1 **Antigenicity and Immunogenicity of Differentially Glycosylated HCV E2**

2 **Envelope Proteins Expressed in Mammalian and Insect Cells**

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ABSTRACT

Development of a prophylactic vaccine for hepatitis C virus (HCV) remains a global health challenge. Cumulative evidence supports the importance of antibodies targeting the HCV E2 envelope glycoprotein to facilitate viral clearance. However, a significant challenge for a B cell-based vaccine is focusing the immune response on conserved E2 epitopes capable of eliciting neutralizing antibodies not associated with viral escape. We hypothesized that glycosylation might influence the antigenicity and immunogenicity of E2. Accordingly, we performed head-to-head molecular, antigenic and immunogenic comparisons of soluble E2 (sE2) produced in (i) mammalian (HEK293) cells, which confer mostly complex and high mannose type glycans; and (ii) insect (Sf9) cells, which impart mainly paucimannose type glycans. Mass spectrometry demonstrated that all 11 predicted N-glycosylation sites were utilized in both HEK293- and Sf9-derived sE2, but that N-glycans in insect sE2 were on average smaller and less complex. Both proteins bound CD81 and were recognized by conformation-dependent antibodies. Mouse immunogenicity studies revealed that similar polyclonal antibody responses were generated against antigenic domains A–E of E2. Although neutralizing antibody titers showed that Sf9-derived sE2 induced moderately stronger responses than HEK293-derived sE2 against the homologous HCV H77c isolate, the two proteins elicited comparable neutralization titers against heterologous isolates. Given that global alteration of HCV E2 glycosylation by expression in different hosts did not appreciably affect antigenicity or overall immunogenicity, a more productive approach to increasing the antibody response to neutralizing epitopes may be complete deletion, rather than just modification, of specific N-glycans proximal to these epitopes.

IMPORTANCE

Development of a vaccine for hepatitis C virus (HCV) remains a global health challenge. A major challenge for vaccine development is focusing the immune response on conserved regions of the HCV envelope protein, E2, capable of eliciting neutralizing antibodies. Modification of E2 by glycosylation might influence the immunogenicity of E2. Accordingly, we performed molecular and immunogenic comparisons of E2 produced in mammalian and insect cells. Mass spectrometry demonstrated that the predicted glycosylation sites were utilized in both mammalian and insect cell E2, although the glycan types in insect cell E2 were smaller and less complex. Mouse immunogenicity studies revealed similar polyclonal antibody responses. However, insect cell E2 induced stronger neutralizing antibody responses against the homologous isolate used in the vaccine, albeit the two proteins elicited comparable neutralization titers against heterologous isolates. A more productive approach for vaccine development may be complete deletion of specific glycans in the E2 protein.

INTRODUCTION

Hepatitis C virus (HCV) is a major public health problem and infects over 71 million people worldwide (1). Infection often develops into chronic hepatitis, which is the most prevalent cause of liver failure and hepatocellular carcinoma. In recent years, new direct acting antivirals (DAAs) have supplanted the use of ribavirin and pegylated interferon alpha as a treatment regimen, reaching cures rates of greater than 90% in HCV genotype 1-infected patients (2, 3). However, significant limitations still exist, including the high cost of DAAs

67 that restricts access in developing countries where the disease burden is greatest (3) and

68 concerns about the development of drug resistance (2, 3). Moreover, therapy-induced HCV

69 clearance does not provide immunity to new infections or eliminate the risk of hepatocellular

70 carcinoma in patients with established cirrhosis. Therefore, an effective preventative vaccine

71 is an important medical and public health need (4).

72   The genetic diversity of HCV of at least seven genotypes that differ up to 30% in

73 nucleotide sequence poses a major challenge to developing a pan-genotypic vaccine. HCV is

74 composed of a nucleocapsid core enveloped by a lipid bilayer in which two surface

75 glycoproteins, E1 and E2, are anchored. During acute infection, a robust neutralizing

76 antibody response correlates with spontaneous resolution of infection (5, 6). Most broadly

77 neutralizing antibodies (bNAbs) recognize conformational epitopes in E2 and some in E1E2

78 (7, 8). Passive immunization with anti-HCV antibodies before HCV challenge can prevent

79 infection against homologous virus challenge in animal models (9, 10), although virus

80 breakthrough can occur and protection against heterologous virus strains remains a problem.

81 Other complicating factors for vaccine development include low immunogenicity of viral

82 envelope proteins and antibody responses directed to regions that display a high mutational

83 rate of change (11). In addition, a number of studies have highlighted the role of N-linked

84 glycosylation in masking or shielding E2 epitopes from recognition by bNAbs (12-16), as

85 also observed for HIV and influenza virus (17). Such glycan shielding may hinder bNAb

86 induction by reducing the exposure of neutralizing epitopes to the humoral immune system,

87 further complicating development of a B cell-based HCV vaccine.

88   There is evidence from a number of studies, mainly involving HIV and influenza, that

89   alteration of glycans on viral envelope glycoproteins can impact immune recognition and

90   immunogenicity. Some studies have used hyperglycosylation, the addition of N-glycans

91   through mutagenesis to create glycan sequons, to mask certain sites associated with

92   non-neutralizing antibodies and antibody escape (18-22). Conversely, targeted removal of

93   glycans through mutagenesis of glycan sequons has been used to improve or alter

94   neutralizing antibody engagement of key epitopes for HIV-1 (23-26), influenza A virus (27),

95   and HCV (13-15). For example, in one study, elimination of glycosylation sites in the

96   vicinity of the CD4-binding site of the HIV-1 envelope glycoprotein (Env) generated an

97   immunogen that more efficiently activated B cells expressing germline precursors of bNAbs

98   (25). However, in another study, targeted deglycosylation of HIV-1 Env failed to generate an

99   antigen capable of eliciting neutralizing antibodies against wild-type viruses in immunized

100   guinea pigs or rhesus macaques (28). In the case of influenza A virus, removal of glycans in

101   the hemagglutinin (HA) protein that shield key epitopes of HA from antibody recognition

102   produced immunogens that elicited bNAbs effective against antigenically distant virus

103   strains (27). In the case of HCV, deletion of specific glycans in HCV E1E2 was found to

104   modulate binding of certain bNAbs (14, 15), although the immunogenicity of these modified

105   E1E2 proteins was not studied.

106   An alternate strategy to modulate presentation of key antibody epitopes is

107   modification of the extent of glycosylation through the use of various expression cell types

108   (29-32), or enzymatic truncation of glycoforms (33). In one study, HIV-1 gp120 was

109   produced in *Spodoptera* insect (Sf9) cells, which impart mainly paucimannose type glycans,

110   and in mammalian (HEK293) cells treated with the α-mannosidase inhibitor kifunensine,

111 which confers only high mannose type glycans (30). These global alterations in

112 glycosylation compared to gp120 produced in untreated HEK293 cells led to increased

113 exposure of the CD4 binding site, which encouragingly translated to increased binding of

114 bNAbs specific for this site. However, the effect on HIV-1 neutralization titers in animals

115 immunized with these modified gp120 glycoproteins was not reported. More recently, a

116 secreted, soluble form of HCV E2 (sE2) produced in *Drosophila* insect (S2) cells was found

117 to be more immunogenic than the corresponding protein produced in HEK293 cells (31).

118 Moreover, S2-derived sE2 elicited higher titers of antibodies capable of neutralizing a

119 diverse panel of HCV genotypes, suggesting that distinct glycosylation patterns should be

120 taken into consideration in development of a recombinant HCV vaccine.

121   To further test the hypothesis that differential glycosylation may influence the

122 antigenicity and immunogenicity of E2, we performed head-to-head molecular, antigenic

123 and immunogenic comparisons of sE2 produced in (i) mammalian (HEK293) cells, which

124 impart high mannose, hybrid and complex glycans; and (ii) insect (Sf9) cells, which confer

125 mainly paucimannosidic glycans. In contrast to Li et al. (31), we found that immunization of

126 mice with mammalian and insect sE2 glycoproteins elicited comparable antibody

127 neutralization titers against heterologous HCV isolates, although Sf9-derived sE2 was a

128 more potent immunogen against the homologous H77c isolate. We discuss possible reasons

129 for the apparent discrepancy between our results and theirs, and conclude that targeted

130 deletion of specific E2 glycans, rather than expression system-dependent modification of all

131 glycans, may be a better strategy for increasing the exposure of virus neutralizing epitopes to

132 the humoral immune system.

133

134   **MATERIALS AND METHODS**

135   **Protein expression, purification, and antibodies.** For mammalian cell expression, a

136   gene encoding HCV E2 from strain 1a H77c (residues 384–661) was cloned into the vector

137   pSecTag2 (Invitrogen) with an N-terminal immunoglobulin κ light chain signal sequence (for

138   secretion) and a C-terminal His$_6$ tag (for purification). The construct was transfected with

139   293fectin into FreeStyle HEK293-F cells (Invitrogen). Recombinant monomeric E2 was

140   purified from culture supernatants by sequential HisTrap Ni^{2+}-NTA and Superdex 200 columns

141   (GE Healthcare). For insect cell expression, the same E2 sequence was fused to the gp67

142   secretion signal sequence of baculovirus vector pAcGP67-B (BD Biosciences) with a

143   C-terminal His$_6$ tag. To generate recombinant baculovirus, this construct was transfected into

144   Sf9 cells together with BaculoGold Linearized DNA (BD Biosciences). Soluble monomeric E2

145   was purified from culture supernatants of Sf9 cells infected with recombinant baculovirus

146   using sequential HisTrap Ni^{2+}-NTA and Superdex 200 columns.

147   Human CD81 large extracellular loop (CD81-LEL; CD81 residues 113–201) coding

148   sequence DNA was synthesized (GenScript) and cloned into the vector pHLsec (Addgene

149   plasmid #99845; a gift from E. Yvonne Jones) which includes a C-terminal His$_6$ tag. The

150   construct was transfected with PEI MAX 40K (Polysciences) into FreeStyle HEK293-F cells.

151   CD81-LEL was purified from culture supernatant by sequential HiTrap Chelating HP

152   Ni^{2+}-NTA and Superdex 75 columns (GE Healthcare). Before biolayer interferometry, purified

153   CD81-LEL was stripped of its C-terminal His$_6$ tag by digestion with carboxypeptidase

154   A-agarose (Sigma).

155   Monoclonal antibody HCV1 was kindly provided by Dr. Yang Wang (MassBiologics,

156   University of Massachusetts Medical School) (37). Monoclonal antibodies against different

157   antigenic domains, CBH-4B and CBH-4G (domain A), HC-1 and HC-11 (domain B), CBH-7

158   (domain C), HC84.24 and HC84.26 (domain D), and HC33.1 and HC33.4 (domain E) were

159   employed and prepared as described (7).

160   **Mass spectrometry.**   Digestion was performed on 80 µg each of HEK293- and

161   Sf9-derived sE2 by denaturing using 6 M guanidine HCl, 1 mM EDTA in 0.1 M Tris, pH 7.8,

162   reduced with a final concentration of 20 mM DTT (65 ^{o}C for 90 min), and alkylated at a final

163   concentration of 50 mM iodoacetamide (room temperature for 30 min). Samples were then

164   buffer exchanged into 1 M urea in 0.1 M Tris, pH 7.8 for digestion. Sequential digestion was

165   performed using trypsin (1/50 enzyme/protein ratio, w/w) for 18 hours at 37°C, followed by

166   chymotrypsin (1/20 enzyme:protein, w/w) with 10 mM CaCl$_2$ overnight at room temperature.

167   LC-UV-MS analyses were performed using an UltiMate 3000 LC system coupled to an LTQ

168   Orbitrap Elite equipped with a heated electrospray ionization (HESI) source and operated in a

169   top 5 dynamic exclusion mode. A volume of 30 µl (representing 9 µg of digested protein) of

170   sample was loaded via the autosampler onto a C18 peptide column (AdvanceBio Peptide 2.7

171   um, 2.1 x 150 mm, Agilent part number 653750-902) enclosed in a thermostatted column oven

172   set to 50 °C. Samples were held at 6 °C while queued for injection. The chromatographic

173   gradient was conducted as described in **Table 1**. Glycan and glycopeptide identification was

174   performed using Byonic software and extracted ion chromatograms used for quantification of

175   glycoforms in Byologic software (Protein Metrics).

176 **Glycoprotein modeling.** Glycans were modeled on the E2 protein structure using

177 Rosetta software (34). Prior to glycan modeling, residues from antigenic domain E were

178 modeled into the structure of E2 core (Protein Data Bank accession code 4MWF) (35) and

179 refined using the FloppyTail method in Rosetta (36). The most abundant glycan at each site

180 was encoded as an IUPAC string and modeled on E2 core using the SimpleGlycosylateMover

181 in Rosetta, followed by the glycan_relax protocol to alleviate clashes involving glycans and

182 energetically unfavorable conformations (using command line flags "-tree_based_min_pack

183 -glycan_relax_refine"). Accessible surface area calculations were performed in Rosetta using

184 the TotalSasa filter, specifying the probe radius using the "-sasa_calculator_probe_radius"

185 command line flag. Percent E2 surface masking by glycans was calculated by dividing the

186 surface accessibility of protein residues in the glycosylated E2 model by the surface

187 accessibility of protein residues in the same model with all glycans removed. Structural

188 visualization was performed in Pymol (www.pymol.org).

189 **Biolayer interferometry.** The interaction of sE2 derived from HEK293 or Sf9 cells

190 with CD81 and human monoclonal antibodies (HMAbs) in IgG format specific for antigenic

191 domains A (CBH-4B, CBH-4G), B (HC-1, HC-11), D (HC84.26, HC84.24), and E (HCV1,

192 HC33.1) (7, 37) was measured using an Octet RED96 instrument and Ni^{2+}-NTA biosensors

193 (Pall ForteBio). The biosensors were loaded with 5 μg/mL of purified His_6-tagged sE2 for

194 600 sec, then stabilized with the chemical crosslinker mixture of 0.1 M

195 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.025 M N-hydroxysuccinimide (GE

196 Life Sciences) for 60 seconds followed by reaction quenching with 1 M ethanolamine, pH

197 8.0 for 60 sec. Association for 300 sec followed by dissociation for 300 sec against a 2-fold

198   concentration dilution series of each antibody was performed. Data analysis was performed

199   using Octet Data Analysis 10.0 software and utilized reference subtraction at 0 nM antibody

200   concentration, alignment to the baseline, interstep correction to the dissociation step, and

201   Savitzky-Golay fitting. Curves were globally fitted based on association and dissociation to

202   obtain K_D values.

203   **Animal immunization.** CD-1 mice were purchased from Charles River. Prior to

204   immunization, sE2 antigens were formulated with SAS adjuvant (Sigma Adjuvant System,

205   Sigma Aldrich) at a 1:1 ratio according to the manufacturer's instructions. On scheduled

206   vaccination days, groups of 6 female mice, age 7–9 weeks, were injected via the

207   intraperitoneal (IP) route with a 50 µg sE2 prime (day 0) and boosted with 10 µg sE2 on days

208   7, 14, 28, and 35. Blood samples were collected prior to each injection with a terminal bleed

209   on day 49. The collected samples were processed for serum by centrifugation and stored at

210   -80 °C until analysis was performed.

211   **Competitive binding ELISA.** An ELISA measured the inhibition by mouse sera of

212   HMAb binding to E2 glycoproteins captured by *Galanthus nivalis* lectin (GNA) (38).

213   Briefly, microtiter plates were coated with GNA and blocked with 2.5% bovine serum

214   albumin (BSA) and 2.5% normal goat serum in 0.1% Tween-PBS, prior to the addition of 2

215   µg/well of HEK293 sE2. Pretitrated mouse serum was added to each well at a saturating

216   concentration. After 1 h, HMAb was added at a concentration corresponding to 65% to 75%

217   of the maximal optical density (OD) level, incubated for 30 min at either room temperature

218   or 40 °C, and then washed. Bound HMAb was detected by incubation with alkaline

219   phosphatase-conjugated goat anti-human IgG (Promega), followed by incubation with

10

220 p-nitrophenyl phosphate (Sigma) for color development. Absorbance was measured at

221 405/570 nm. This assay was carried out in triplicate, a minimum of two times for each

222 HMAb.

223   **Cell lines and pseudoparticle plasmid construction.** The human embryonic

224 kidney 293T (HEK293T) and Hep3B cells were purchased from ATCC (www.atcc.org).

225 Both HEK293T and Hep3B cells were grown in Dulbecco's modified essential medium

226 (DMEM) (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) and

227 1% penicillin-streptomycin (Thermo Fisher Scientific). The human hepatoma cell line,

228 HuH7 was purchased from ECACC and routinely tested for mycoplasma contamination.

229 HuH7 cells were grown in Dulbecco's modified essential medium (DMEM) (Thermo Fisher

230 Scientific) supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino

231 acids (NEAA) (Thermo Fisher Scientific). All were maintained in a humidified 37 °C, 5%

232 CO_2 incubator. The different genotype constructs for gt1a (H77c, UNKP1.1.1), gt1b

233 (UNKP1.20.3, UNKP1.21.2), gt2a (J6, JFH), gt2b (UNKP2.4.1), gt2i (UNKP2.1.2), gt3

234 (UNKP3.2.2), gt4 (UNKP4.2.1), gt5 (UNKP5.1.1), and gt6 (UNKP6.1.1) were described

235 previously (39, 40). The MLV Gag-Pol packaging vector (phCMV-5349) and luciferase

236 reporter plasmid (pTG126) have been reported (40). All plasmids were grown and purified

237 using the EndoFree Plasmid Maxi Kit (Omega Bio-tek). Concentration and purity were

238 verified by restriction analysis, sequencing and spectrophotometry.

239   **Pseudoparticle generation and titration.** HCV pseudoparticles (HCVpp) were

240 generated by co-transfection of HEK293T cells with the MLV Gag-Pol packaging vector,

241 luciferase reporter plasmid, and plasmid expressing HCVE1E2 using Lipofectamine 3000

242 (Thermo Fisher Scientific) as described previously (40). No-envelope control (empty

243 plasmid) was used as negative control in all experiments. Supernatants containing

244 HCVE1E2 pseudoparticles were harvested at 48 h and 72 h post-transfection, and filtered

245 through 0.45 μm pore-sized membranes. The generated HCVpp is capable of achieving a

246 single-round infection in Hep3B or HuH7 target cells and contains a luciferase reporter gene

247 that can be expressed in infected cells. Titration of HCVpp was then determined by infecting

248 Hep3B cells with a serial dilution.

249   **HCVpp neutralization assays.** For infectivity and neutralization testing of HCVpp,

250 either 10^4 Hep3B cells per well or 1.5 x 10^4 HuH7 cells per well were plated in white 96-well

251 tissue culture plates (Corning) and incubated overnight at 37 °C. The following day, HCVpp

252 were mixed with appropriate amounts of antibody and then incubated for 1 h at 37 °C before

253 adding to Hep3B or HuH7 cells. After 72 h at 37 °C, either 100 μl Bright-Glo (Promega) was

254 added to each well and incubated for 2 min or cells were lysed with Cell lysis buffer

255 (Promega E1500) and placed on a rocker for 15 min. Luciferase activity was then measured

256 in relative light units (RLUs) using either a SpectraMax M3 microplate reader (Molecular

257 Devices) with SoftMax Pro6 software (Bright-Glo protocol) or wells were individually

258 injected with 50uL luciferase substrate and read using a FLUOstar Omega plate reader

259 (BMG Labtech) with MARS software. Infection by HCVpp was measured in the presence of

260 anti-HCV E2 MAbs, tested animal sera, pre-immune animal sera, and non-specific IgG at the

261 same dilution. Each sample was tested in duplicate or triplicate. Neutralizing activities were

262 reported as 50% inhibitory dilution (ID_{50}) values and were calculated by nonlinear regression

263 (Graphpad Prism Version 7), using lower and upper bounds (0% and 100% inhibition) as

264   constraints to assist curve fitting.

265   **Statistical analysis.**   Comparison of neutralization titers between groups (ID_{50}

266   values; **Figures 8 and 9**) was performed using Kruskal-Wallis ANOVA with Dunn's multiple

267   comparison test. Data analysis was not blinded. Differences were considered statistically

268   significant at $p < 0.05$. Comparison of serum antibody percent E2 binding competition

269   between groups (Figure 7) was performed using t-test. Statistical analyses were performed

270   using GraphPad Prism 7 software.

271

272   **RESULTS**

273   **Expression of soluble HCV E2 glycoprotein in HEK293 and Sf9 cells.** In order to

274   investigate the effect of different glycosylation patterns on the antigenicity and

275   immunogenicity of HCV E2, we produced a soluble form of E2 (sE2) lacking the

276   hydrophobic C-terminal transmembrane anchor in mammalian (HEK293) and insect (Sf9)

277   cells, which are known to attach different N-glycan moieties to proteins (42, 43). For

278   expression of sE2 in HEL293 cells, a DNA fragment encoding amino acids 384–661 of strain

279   1a HCV H77c polyprotein was cloned into the pSecTag2 vector under control of the CMV

280   promoter. The same DNA fragment was used for expression of sE2 in Sf9 insect cells using

281   the pAcGP67-B vector in which expression is controlled by the polyhedrin promoter (**Figure

282   1A**). Secreted, monomeric sE2 proteins bearing C-terminal His_6 tags were purified from

283   culture supernatants by immobilized Ni^{2+} affinity chromatography, followed by size

284   exclusion chromatography. Superdex 200 elution profiles for HEK293- and Sf9-dervied sE2

285   showed that both proteins formed oligomers and high molecular aggregates, in addition to

13

286   monomers (**Figure 1B and C**). In both cases, we isolated the peak corresponding to

287   monomeric sE2. Only monomeric HEK293- and Sf9-dervied sE2 proteins were used for all

288   subsequent analytical and immunological experiments. As shown in **Figure 1D**, the

289   HEK293- and Sf9-derived sE2 proteins migrated in non-reducing SDS-PAGE at apparent

290   molecular weights of ~75 kDa and ~40 kDa, respectively, compared to a calculated

291   molecular weight of ~34 kDa based on primary amino acid sequence. Thus, sE2 is

292   glycosylated differently by mammalian and insect cells, as expected (42, 43). In addition, we

293   examined HEK293- and Sf9-derived sE2 in SDS-PAGE under reducing conditions and

294   carried out Western blot analysis using HMAb HC33.1 (**Figure 1E**). HC33.1 recognizes a

295   linear epitope of HCV E2 (domain E) comprising residues 412–423 (7). The Western blot

296   revealed major bands at ~60 kDa and ~40 kDa for reduced HEK293- and Sf9-derived sE2,

297   respectively, which is similar to what was observed under non-reducing conditions (**Figure

298   1D**). There was no indication of significant proteolysis of either HEK293- or Sf9-derived

299   sE2 (**Figure 1E**). Moreover, reactivity with HC33.1 confirmed the identity of both purified

300   proteins as HCV E2. We therefore conclude that Sf9-derived sE2 is intact and not more

301   susceptible to proteolysis than HEK293-derived sE2, despite differences in glycosylation. To

302   pinpoint these differences, we used mass spectrometry (MS) to identify the nature of sugars

303   attached to HEK293- and Sf9-dervied sE2.

304   **N-glycosylation analysis of HCV E2 expressed in HEK293 and Sf9 cells.** Three

305   major types of mature N-glycans may be attached to glycoproteins such as HCV E2

306   produced in mammalian cells: (i) high mannose glycans, consisting of up to six mannose

307   (Man) residues attached to a trimannosyl chitobiose core ($Man_3GlcNAc_2$); (ii) complex type

14

308   glycans, composed primarily of N-acetylglucosamine (GlcNAc) and galactose (Gal)

309   residues attached to the Man₃GlcNAc₂ core, with or without sialic acid (NeuAc) residues,

310   where a fucose (Fuc) residue may be added to the first GlcNAc of the core; and (iii) hybrid

311   type glycans, which consist of Man and lactosamine (GlcNAcGal) residues, with or without

312   NeuAc, attached to the Man₃GlcNAc₂ core (42, 43). Insect expression systems, on the other

313   hand, commonly express paucimannose structures as well as core α1,3 fucosylation absent in

314   mammalian expression systems.

315   We used MS to characterize the glycan moieties attached to the 11 potential

316   N-glycosylation sites of HCV E2 derived from HEK293 and Sf9 cells. The composition and

317   microheterogeneity of the glycans attached to each N-linked site were determined by

318   LC-UV-MS/MS analyses of enzymatic digests (trypsin and chymotrypsin sequential digest)

319   of reduced and alkylated HEK293- and Sf9-derived sS2. Glycopeptide identifications were

320   made based on MS/MS fragmentation, followed by relative abundance calculation using

321   extracted ion chromatograms of the parent ion. Representative MS spectra of glycopeptide

322   447–455 of HEK293- and Sf9-derived sE2 are shown in **Figure 2**. Doubly charged

323   glycopeptides observed above 1% relative abundance are depicted to demonstrate the

324   general trend of glycans of larger composition observed in HEK293-derived sE2. A complete

325   list of the glycan compositions identified at each glycosylation site of HEK293-derived

326   versus Sf9-derived sE2, as well as the corresponding glycan populations down to 0.1%

327   relative abundance based on extracted ion chromatograms of the parent ions, is presented in

328   **Supplementary Table 1**. These 11 glycosylation sites are located at E2 positions 417

329   (designated N1), 423 (N2), 430 (N3), 448 (N4), 476 (N5), 532 (N6), 540 (N7), 556 (N8), 576

330   (N9), 623 (N10), and 645 (N11).

331   Multiple complex and high mannose type glycans were identified at 11 of 11

332   N-linked sites of HEK293-derived sE2 (**Supplementary Table 1**). As expected for a

333   glycoprotein expressed in insect cells (42, 43), the major glycoforms present on Sf9-derived

334   sE2 were paucimannose N-glycans, with or without one or two core Fuc residues. Complex

335   N-glycans were detected at all sites except N7 in Sf9-derived sE2, albeit with a lower degree

336   of extension and branching on average than in HEK293-derived sE2. Complex type glycans

337   accounted for only 1–10% of the total identified N-glycans in Sf9-derived sE2

338   (**Supplementary Table 1**). The most abundant glycoform at each site observed in LC-MS

339   analysis of HEK293- and Sf9-derived sE2 is depicted in **Figure 3**.

340   For both HEK293- and Sf9-derived sE2, the same nine N-glycan sites are near fully

341   (>95%: N1, N2, N4, N5, N6, N8 and N9) or highly (>75%: N3 and N7) glycosylated (**Figure**

342   **4**). Of note, the two exceptions (N10 and N11) exhibit relatively low levels of glycan

343   occupancy in both mammalian and insect cell-derived sE2. This concordance in extent of

344   glycosylation for sE2 from two hosts with significantly different protein N-glycosylation

345   pathways suggests that factors intrinsic to E2 itself, such as differential accessibility of

346   individual glycosylation sites to the cellular N-glycosylation biosynthetic machinery, may

347   explain the observed site-specific differences in percent glycosylation (**Figure 4**).

348   **Molecular modeling of N-glycosylation sites.** To compare the structural impact of

349   N-glycosylation in sE2 derived from HEK293 versus Sf9 cells, we modeled the most

350   abundant glycoform at each site on the E2 core structure (**Figure 5**). This analysis

351   highlighted the differential glycan sizes observed in the two expression systems, particularly

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352 those proximal to key epitopes such as N6 (**Figure 5A and B**), which is adjacent to the

353 ARC3 binding site in antigenic domain B (7) and is markedly larger for HEK293- versus

354 Sf9-expressed sE2. These models were used to calculate protein surface occlusion from

355 glycosylation (**Table 2**). Though a solvent-sized probe (1.4 Å radius) detected little

356 difference between E2 surface occlusion in the models, larger probe radii (5 and 10 Å),

357 which are more representative of antibody complementarity-determining region (CDR)

358 loops, revealed relatively high E2 occlusion (40–65% of the protein surface), with

359 HEK293-derived sE2 having somewhat higher levels of masked surface than Sf9-derived

360 sE2 (9% higher for 10 Å probe). This is similar to previous observations comparing

361 glycosylation of HEK293- versus Sf9-expressed HIV gp120, where approximately 10%

362 greater surface occlusion for HEK293-expressed gp120 was calculated using a 10 Å probe

363 radius (30). However, it should be noted that this analysis does not include E2 regions

364 missing from the crystal structure (e.g. HVR1), E2 dimerization with E1, and conformational

365 mobility of glycans and E2 residues. Nevertheless, the calculated 9% reduction in protein

366 surface coverage imparted by the Sf9 cell production system could potentially affect the

367 exposure of antibody binding sites proximal to glycans.

368 **CD81 binding and antigenicity of HCV E2 expressed in mammalian versus**

369 **insect cells.** To determine whether differential glycosylation affected binding to the CD81

370 entry receptor, we used biolayer interferometry (BLI) to measure the affinity of HEK293-

371 and Sf9-derived sE2 for recombinant CD81 (**Figures 6A and B**). Purified sE2 proteins were

372 directionally coupled to a Ni^{2+}-NTA biosensor surface through their C-terminal His_6 tags.

373 HEK293-derived sE2 bound CD81 with a K_D of 510 nM compared to 440 nM for

17

374   Sf9-derived sE2 (**Table 3**). These very similar K_D values demonstrate that both glycoproteins

375   are functional with respect to entry receptor binding. Moreover, this result indicates that the

376   proteins are properly folded since alanine-scanning mutagenesis has shown that the CD81

377   binding site comprises residues from several non-contiguous segments of the E2 polypeptide

378   chain (i.e. it is conformational in nature) (7, 8).

379   To determine whether differential glycosylation affected epitope presentation, we

380   used BLI to measure the binding of HEK293- and Sf9-expressed sE2 to a panel of eight

381   HMAbs targeting four antigenic domains on E2: CBH-4B and CBH-4G (domain A), HC-1

382   and HC-11 (domain B), HC84.26 and HC84.24 (domain D), and HCV1 and HC33.1 (domain

383   E) (7, 37) (**Figure 6C–L**). Of note, this provides a direct comparative view of 293- versus

384   Sf9-expressed sE2, while absolute measured affinity (K_D) values may reflect effects of

385   bivalent IgG binding. Five of these HMAbs bound both sE2 proteins with very similar K_Ds,

386   all in the nanomolar range: HC84.26 (1.8 nM for HEK293-derived sE2; 2.7 nM for

387   Sf9-derived sE2), HCV1 (36 and 9.3 nM), HC33.1 (50 and 47 nM), HC-1 (65 and 69 nM),

388   and HC-11 (7.0 and 25 nM) (**Table 3**). Importantly, the ability of conformation-dependent

389   HMAbs HC84.26, HC-1, and HC-11 (7) to recognize both versions of sE2 with high affinity

390   indicates that both glycoproteins are correctly folded, in agreement with our CD81 binding

391   results. The other three HMAbs bound HEK293-derived sE2 more tightly than Sf9-derived

392   sE2: HC84.24 (1.9 nM for HEK293-derived sE2; 1200 nM for Sf9-derived sE2), CBH-4B

393   (11 and 120 nM), and CBH-4G (120 nM for HEK293-derived sE2; no apparent binding to

394   Sf9-derived sE2) (**Table 3**). We attribute these affinity differences to differential

395   glycosylation, given that glycans cover ~50% of the surface of sE2 (**Figure 2**) and so are

18

396   likely to influence HMAb binding to at least some epitopes, either directly or indirectly (see

397   Discussion). These results demonstrate that increasing protein surface exposure by reducing

398   glycan size did not translate into enhanced antigenicity, at least for the eight HMAbs tested

399   here.

400   **Immunization of mice with sE2 induces serum antibodies that recognize**

401   **broadly neutralizing epitopes.** To compare the antibody responses in mice immunized

402   with the HEK293- and Sf9-derived sE2 proteins, groups of CD-1 mice (n=5) were

403   immunized with 50 μg of purified protein formulated in SAS adjuvant (oil-in-water

404   emulsion), followed by four 10 μg boosts over a course of five weeks. Serum samples were

405   collected one week after the fifth immunization (D=42), and sera tested for epitope-specific

406   responses by competitive binding ELISA using HMAbs derived from HCV-infected

407   individuals that represent the five antigenic domains of E2 (7-10): CBH-4B (domain A),

408   HC-1 (domain B), CBH-7 (domain C), HC84.26 (domain D), and HC33.3 (domain E)

409   (**Figure 7**). Based on binding competition, antibodies were elicited to all five antigenic

410   domains in both groups of mice. Both groups had relatively high levels of serum competition

411   with the antigenic domain B HMAb HC-1; this domain is associated with broadly

412   neutralizing antibodies and overlaps with the CD81 binding site (7). Comparing the two

413   groups of immunized mice, no significant differences were observed for domain-specific

414   serum antibody competition (t-test, GraphPad Prism 7).

415   **Viral neutralization against homologous and heterologous isolates of HCV.** In

416   order to assess the relative potency of the HEK293- and Sf9-derived sE2 immunogens,

417   mouse sera were tested for neutralizing antibody (nAb) titers against homologous and

19

418 heterologous isolates of HCV E1E2 using the HCVpp neutralization assay. The values are

419 expressed as serum dilution levels corresponding to 50% neutralization (ID_{50}). As shown in

420 **Figure 8**, the mean ID_{50} values of nAb against the homologous isolate (H77c) (i.e. same

421 E1E2 sequences as used in the vaccine) were 5-10 fold higher for Sf9-derived versus

422 HEK293-derived sE2 after the third (D=28), fourth (D=35), and fifth (D=42) immunizations,

423 with significant differences between groups at Days 28 and 42. However, the difference

424 diminished two weeks after the last immunization (D=49) to give approximately the same

425 values.

426   To assess the breadth of neutralization, mouse sera (Day 49) were subsequently

427 tested against a diverse panel of six HCV genotypes: gt1a, gt1b, gt2a, gt2b, gt2i, gt3a, gt4a,

428 gt5a, and gt6a, as previously described (40). As shown in **Figure 9**, animals immunized with

429 both HEK293- and Sf9-derived sE2 were able to elicit bNAbs against all of the HCVpps

430 tested in the panel, although genotypes 1a and 1b had the highest ID_{50} titers overall. The ID_{50}

431 values against the homologous H77c isolate were moderately, though not significantly,

432 higher for Sf9-derived sE2. This confirms the relative H77c neutralization titers observed at

433 this timepoint in **Figure 8**, which was performed separately using different target cells

434 (Hep3B cells, versus HuH7 cells for **Figure 9**). For most of the other isolates, there were

435 modest, though insignificant increases of ID_{50}s for the HEK293 sE2 immunized group, with

436 the exception of the J6 isolate where a significant increase for the HEK293 sE2 titers was

437 observed. It is possible that this modest decrease in breadth could be due to a more

438 pronounced nAb response to HVR1 or other variable regions induced by Sf9-expressed sE2

439 versus HEK293-expressed sE2. HVR1 has been reported as an immunodominant site for

20

440   nAbs, although the nAb response is largely strain-specific (44-47). Therefore, the lower level

441   of ID_{50} values for the other heterologous HCVpp genotypes may reflect lack of nAb

442   cross-recognition of the HVR1 sites due to sequence variation in this region.

443

444   **DISCUSSION**

445   In this study, we asked whether reducing the overall glycosylation of HCV sE2 (gt1a,

446   H77) by producing this protein in *Spodoptera* insect (Sf9) cells improved its antigenicity

447   and/or immunogenicity compared to sE2 produced in mammalian (HEK293) cells. Mass

448   spectrometry showed that all 11 predicted N-glycosylation sites were utilized in sE2 from

449   both HEK293 and Sf9 cells. Although most sites were fully or nearly fully glycosylated, two

450   sites (N10 and N11) showed low (<50%) glycan occupancy. As expected, N-glycans in

451   insect sE2 were, on average, smaller and less complex than N-glycans in mammalian sE2.

452   After modeling the most abundant glycoform at each site on the E2 structure (35), we

453   quantified glycan coverage by calculating the amount of protein surface exposed to a

454   spherical probe the size of an antibody binding site. Our analysis revealed that 64% of the

455   surface of HEK293-derived sE2 was occluded by N-glycans compared to 55% for

456   Sf9-derived sE2, corresponding to a 9% reduction in glycan shielding in insect

457   cell-expressed material. However, this reduction was not associated with improved HMAb

458   binding (**Table 3**). The ability of CD81, HC84.26, HCV1, HC33.1, HC-1 and HC-11 to bind

459   HEK293- and Sf9-derived sE2 equally well indicates that E2 recognition by these six ligands

460   does not require complex type glycans.

21

461   By contrast, HC84.24, CBH-4B and CBH-4G bound Sf9-derived sE2 less well than

462   HEK293-derived sE2. It is not particularly surprising that some HMAbs bind these two

463   glycoproteins with different K_Ds given that: 1) Sf9- and HEK293-derived sE2 are

464   glycosylated quite differently; 2) glycans cover ~50% of the surface of E2 (**Figure 5**) and so

465   are likely to influence HMAb binding to at least some epitopes, either directly by forming

466   part of such epitopes or indirectly by being proximal to them; 3) the glycosylation profile of

467   natural E2 (i.e. E2 produced in HCV-infected human liver cells) is probably more similar to

468   that of HEK293-derived sE2 than of Sf9-derived sE2; and 4) the HMAbs used in this study

469   were derived from individuals naturally infected with HCV. A previous global epitope

470   mapping study (7) found that mutation of certain E2 glycan sites, including glycan N10

471   which is located in the E2 back layer near the mapped CBH-4B and CBH-4G binding sites

472   (**Figure 5**) and shows reduced occupancy for Sf9-expressed sE2 (**Figure 4**), affects E2

473   binding for these and other HMAbs. More revealing with respect to structural and functional

474   integrity is the ability of Sf9- and HEK293-derived sE2 to bind the CD81 receptor with

475   nearly identical K_Ds (**Figure 6A and B**).

476   As observed for recombinant HIV-1 envelope trimer produced in HEK293 cells (23),

477   our LC-UV-MS/MS analysis of HEK293-derived sE2 revealed the presence of

478   under-processed N-glycans that remain in oligomannose form (Man$_{5-9}$GlcNAc$_2$), possibly

479   because the high density of glycans on the E2 surface imposes steric constraints that limit the

480   actions glycan-processing enzymes in the Golgi compartment. We detected high mannose

481   N-glycans at all 11 N-linked sites of HEK293-derived sE2. However, except at sites N8 and

482   N11, the large majority of total identified glycoforms at the other nine N-linked sites were

483   complex type glycans, which are a hallmark of passage through the Golgi apparatus. In this

484   respect, the overall glycosylation profile of HEK293-expressed sE2 resembles that of E1E2

485   heterodimers associated with HCV pseudoparticles (HCVpp), which also display a majority

486   of complex type glycans (16, 48). By contrast, E1E2 heterodimers associated with cell

487   culture-derived HCV (HCVcc) contain a more balanced mixture of high mannose and

488   complex type glycans. This difference suggests that both HEK293-expressed sE2 and

489   HCVpp-associated E1E2 are more accessible to Golgi glycan-processing enzymes than

490   HCVcc-associated E1E2, possibly because, in the HCVcc system, the E1E2 glycoprotein is

491   associated with nascent viral particles as it travels through the secretory pathway, thereby

492   restricting its exposure to Golgi enzymes.

493   In a previous study of HCV sE2 produced in mammalian (CHO) cells (49), complex

494   glycans were detected at only 2 of 11 N-linked sites (N2 and N3); all 9 other sites were

495   occupied by high or paucimannose N-glycans. By contrast, we identified complex glycans at

496   all 11 N-linked sites. Moreover, a complex glycan was the most abundant glycoform at 6 of

497   these 10 sites (N1, N3, N4, N5, N6, and N9) (**Figure 3**). These surprisingly large differences

498   in glycosylation profiles may result from the use of different mammalian cell lines for sE2

499   expression: HEK293 here versus CHO in the earlier study (49). The substantially higher

500   content of complex N-glycans in HEK293-derived sE2 indicates more extensive processing

501   of oligomannoses during passage of this particular glycoprotein through the Golgi

502   compartment of HEK293 than CHO cells.

503   We found that differences in N-glycosylation of sE2 produced in HEK293 versus Sf9

504   cells did not appreciably alter recognition by most HMAbs. Immunogenicity studies in mice

505   showed that similar polyclonal antibody responses were elicited against domains A–E by

506   competition binding analysis. Neutralizing antibody titers showed that Sf9-derived sE2 was

507   a more potent immunogen than HEK293-derived sE2 when tested against the homologous

508   H77c isolate. However, both HEK293- and Sf9-derived sE2 elicited robust neutralizing

509   antibody titers when tested against a diverse panel of HCVpp genotypes, although the overall

510   titers trended higher for HEV293-derived sE2.

511   Our results differ markedly from those reported previously in a comparison of

512   HEK293- and *Drosophila* S2-derived sE2 (31). In that study, the insect material was found to

513   be a more potent immunogen against a diverse panel of heterologous isolates. Whether or not

514   there is a difference in glycosylation between E2 proteins derived from *Drosophila* versus

515   *Spodoptera* cells that could affect immunogenicity is an open question. At present, most

516   knowledge of insect glycobiology comes from the *Drosophila* model (43), and it cannot be

517   excluded that glycosylation in *Spodoptera* may differ in certain aspects. However, our

518   LC-UV-MS/MS analysis of Sf9-expressed sE2 revealed a glycosylation pattern dominated

519   by paucimannose N-glycans, with or without one or two core fucose residues, which is

520   entirely consistent with what would be expected for sE2 produced in S2 cells (42, 43).

521   Therefore, it is unlikely that differences in N-glycosylation can explain the apparent ability

522   of S2-derived sE2, but not Sf9-derived sE2, to elicit higher titers of bNAbs against diverse

523   HCV genotypes than HEK293-derived sE2 (31).

524   Several other factors may be responsible for the discrepancy between our results and

525   those of Li et al. (31). One is the different mouse strains used here (CD-1) and in the Li et al.

526   study (BALB/c). Although CD-1 mice are outbred and have more genetic diversity than

527   inbred BALB/c mice, this likely did not contribute to the differences seen between the two

528   studies because in this study the B cell responses to sE2 are similar using CD-1 and BALB/c

529   mice (data not shown). It is also possible that the different adjuvants used by us (SAS) and Li

530   et al. (31) (alum/CpG) significantly affected the immune response to otherwise very similar

531   insect cell-derived sE2 glycoproteins. For example, in a study comparing enzymatically

532   demannosylated HIV-1 gp120 produced in CHO cells with its untreated counterpart,

533   demannosylation was found to increase immunogenicity in an adjuvant-dependent manner.

534   Thus, when administered in alum, demannosylated gp120 was more immunogenic than the

535   untreated glycoprotein, but this difference disappeared when Quil-A, a saponin-based

536   adjuvant, was used (50). In this study, we used SAS as the adjuvant, which is an oil-in-water

537   emulsion compared to alum/CpG used by Li et al. (31). It has been previously reported that

538   innate and adaptive immune responses can differ between the two adjuvant systems

539   depending on the antigen used, route of administration, and schedule (51). Nevertheless,

540   both adjuvant systems are capable of eliciting robust B cell responses so this also may not

541   account for the breadth of neutralization differences seen between the two studies. Another

542   difference with the Li et al. study and ours is that our sE2 protein was derived from the gt1a

543   isolate, H77, while their sE2 was from the gt1b isolate, Con1b. These have 22% sequence

544   divergence at the amino acid level, which may impact immunogenicity of specific epitopes

545   or E2 overall; recent studies have demonstrated that HVR1 (amino acid divergence is 59% in

546   that region between H77 and Con1b, with 16 out of 27 amino acids changed) and other E2

547   sites are responsible for differences in neutralization sensitivity for sets of E2-targeting

548   bnAbs (52, 53). Finally, we tested neutralization using HCVpp, whereas Li et al. (31) used

549 HCVcc. This difference is potentially significant because, as noted above, HCVpp and

550 HCVcc have different glycosylation profiles (16, 48). Any one or any combination of the

551 above differences could account for the discrepancies between the two studies.

552   While conceptually very appealing, the idea that altering glycans on viral envelope

553 glycoproteins can actually yield superior immunogens for vaccine development remains to

554 be demonstrated, despite considerable effort by a number of laboratories and some promising

555 results. For example, although HIV-1 gp120 produced in Sf9 cells displayed increased

556 binding to antibodies targeting the CD4 binding site (30), the new generation of HIV-1

557 envelope trimers for vaccine use is expressed in mammalian, not insect, cells (54). Similarly,

558 antigens based on HIV-1 envelope glycan deletion mutants have proved to be less effective

559 than, or at best equivalent to, wild-type antigens at generating neutralizing antibody

560 responses possessing both breadth and potency (17, 28, 55, 56). At present, selective

561 deglycosylation of HIV-1 envelope trimers is under investigation as a strategy for activating

562 naïve B cells expressing germline precursors of antibodies that target the CD4 binding site

563 (25, 26). Given our findings that global alteration of HCV E2 glycosylation by expression in

564 different cellular hosts did not appreciably affect antigenicity or overall immunogenicity,

565 future efforts will focus on complete deletion, rather than only modification, of specific

566 N-glycans in order to increase the exposure of virus neutralizing epitopes on E2 to the

567 humoral immune system. For example, the removal of N-glycans at E2 positions 417 (N1)

568 and 532 (N6) has led to increased sensitivity of the mutant isolates to neutralization by MAbs

569 to antigenic domain E and B, respectively (14, 15). A focused effort to eliminate these and

570   other glycans that affect antibody binding to domains B, D and E might lead to an improved

571   immunogen to elicit their associated bNAbs.

572

573

588   **FIGURE LEGENDS**

589   **Figure 1.** Expression of soluble HCV E2 (sE2) derived from mammalian (HEK293) and

590   insect (Sf9) cells. (A) Schematic representation of sE2 expression cassettes used in

591   mammalian (HEK293-sE2) and insect cell (Sf9-sE2) vectors. A sequence encoding sE2

592   (amino acids 384–661) from genotype 1a (H77) was cloned into the vector pSecTag2

593   in-frame with an immunoglobulin light chain κ signal peptide sequence and fused with a

594   C-terminal His$_6$ tag. Recombinant plasmid was used to transfect HEK293F cells. The same

595   sequence was cloned into the baculovirus expression vector pAcGP67-B with a polyhedrin

596   (Polh) promoter. The construct was transfected into Sf9 cells together with BaculoGold

597   Linearized to produce sE2. (B) Superdex 200 size exclusion chromatography profile of

598   HEK293-derived sE2 following HisTrap Ni^{2+}-NTA affinity chromatography. Peak (red

599   arrow) corresponding to monomeric sE2 was isolated for analytical and immunization

600   studies. (C) Superdex 200 chromatography profile of Sf9-derived sE2 following HisTrap

601   Ni^{2+}-NTA chromatography. Peak (red arrow) corresponding to monomeric sE2 was isolated

602   for all further experiments. Peak marked by red arrow corresponds to monomeric sE2. (D)

603   Non-reducing SDS-PAGE (10%) of purified HEK293- and Sf9-derived sE2 proteins used for

604   analytical characterization and immunization studies (Coomassie blue G-250 staining). (E)

605   Western blot analysis of purified HEK293- and Sf9-derived sE2 proteins. Proteins were

606   separated on SDS-PAGE (4–15%) after reduction by β-mercaptoethanol and boiling for 10

607   min. Proteins were then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories).

608   The membrane was probed with anti-HCV E2 HMAb HC33.1 (7) at 4.5 μg/ml, followed by

609   secondary goat anti-human IgG-HRP conjugate at 1:5000 dilution.

29

610

Figure 2. Representative ESI-MS of chymotryptic peptide FNSSGCPER (amino acids 447–

455) from HCV sE2. (A) Glycopeptide heterogeneity observed on FNSSGCPER peptide

from HEK293-expressed sE2 (RA ≥ 1% listed in spectrum and RA ≥ 0.1% listed in table).

(B) Glycopeptide heterogeneity observed on FNSSGCPER peptide in Sf9-expressed E2 (RA

≥ 1% listed in spectrum and RA ≥ 0.1% listed in table). Note that only doubly charged

glycopeptides are labeled in the spectrum for clarity for both panels (A) and (B).

617

Figure 3. Schematic representation of HCV sE2 chymotryptic glycopeptides depicted with

the most abundant glycan observed during LC-MS analysis of HEK293 (top glycoform) and

Sf9 (bottom glycoform) sE2 proteins. In cases where the most abundant proteoform was

aglycosylated, the most abundant glycosylated form was used (**Supplementary Table 1**).

"…" represents flanking amino acid sequence not shown for brevity. N-acetylglucosamine,

blue squares; fucose, red triangles; mannose, green circles; galactose, yellow circles; sialic

acid, purple diamonds.

625

Figure 4. Comparison of percent glycosylation of N-glycan sites for mammalian

cell-expressed (HEK293) and insect cell-expressed (Sf9) sE2 glycoproteins. Most N-glycan

sites are fully or nearly fully glycosylated, with the exception of N10 (N623) and N11

(N645), where N-glycan sites have relatively lower glycan occupancy for Sf9-derived eE2,

whereas glycan N3 (N430) has lower glycan occupancy in HEK293 cells.

631

632 **Figure 5.** Modeled structural impact of glycosylation for HEK293- versus Sf9-expressed

633 sE2. Structures of the most abundant glycoforms for HEK293 (A, C) and Sf9 (B, D) sE2

634 were modeled using Rosetta (36) onto the E2 core crystal structure (PDB code 4MWF) (35).

635 Glycans are shown as tan, slate, and yellow sticks and labeled, and E2 is shown as surface

636 and cartoon, with views of E2 front layer (A, B) and back layer (C, D). For reference,

637 neutralizing antibody footprints on E2 are colored blue, magenta, and green, based on the

638 epitope-bound crystallographic structures of HC33.1 (PDB code 4XVJ), AR3C (PDB code

639 4MWF), and HC84.26.5D (PDB code 4Z0X) antibodies, respectively. Colored E2 residues

640 indicate those within 5.0 Å of bound antibody, and shared E2 contact residues between

641 antibodies are colored according to antibody with highest E2 residue burial. Residues from

642 antigenic domain E were modeled at the N-terminus of the E2 core structure using Rosetta

643 (36). Glycan N5, which is within a region that is missing from the E2 core crystal structure, is

644 not shown.

645

646 **Figure 6**. BLI analysis of CD81 and antibody binding to HCV sE2 from HEK293 and Sf9

647 expression systems. (A) Sensograms (left) for CD81 binding to immobilized

648 HEK293-derived sE2. CD81 concentrations were 5000, 4000, 2500, 2000, 1250, 1000, 625,

649 500, 312.5, 250, 156.25, 125, 78.125, 62.5, 39.06 nM. Steady state analysis graph (right)

650 gave K_D = 510 ± 22 nM. (B) Sensograms (right) for CD81 binding to immobilized

651 Sf9-derived sE2. CD81 concentrations were 5000, 4000, 2500, 2000, 1250, 1000, 625, 500,

652 312.5, 250, 156.25, 125, 78.125, 62.5, 39.06 nM. Steady state analysis graph (right) gave K_D

653 = 440 ± 38 nM. (C) Sensograms (left) for HC84.26 (domain D-specific HMAb) binding to

31

654   immobilized HEK293-derived sE2. HC84.26 concentrations were 5, 2.5, 1.25, 0.625, 0.3125

655   and 0.156 nM. Steady state analysis graph (right) gave $K_D = 1.8 \pm 0.5$ nM. (D) Sensograms

656   (left) for HC84.26 binding to immobilized Sf9-derived sE2. HC84.26 concentrations were

657   10, 5, 2.5, 0.625, 0.3125 and 0.156 nM. Steady state analysis graph (right) gave $K_D = 2.7 \pm$

658   0.8 nM. (E) Sensograms (left) for HC84.24 (domain D-specific HMAb) binding to

659   immobilized HEK293-derived sE2. HC84.24 concentrations were 20, 15, 10, 7.5, 5, 3.75,

660   2.5, 1.875, 0.9375, 0.625, 0.469, 0.3125, 0.234 and 0.156 nM. Steady state analysis graph

661   (right) gave $K_D = 1.9 \pm 0.3$ nM. (F) Sensograms (left) for HC84.24 binding to immobilized

662   Sf9-derived sE2. HC84.24 concentrations were 1000, 750, 500, 375, 250, 187.5, 125, 93.8,

663   62.5, 46.9, 31.6 and 23.4 nM. Steady state analysis graph (right) gave $K_D = 1200 \pm 290$ nM.

664   (G) Sensograms (left) for CBH-4B (domain A-specific HMAb) binding to immobilized

665   HEK293-derived sE2. CBH-4B concentrations were 100, 50, 25, 12.5, 6.25 and 3.125 nM.

666   Steady state analysis graph (right) gave $K_D = 11 \pm 1.7$ nM. (H) Sensograms (left) for

667   CBH-4B binding to immobilized Sf9-derived sE2. CBH-4B concentrations were 50, 25,

668   12.5, 6.25 and 3.13 nM. Steady state analysis graph (right) gave $K_D = 120 \pm 11$ nM. (I)

669   Sensograms (left) for HCV1 (domain E-specific HMAb) binding to immobilized

670   HEK293-derived sE2. HCV1 concentrations were 200, 150, 100, 75, 50, 37.5 and 25 nM.

671   Steady state analysis graph (right) gave $K_D = 36 \pm 3.0$ nM. (J) Sensograms (left) for HCV1

672   binding to immobilized Sf9-derived sE2. HCV1 concentrations were 25, 20, 15, 10, 5, 2.5,

673   1.25 and 0.078 nM. Steady state analysis graph (right) gave $K_D = 9.3 \pm 2.0$ nM. (K)

674   Sensograms (left) for HC-1 (domain B-specific HMAb) binding to immobilized

675   HEK293-derived sE2. HC-1 concentrations were 200, 175, 150, 75, 50, 37.5, 25, 12.5, 6.25,

676 3.125, 1.56, 0.78, 0.39 and 0.195 nM. Steady state analysis graph (right) gave $K_D = 65 \pm 10$

677 nM. (L) Sensograms (left) for HC-1 binding to immobilized Sf9-derived sE2. HC-1

678 concentrations were 200, 175, 150, 75, 50, 37.5, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and

679 0.195 nM. Steady state analysis graph (right) gave $K_D = 69 \pm 6.5$ nM.

680

681 **Figure 7.** Competition binding analysis of immune sera corresponding to domains A–E. (A)

682 HEK293-derived sE2 immunized CD-1 mice. (B) Sf9-derived sE2 immunized CD-1 mice.

683 Sera from day 42 were tested for binding competition using antibodies representing antigenic

684 domains A–E (7), with mean percent competition shown with black bars. All values were

685 normalized by subtracting percent competition values from control (pre-immune) sera,

686 which were measured individually for each mouse. Antibodies tested were CBH-4B (domain

687 A), HC-1 (domain B), CBH-7 (domain C), HC84.26 (domain D), and HC33.4 (domain E).

688

689 **Figure 8.** Kinetics and magnitude of nAb titers against homologous H77 strain. HCV

690 pseudoparticles (HCVpp) were generated by co-transfection of HEK293T cells with HCV

691 E1E2, MLV Gag-Pol packaging vector, and luciferase reporter plasmid as previously

692 described (40). Titrations of HCVpp were performed on Hep3B cells, and luciferase activity

693 measured in relative light units (RLUs) using SpectraMax M3. Percent neutralization was

694 calculated as $100 \times [1 - yRLUtest/RLUcontrol)]$. nAb titers in animal sera are reported as

695 50% inhibitory dilution (ID_{50}) values, calculated using nonlinear curve fitting in GraphPad

696 Prism. Neutralization kinetics were determined by inhibition of homologous HCVpp (H77)

697 using serial dilutions of serum collected on days 28, 35, 42 and 49. Blue dots represent serum

698   samples from HEK293 sE2 immunized mice, green dots represent samples from Sf9 sE2

699   immunized mice, and black bars indicate geometric means. One Sf9 E2 immunized mouse

700   had insufficient day 35 sera available for testing, thus four rather than five points are shown

701   for that group and day. P-values were determined using GraphPad Prism 7 with

702   Kruskal-Wallis ANOVA, and significant p-values between immunized groups are indicated

703   (*: $p < 0.05$).

704

705   **Figure 9.** Breadth of neutralization against HCV genotypes 1–6. HCVpp generated with

706   functional E1E2 clones derived from six diverse HCV genotypes, gt1a (H77c, UNKP1.1.1),

707   gt1b (UNKP1.20.3, UNKP1.21.2), gt2a (J6, JFH), gt2b (UNKP2.4.1), gt2i (UNKP2.1.2), gt3

708   (UNKP3.2.2), gt4 (UNKP4.2.1), gt5 (UNKP5.1.1), and gt6 (UNKP6.1.1), were assessed for

709   their neutralization sensitivity (50% neutralization titer; ID_{50}) to CD-1 mouse serum samples

710   immunized with HEK293-derived sE2 (blue dots) and Sf9-derived sE2 (green dots), using

711   HuH7 target cells. Geometric mean ID_{50} values are shown as black bars. P-values were

712   determined using GraphPad Prism 7 with Kruskal-Wallis ANOVA, and significant p-values

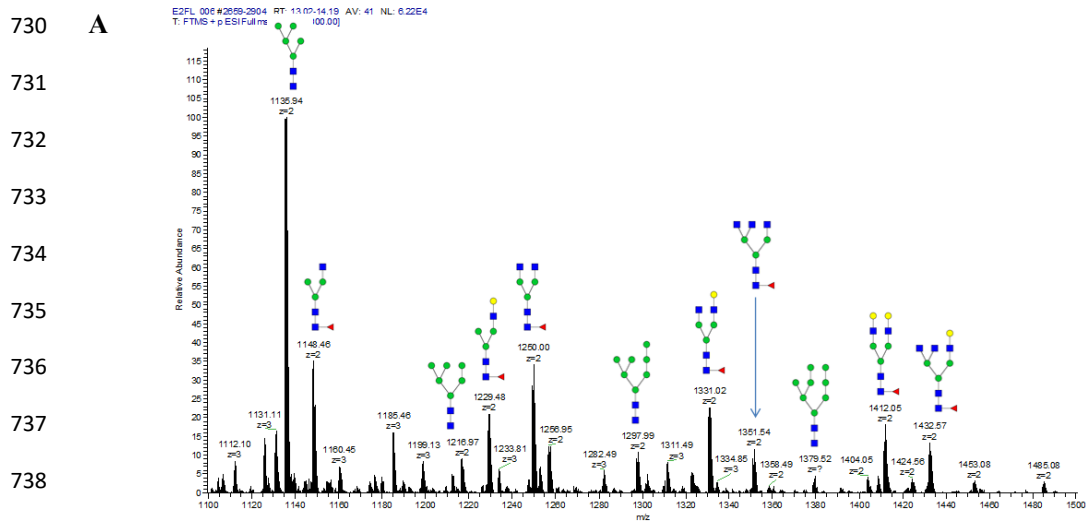713   between immunized groups are indicated (*: $p < 0.05$).

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34

715 **Figure 1.**

716 **A**

721 **B**
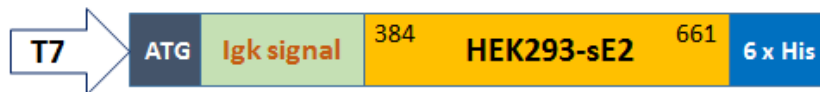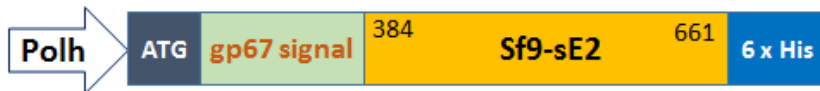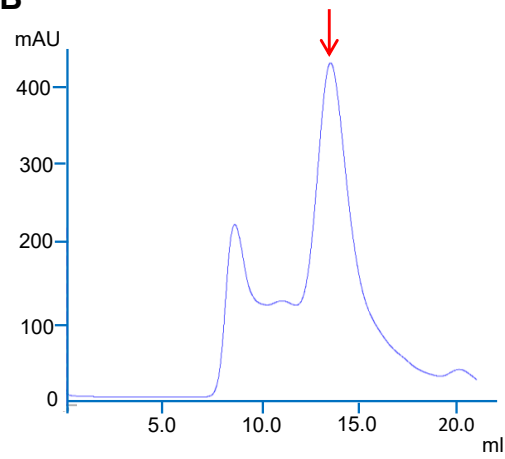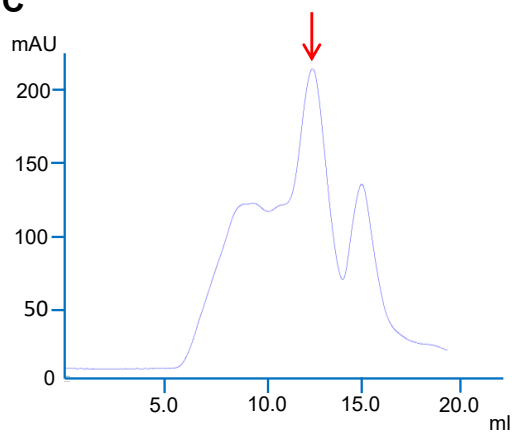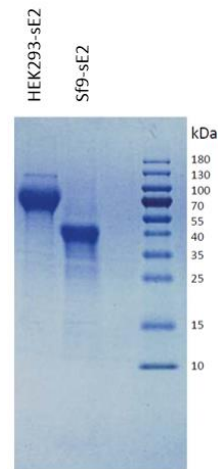
724 **C**

D

E

35

729 **Figure 2.**

730 **A**
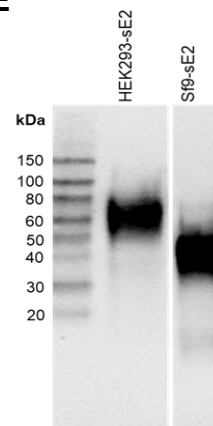
Glycopeptide % relative abundance observed on FNSSGCPER peptide in HEK expressed E2.

														Aglycosylated
17.4	1.47	1.27	0.29	0.49	4.95	10.2	2.27	10.5	10.6	4.43	23.1	8.74	3.86	0.36

744 **B**
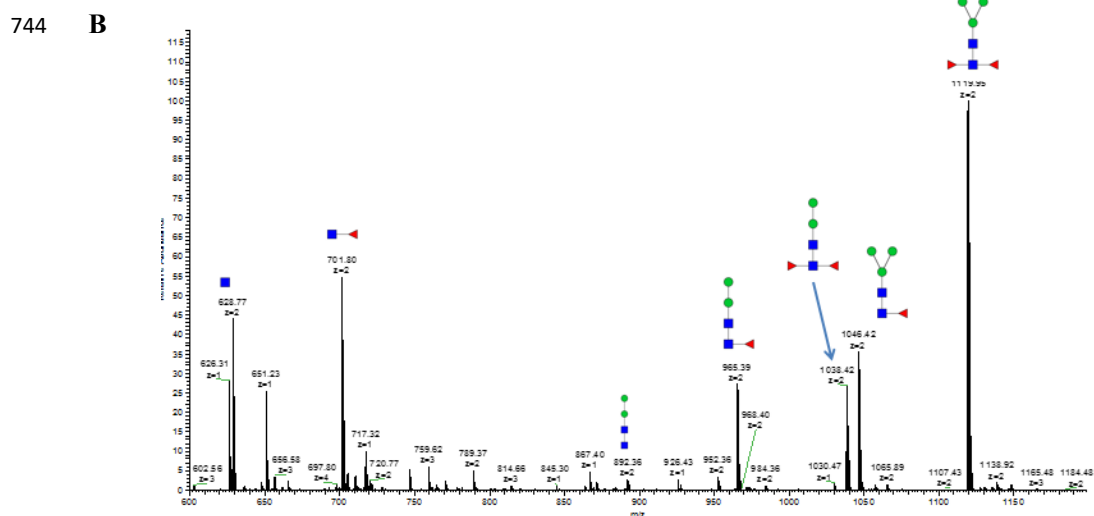
Glycopeptide % relative abundance observed on FNSSGCPER peptide in Sf9 expressed E2.

												Aglycosylated
0.35	0.09	5.7	9.65	1.39	7.22	0.39	0.91	10.66	0.28	12.74	49.85	0.78

36

745 **Figure 3.**
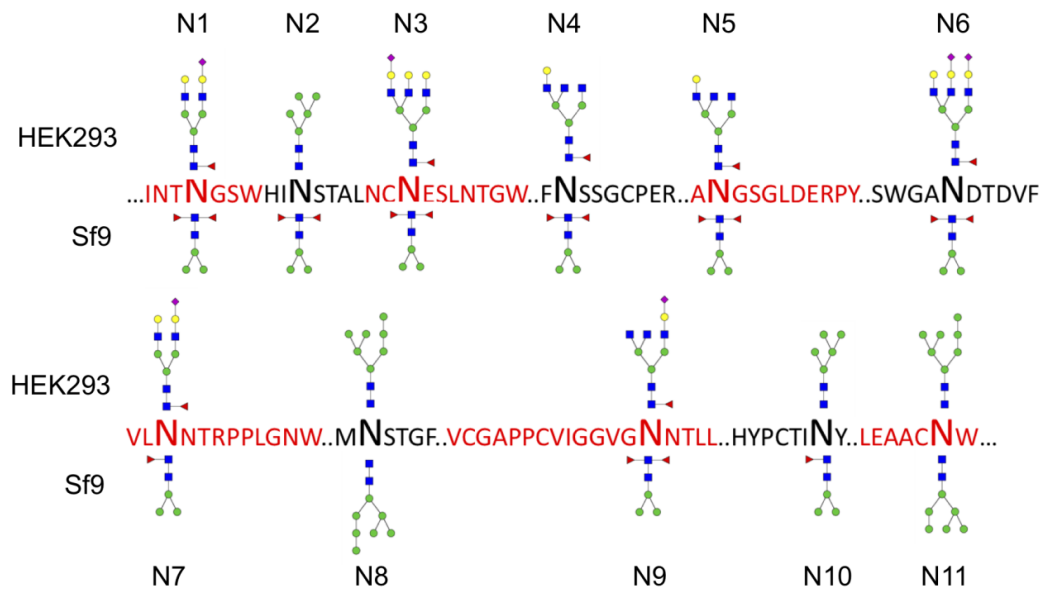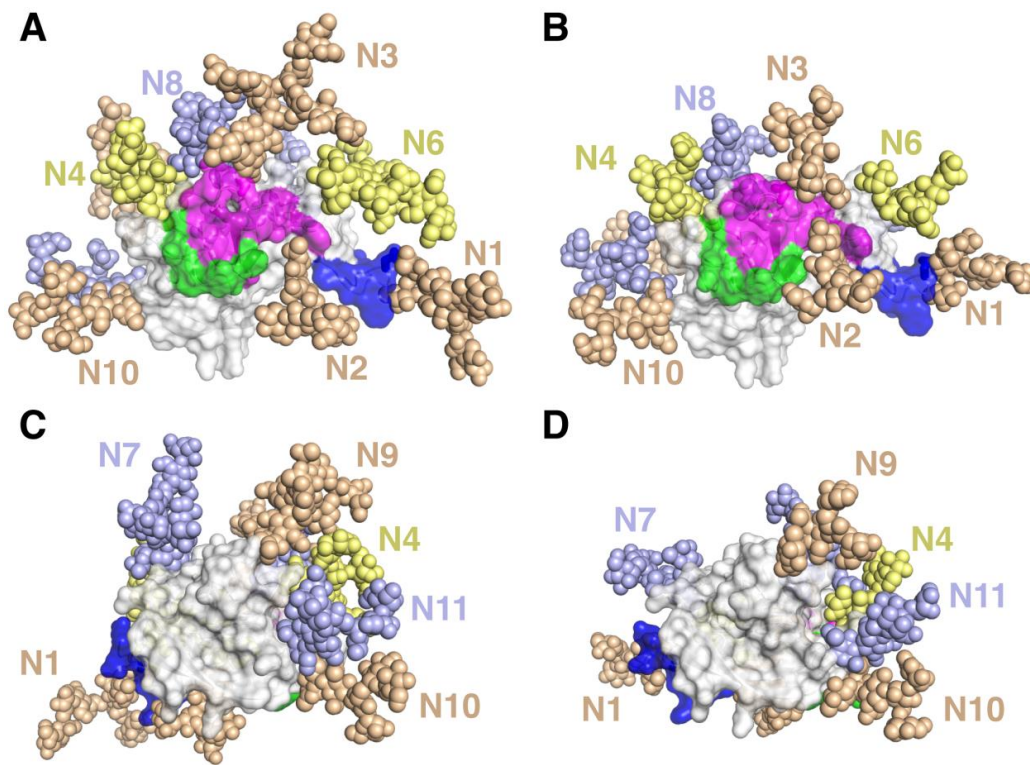

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748   **Figure 4.**

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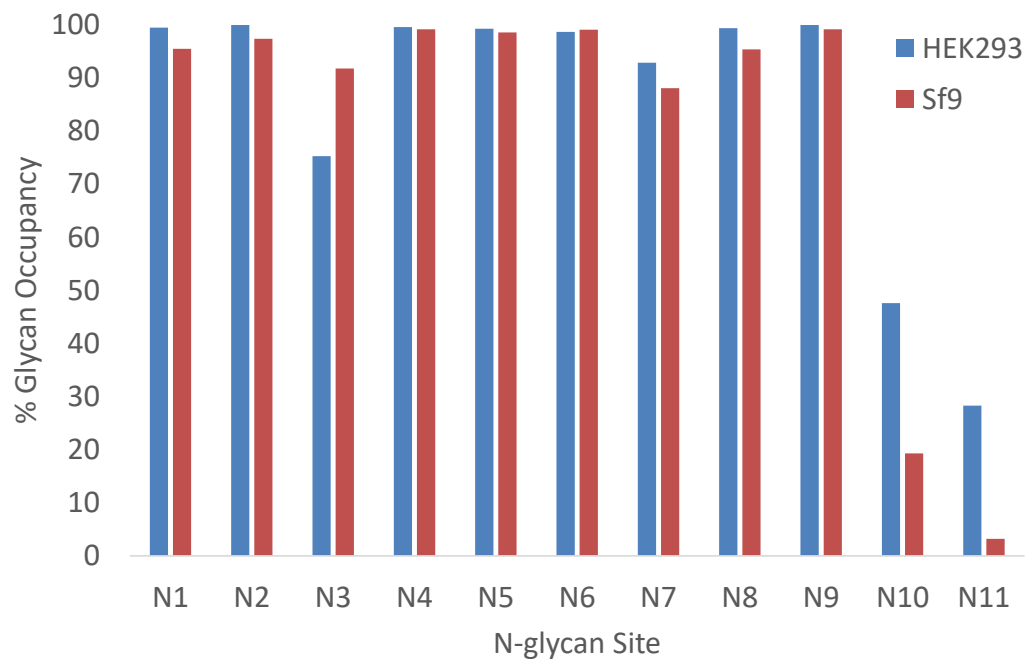
751   **Figure 5.**

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755 **Figure 6.**

A HEK293 E2 vs CD81

R² = 0.997
K_D = 510 ± 22 nM

B Sf9 E2 vs CD81

R² = 0.984
K_D = 440 ± 38nM

C HEK293 E2 vs HC84.26

R² = 0.965
K_D = 1.8 ± 0.5 nM

D Sf9 E2 vs HC84.26

R² = 0.965
K_D = 2.7 ± 0.7 nM

E HEK293 E2 vs HC84.24

R² = 0.962
K_D = 1.9 ± 0.3 nM

F Sf9 E2 vs HC84.24

R² = 0.980
K_D = 1200 ± 29 nM

G HEK293 E2 vs CBH-4B

R² = 0.971
K_D = 11 ± 1.7 nM

H Sf9 E2 vs CBH-4B

R² = 0.926
K_D = 120 ± 11 nM

I HEK293 E2 vs HCV1

R² = 0.985
K_D = 36 ± 3.0 nM

J Sf9 E2 vs HCV1

R² = 0.946
K_D = 9.3 ± 2.0 nM

K HEK293 E2 vs HC-1

R² = 0.980
K_D = 65 ± 1.0 nM

L Sf9 E2 vs HC-1

R² = 0.993
K_D = 69 ± 6.5 nM

40

756 **Figure 7.**

A   HEK293 sE2 immunized mice

B   Sf9 sE2 immunized mice

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759   **Figure 8.**

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762 **Figure 9.**
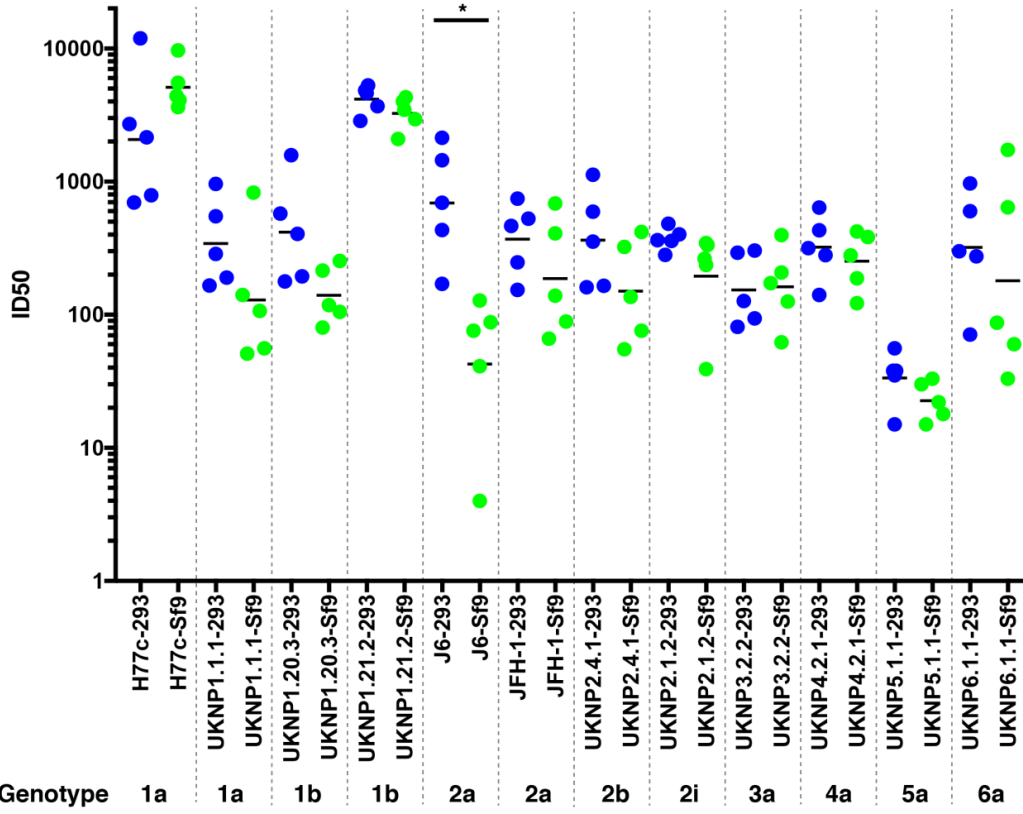
763 Genotype
764

Table 1. Analytical gradient

Time	(mL/min)	% B (0.1 % FA in ACN)	Divert Valve	MS Data Collection
0	0.25	1	Waste	No
2	0.25	1	MS	Yes
6	0.25	10	MS	Yes
70	0.25	35	Waste	No
72	0.25	90	Waste	No
77	0.25	90	Waste	No
79	0.25	1	Waste	No
81	0.25	1	Waste	No
83.5	0.25	10	Waste	No
91.5	0.25	45	Waste	No
93	0.25	90	Waste	No
99	0.25	90	Waste	No
101	0.25	1	Waste	No
115	0.25	1	Waste	No

765

766   **Table 2.** Calculated E2 percent surface occlusion by most abundant N-glycans from HEK293

767   and Sf9 expression systems modeled onto the E2 core structure.

Probe Radius (Å)	HEK293	Sf9
1.4	20%	21%
5	49%	43%
10	64%	55%

768

769

Table 3. Affinities of HEK293 sE2 and Sf9 sE2 for CD81 and HMAbs

Ligand	HEK293 sE2		Sf9 sE2	
	K_D (nM)	R^2	K_D (nM)	R^2
CD81	510 ± 22	0.997	440 ± 38	0.984
HC84.26[a]	1.8 ± 0.5	0.965	2.7 ± 0.8	0.965
HC84.24[a]	1.9 ± 0.3	0.962	1200 ± 29	0.980
CBH-4B[b]	11 ± 1.7	0.971	120 ± 11	0.926
CBH-4G[b]	120 ± 33	0.949	no binding	–
HCV1[c]	36 ± 3.0	0.985	9.3 ± 2.0	0.946
HC33.1[c]	50 ± 9.9	0.979	47 ± 9.9	0.927
HC-1[d]	65 ± 1.0	0.980	69 ± 6.5	0.993
HC-11[d]	7.0 ± 1.3	0.975	25 ± 4.4	0.974

[a]Specific for antigenic domain D. [b]Specific for antigenic domain A.
[c]Specific for antigenic domain E. [d]Specific for antigenic domain B.

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