was conducted as described previously (19). Identification of glycosylated peptides containing the glycan sequon substitution was performed using Byonic software, and extracted ion chromatograms were used for estimating the relative abundance of the glycosylated peptides in Byologic software (Protein Metrics).

**Animal immunization.** CD-1 mice were purchased from Charles River Laboratories, and immunizations were performed by Integrated Biotherapeutics, Inc. (IBT, Rockville, MD) and monitored by the IBT IACUC (NIH Assurance #D17-00974) in compliance with the USDA Animal Welfare Act. Prior to immunization, sE2 antigens were formulated with polyphosphazene adjuvant. Poly[di(carboxylatoxy)phosphazene] (PCPP) (molecular weight, 800,000 Da) (62) was dissolved in PBS (pH 7.4) and mixed with sE2 antigen solution at a 1:1 (prime) or 1:5 (wt/wt) (boost immunization) antigen/adjuvant ratio to provide for a 50-μg PCPP dose per animal. The absence of aggregation in adjuvanted formulations was confirmed by dynamic light scattering (DLS; single peak, z-average hydrodynamic diameter of 60 nm).
The formation of sE2 antigen-PCPP complex was proven by asymmetric flow field flow fractionation (AF4) as described previously (63). On scheduled vaccination days, groups of 6 female mice, aged 7 to 9 weeks, were injected via the intraperitoneal (i.p.) route with 50 μg of sE2 (prime; day 0) and boosted with 10 μg of sE2 on days 7, 14, 28, and 42. Blood samples were collected prior to each injection with a terminal bleed on day 56. The collected samples were processed for serum by centrifugation and stored at −80°C until analysis was performed.

Serum peptide and protein ELISA. Domain-specific serum binding was tested using ELISAs with C-terminal biotinylated peptides from H77C AS412 (aa 410 to 425; sequence N IQLINTNGSWHINST) and AS434 (aa 434 to 446; sequence NTGWLAGLFYQHK), using 2 μg/ml coating concentration. Recombinant sE2 and E1E2 proteins were captured onto GNA-coated microtiter plates. Endpoint titers were calculated by curve fitting in GraphPad Prism software, with the endpoint optical density (OD) defined as four times the highest absorbance value of day 0 serum.

HCV pseudoparticle generation. HCV pseudoparticles (HCVpp) were generated as described previously (19) by cotransfection of HEK293T cells with the murine leukemia virus (MLV) Gag-Pol packaging vector, luciferase reporter plasmid, and plasmid expressing HCV E1E2 using Lipofectamine 3000 (ThermoFisher Scientific). An envelope-free control (empty plasmid) was used as a negative control in all experiments. Supernatants containing HCVpp were harvested at 48 h and 72 h posttransfection and filtered through 0.45-μm-pore-size membranes. Concentrated HCVpp were obtained by ultracentrifugation of 33 ml of filtered supernatants through a 7-ml 20% sucrose cushion using an SW 28 Beckman Coulter rotor at 25,000 rpm for 2.5 h at 4°C, according to a previously reported protocol (26).

HCVpp serum binding. For measurement of serum binding to HCVpp, 100 μl of 0.45-μm-pore-size filtered HCVpp isolates were directly coated onto Nunc-Immuno MaxiSorp (Thermo Scientific) microwells overnight at 4°C. Microwells were washed three times with 300 μl of 1 × PBS and 0.05% Tween 20 in between steps. Wells were blocked with Pierce protein-free blocking buffer (Thermo Scientific) for 1 h. Serum sample dilutions made in blocking buffer were added to the microwells and incubated for 1 h at room temperature. Abs were detected with secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG(H+L) (ab97023; Abcam) and developed with TMB (3,3′,5,5′-tetramethylbenzidine) substrate solution (Bio-Rad). The reaction was stopped with 2 M sulfuric acid. A Molecular Devices M3 plate reader was used to measure absorbance at 450 nm. Endpoint titers were calculated by curve fitting in GraphPad Prism software, with the endpoint OD defined as four times the highest absorbance value of day 0 serum.

HCVpp neutralization assays. For infectivity and neutralization testing of HCVpp, 1.5 × 10⁴ Huh7 cells per well were plated in 96-well tissue culture plates (Corning) and incubated overnight at 37°C. The following day, HCVpp were mixed with appropriate amounts of antibody and then incubated for 1 h at 37°C before they were added to Huh7 cells. After 72 h at 37°C, either 100 μl of Bright-Glo (Promega) was added to each well and incubated for 2 min or cells were lysed with cell lysis buffer (E1500; Promega) and placed on a rocker for 15 min. Luciferase activity was then measured in relative light units (RLU) either by using a SpectraMax M3 microplate reader (Molecular Devices) with SoftMax Pro6 software (Bright-Glo protocol) or by individually injecting wells with 50 μl of luciferase substrate and using a FLUOstar Omega plate reader (BMG Labtech) with MARS software. Infection by HCVpp was measured in the presence of anti-E2 MAbs, tested animal sera, preimmune animal sera, and nonspecific IgG at the same dilution. Each sample was tested in duplicate or triplicate. Neutralizing activities were reported as the 50% inhibitory concentration (IC₅₀) and values were calculated by nonlinear curve fitting (GraphPad Prism), using lower and upper bounds (0% and 100% inhibition) as constraints to assist curve fitting.

Statistical comparisons and correlations. P values between group endpoint titers and group ID₅₀ values were calculated using Kruskal-Wallis one-way analysis of variance (ANOVA), with Dunn’s multiple-comparison test, in GraphPad Prism software. Pearson correlations and correlation significance P values were calculated in R (www.r-project.org).

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