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Identification of novel neutralizing determinants for protection against HCV

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Abstract

Background and Aims: HCV evasion of neutralizing antibodies (nAb) results in viral persistence and poses challenges to the development of an urgently needed vaccine. N-linked glycosylation of viral envelope proteins is a key mechanism for such evasion. To facilitate rational vaccine design, we aimed to identify determinants of protection of conserved neutralizing epitopes.

Approach and Results: Using a reverse evolutionary approach, we passaged genotype 1a, 1b, 2a, 3a, and 4a HCV with envelope proteins (E1 and E2) derived from chronically infected patients without selective pressure by nAb in cell culture. Compared with the original viruses, HCV recombinants, engineered to harbor substitutions identified in polyclonal cell culture–passaged viruses, showed highly increased fitness and exposure of conserved neutralizing epitopes in antigenic regions 3 and 4, associated with protection from chronic infection. Further reverse genetic studies of acquired E1/E2 substitutions identified positions 418 and 532 in the N1 and N6 glycosylation motifs, localizing to adjacent E2 areas, as key regulators of changes of the E1/E2 conformational state, which governed viral sensitivity to nAb. These effects were independent of predicted glycan occupancy.

Conclusions: We show how N-linked glycosylation motifs can trigger dramatic changes in HCV sensitivity to nAb, independent of glycan occupancy.

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These findings aid in the understanding of HCV nAb evasion and rational vaccine design, as they can be exploited to stabilize the structurally flexible envelope proteins in an open conformation, exposing important neutralizing epitopes. Finally, this work resulted in a panel of highly fit cell culture infectious HCV recombinants.

INTRODUCTION

Viral evasion from neutralizing antibodies (nAb) compromises vaccine efficacy, leads to failure of treatment with monoclonal antibodies (mAb), and allows viruses to establish recurring and chronic infections. HCV has circulated in the human population for hundreds of years,[1] allowing for extensive host adaptation with development of efficient strategies to evade nAb contributing to viral persistence and posing an obstacle to the development of an urgently needed vaccine[2–4] (www.who.int/publications/i/item/9789240027077). A prophylactic vaccine is likely required to achieve the WHO target to eliminate viral hepatitis as a major public health threat (https://apps.who.int/iris/bitstream/handle/10665/246177/WHO-HIV-2016.06-eng.pdf).

Worldwide, approximately 1.5 million individuals are infected with HCV annually. In 80% of cases the virus persists, resulting in at least 58 million chronic infections with an increased risk for liver cirrhosis and HCC, ultimately resulting in about 290,000 deaths annually[2] (www.who.int/publications/i/item/9789240027077). Antivirals have limited impact on this epidemic due to lack of symptoms before development of severe and often irreversible liver disease and lack of screening programs. Other reasons are high treatment cost, re-infections, and emerging antiviral resistance.[2,4]

HCV is a small enveloped positive single-stranded RNA virus within the *Flaviviridae* family. Due to the high error rate of its RNA polymerase and its long evolution in humans, HCV shows great genetic heterogeneity.[1,5] The eight genotypes and > 90 subtypes differ in 30% and 20% of their sequence, respectively.[6,7] Genotypes 1, 2, and 3 cause >80% of infections worldwide, while genotype 4, 5, and 6 infections cluster in the Middle East, South Africa and South East Asia, respectively.[5] Genotypes 7 and 8 were so far detected in few individuals. In contrast to the HCV nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), Core and the envelope glycoproteins E1 and E2 act as structural proteins incorporated in the virion. E1 [amino acid (aa) 192–383] and E2 (aa 384–746) mediate viral entry via a receptor complex, including cluster of differentiation 81 (CD81) and scavenger receptor class B type 1 (SR-BI), and are the main target of nAb[8] (unless otherwise indicated aa positions refer to the H77 reference polyprotein; GenBank accession number AF009606). E2 core structures revealed a central beta-sandwich flanked by a front layer (aa 424–459) and a back layer (aa 597–645).[9,10] Recently solved E2 ectodomain structures included conformationally more flexible regions such as hypervariable region 1 (HVR1, aa 384–410), antigenic site 412 (AS412 or epitope I, aa 412–423), and the CD81 binding loop (aa 519–535) (Figure S1).[11,12] The CD81 binding site comprises AS412, the front layer, the CD81 binding loop, and parts of the back layer.[11–13]

NAb, induced by vaccination or natural infection, are important immune correlates of protection against viral infections. For HCV, early emergence of broadly nAb (bnAb), neutralizing diverse HCV variants, is associated with protection against chronic infection in humans.[14-16] Important conserved linear and conformational neutralizing epitopes localize to the E2 CD81 binding surface, suggesting blocking of E2-CD81 interactions as correlate for efficient viral neutralization.[3,8] Viral escape from nAb targeting the main conserved linear epitope AS412 occurs rapidly by point mutations in the target sequence.[17-20] In contrast, clinical protection from chronic infection was associated with nAb targeting conserved conformational epitopes[21,22] in (i) antigenic region 3 (AR3) in E2 (aa 426–443 and 529–553, thus formed by the E2 front layer and the CD81 binding loop regions and overlapping with the CD81 receptor binding site) or (ii) antigenic region 4 (AR4), likely localizing to E2 but requiring E1 for stable presentation (described to involve E2 aa 657, 692, and 698 in functional analysis and aa 623, 646/8/9, 667, 671/6, and 696/8 in a recent E1/E2 structure, thus formed by the E2 back layer and stem).[8,23-26] Other neutralizing epitopes localize to antigenic region 2 (AR2) in the E2 back layer (involving aa 625 and 628) and antigenic region 5 (AR5), localizing to the E1/E2 interface (involving aa 639 and 665).

Conserved neutralizing epitopes are likely occluded in the E1/E2 complex, possibly explaining the suboptimal capacity of HCV envelope protein–based vaccines to induce bnAb as well as the high propensity of HCV to escape nAb and to establish chronic infections.[27,28] Epitope protection was suggested to be mediated by steric shielding by extensive N-linked glycosylation of the HCV envelope proteins,[29] as observed for other enveloped viruses.[30,31] In addition, N-linked glycosylation might stabilize a closed, neutralization-resistant conformational state of the envelope

proteins.[32] HCV has 4 and 11 conserved N-linked glycosylation sites in E1 and E2, respectively.[29]

To facilitate rational HCV vaccine design, we aimed to identify determinants of protection of conserved neutralizing epitopes using a comprehensive reverse evolutionary approach. As envelope protein sequences from patient-derived HCV have been under selective pressure imposed by nAb, we hypothesized that relieving such pressure in cell culture might inform on genetic determinants of neutralization resistance. Therefore, we passaged cell culture infectious HCV with patient-derived envelope protein sequences of genotype 1a, 1b, 2a, 3a, and 4a without selective pressure by nAb. We evaluated passaged viruses for fitness in viral kinetic assays and for exposure of conserved neutralizing epitopes using mAb targeting AR2, AR3, AR4, and AR5 in neutralization assays. We aimed to reveal mechanisms inducing the observed highly fit and highly neutralization sensitive phenotypes by studying genetic changes and changes of the envelope protein conformational state (E1/E2 state) in passaged viruses in reverse genetic studies, as well as by viral breathing and receptor blocking assays.

EXPERIMENTAL PROCEDURES

Cell culture infectious HCV recombinants

Original recombinants subjected to reverse evolution *in vitro* were based on the Japanese Fulminant Hepatitis 1 (JFH1) strain with Core-NS2 of genotype(isolate) 1a (H77),[33] 1b(J4),[34] 2a(J6),[35] 3a(S52),[34] 4a(ED43),[33] and 6a(HK6a).[34] The 2a(J6), 3a(S52) and 6a(HK6a) recombinants harbored mutations in NS5A and NS5B, previously identified in a high-yield 5a(SA13) variant.[36] Viruses harbored Core-NS2 consensus sequences derived from chronically infected patients, determined following one viral passage in a chimpanzee[37] or in the case of 1a(H77) directly from the patient. All cell culture infectious genotype 6a HCV depend on two cell culture adaptive mutations in E1 and E2.[34] Mutations identified in serial passage were introduced into original recombinants with restriction enzyme and ligation-based cloning or the QuikChange II XL site-directed mutagenesis kit (Agilent). Mutated 1a(H77) and 4a(ED43) were engineered using subclones, generated with the TOPO-XL Cloning kit (Invitrogen) based on real-time PCR fragments spanning the complete HCV open reading frame (ORF) amplified as described subsequently, and the In-Fusion HD cloning kit (Takara Bio). The complete HCV sequences of final plasmids were confirmed (Macrogen).

Huh7.5 cell culture

Huh7.5 cells were cultured as described.[38] When HCV-infected cells were split, HCV-containing supernatants were harvested and stored at −80°C, and viral spread was monitored by HCV NS5A immunostaining using primary antibody anti-NS5A 9E10[35] and secondary antibody Alexa Fluor 594 goat anti-mouse IgG (H+L) (Invitrogen).[38]

Serial passage of HCV recombinants

For passage in flasks, 10^6 cells were seeded into T25 flasks and the next day inoculated with supernatant obtained from the preceding culture at the peak of infection, as determined by immunostaining. For passage in 96-well plates, 6000 cells/well were seeded and inoculated as described.[36]

Transfection with HCV recombinants

Transfections were done as described.[36] Plasmids were linearized with XbaI (New England Biolabs), and HCV-RNA transcripts were generated using T7 RNA polymerase (Promega). Approximately 2.5 µg transcript was transfected using 5 µl Lipofertamine 2000 (Invitrogen) in Opti-MEM (Gibco/Invitrogen) into Huh7.5 cells plated in 6-well plates at 350,000 cells/well the previous day. For each experiment, the same amount of RNA transcripts was transfected for all recombinants. RNA-Lipofectamine transfection complexes were incubated on cells for 6 h.

Production of virus stocks

For virus stock production, 3×10^6 cells were seeded into T80 flasks and inoculated the following day with supernatant obtained from the preceding culture (transfection or first passage) at the peak of infection. Cell culture supernatant was collected at peak of infection.

HCV kinetic experiments

A total of 10^6 cells were seeded in T25 flasks and infected the following day at multiplicity of infection 0.003 with transfection supernatants. Cultures were followed by immunostaining and determination of HCV infectivity titers.

Determination of supernatant HCV infectivity, RNA, and Core titers

HCV infectivity titers were determined as focus forming units (FFU)/ml.[39] Cells were plated at 6000 cells/well onto 96-well plates (Nunc) and the next day infected with serially diluted supernatants. After 48 h, HCV NS5A–positive cells were visualized by immunostaining, and FFU were counted using an Immunospot series 5 UV

analyzer (CTL Europe GmbH).[39] HCV RNA and Core titers were determined by TaqMan real-time PCR assay[34] and ARCHITECT HCV Ag assay (Abbott),[40] respectively.

HCV sequence analysis

HCV RNA was purified from supernatants using Trizol LS (Life technology) and RNeasy MinElute kit (Qiagen). Reverse transcription was done with Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific) and a genotype 2a specific primer.[41,42] PCR was done to amplify the ORF or the Core-E2 region with Q5 Hot start High-Fidelity DNA polymerase and genotype-specific primers.[42]

For serially passaged viruses and viruses from kinetic experiments, ORF amplicons were subjected to next-generation sequencing (NGS) in house on the Miseq platform.[41,42] For virus stocks, the E1/E2 sequence was confirmed using Sanger sequencing (Macrogen) or NGS.

HCV neutralization assays

Assays were done using polyclonal C211[32] or mAb AR2A, AR3A, AR4A, and AR5A[23,24] as described.[38] One day before neutralization, 6000 cells/well were plated in poly-D-lysine-coated 96-well plates. Viruses were incubated with antibodies for 1 h at 37°C before inoculation of cells. After 3 h at 37°C, cells were washed, and fresh media was added. Forty-eight hours after infection, cultures were immunostained for HCV NS5A, as described for infectivity titrations.[38]

Inhibition of HCV entry by receptor blocking

In general, experiments were done as described.[34] Briefly, 6000 cells/well were plated in poly-D-lysine-coated 96-well plates. The next day, cells were incubated for 1 h with anti-CD81 primary antibody (JS-81), control antibody (553447) (both BD Pharmingen), anti-SR-BI (C16-71), or control antibody (D)[43] with a concentration from 10 to 0.0006 µg/ml. Then, viruses were added and incubated for 3 h. Cells were washed and fresh media was added. Forty-eight hours after infection, cultures were immunostained for HCV NS5A.

HCV breathing experiments

In general, experiments were carried out as described.[32] A total of 6000 cells/well were plated in poly-D-lysine-coated 96-well plates. The next day, AR3A mAb at different concentrations and 600 FFU of HCV were incubated for 1 h at 4°C, 37°C, and 40°C and added to cells, followed by a spinoculation at 500 relative centrifugal force. After 3 h of incubation, cells were washed, and fresh media was added. After 48 h at 37°C, cultures were immunostained for HCV NS5A. EC50 were calculated with GraphPad Prism, as described for neutralization assays.

HCV temperature stability assays

Frozen viruses were thawed and incubated for 0, 2, 4, 8, or 24 h at 4°C, 37°C, or 40°C in three replicate experiments before inoculation of 6000 cells/well plated in 96-well plates seeded the day before. After 48 h at 37°C cultures were immunostained for HCV NS5A. For each experiment, FFU counts were determined in three replicates.

Antibodies

See Table S1. Antibodies used in this study.

RESULTS

HCV reverse evolution in the absence of nAb resulted in increased viral fitness and genetic changes in the envelope proteins

To identify determinants of neutralizing epitope protection, we adopted a reverse evolutionary approach by in vitro serial passage of cell culture infectious HCV harboring envelope protein sequences from patients chronically infected with HCV of genotype(isolate) 1a(H77), 1b(J4), 2a(J6), 3a(S52), and 4a(ED43).[34] We also passaged 6a (HK6a), containing vital culture adaptive substitutions F349S and N417T in E1 and E2, respectively, likely responsible for its high neutralization sensitivity.[34] We previously passaged 5a(SA13), which acquired T385P in E2, mediating an up to 10-fold increase in neutralization sensitivity.[36] Here, serial passage resulted in an up to 2.5-log10 increase in peak infectivity titers to about 6 log10 FFU/ml (Figure S2, Figure 1A). All passaged viruses had acquired polyprotein substitutions, including envelope protein substitutions (Figure 1A,B, Tables S2–S7). Thus, HCV evolution without nAb pressure resulted in increased viral fitness and genetic changes.

Engineered HCV variants with substitutions selected in the absence of nAb showed high fitness and increased sensitivity to bnAb

Compared with the respective original viruses, "mutated" viruses, engineered to harbor substitutions identified in >70% of passaged viruses (Figure 1B, Tables S2–S7), showed a similar increase in viral fitness as the
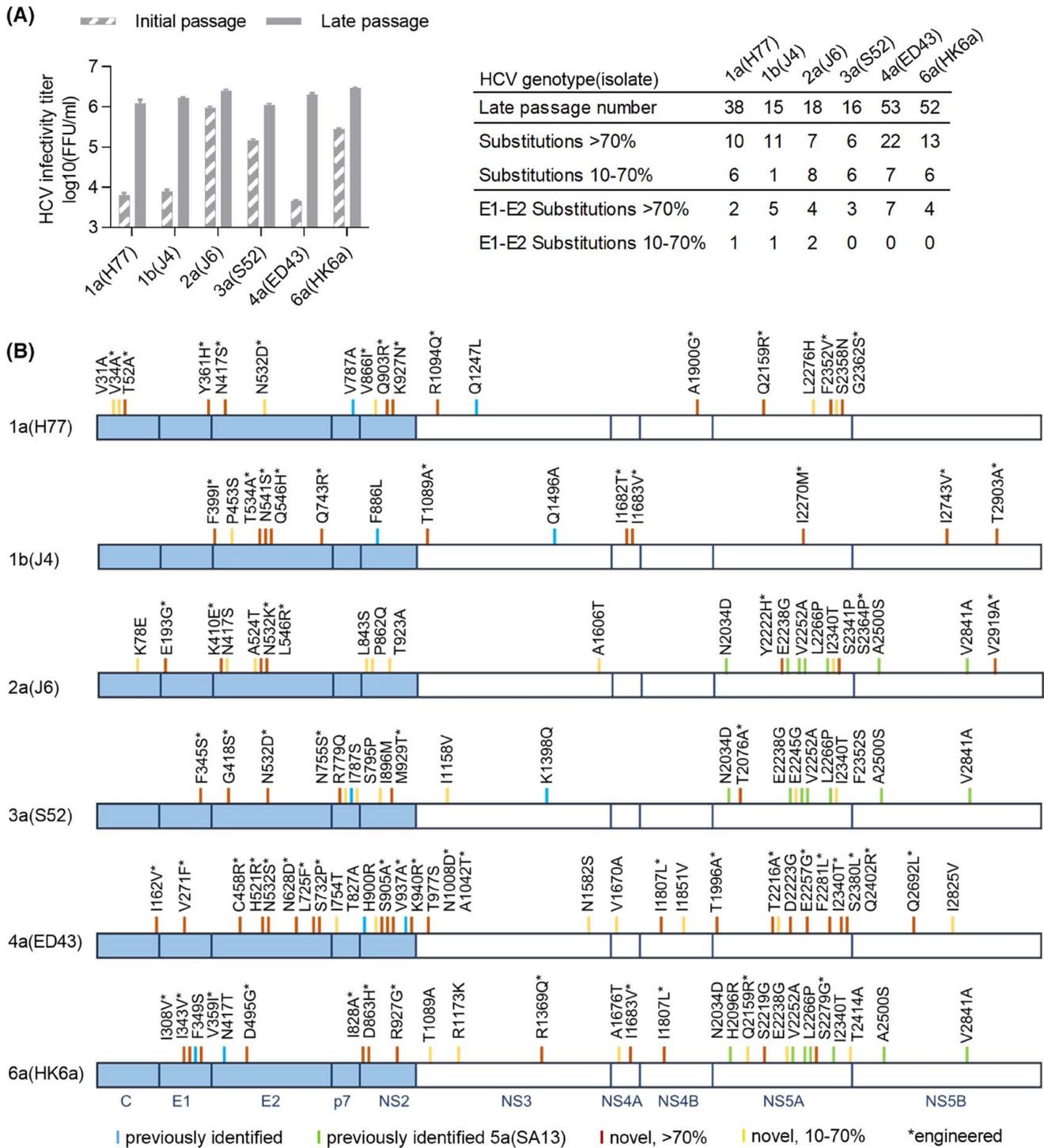
FIGURE 1 Reverse evolution of HCV without neutralizing antibody (nAb) pressure resulted in increased HCV infectivity titers and multiple genetic changes. HCV recombinants based on the Japanese Fulminant Hepatitis 1 (JFH1) strain with Core-NS2 of genotype(isolate) 1a(H77), 1b (J4), 2a(J6), 3a(S52), 4a(ED43), and 6a(HK6a) were serially passaged in Huh7.5 cells. (A) Peak HCV infectivity titers in initial and late passages were determined as log10 focus forming units (FFU)/ml and are means of three replicate determinations with SEM. The late passage (passage number indicated for each recombinant) was analyzed by next-generation sequencing (NGS): substitutions >70%, number of polyprotein substitutions showing >70% frequency in NGS; E1–E2 substitutions >70%, number of envelope protein substitutions showing >70% frequency in NGS; substitutions 10%–70%, number of polyprotein substitutions showing 10%–70% frequency in NGS; E1–E2 substitutions 10%–70%, number of envelope protein substitutions showing 10%–70% frequency in NGS. (B) Schematic overview of JFH1-based genotype 1, 2, 3, 4, and 6 genomes with substitutions identified following passage in cell culture. Blue bars indicate previously identified substitution engineered in originally developed viruses.[33-35] Green bars indicate substitution previously identified in NS5A and NS5B of a high-yield 5a(SA13) virus.[36] Red bars indicate novel substitutions with >70% frequency in NGS. Yellow bars indicate novel substitutions with 10%–70% frequency in NGS. *Novel substitutions with >70% frequency in NGS were engineered, except for 1a(H77), for which V34A, N532D, and V866I present in 10%–70%, as well as N2034D and S2996G found in two of six subclones, were engineered for technical reasons. Substitution numbers relate to the H77 polyprotein reference sequence (GenBank accession number AF009606).
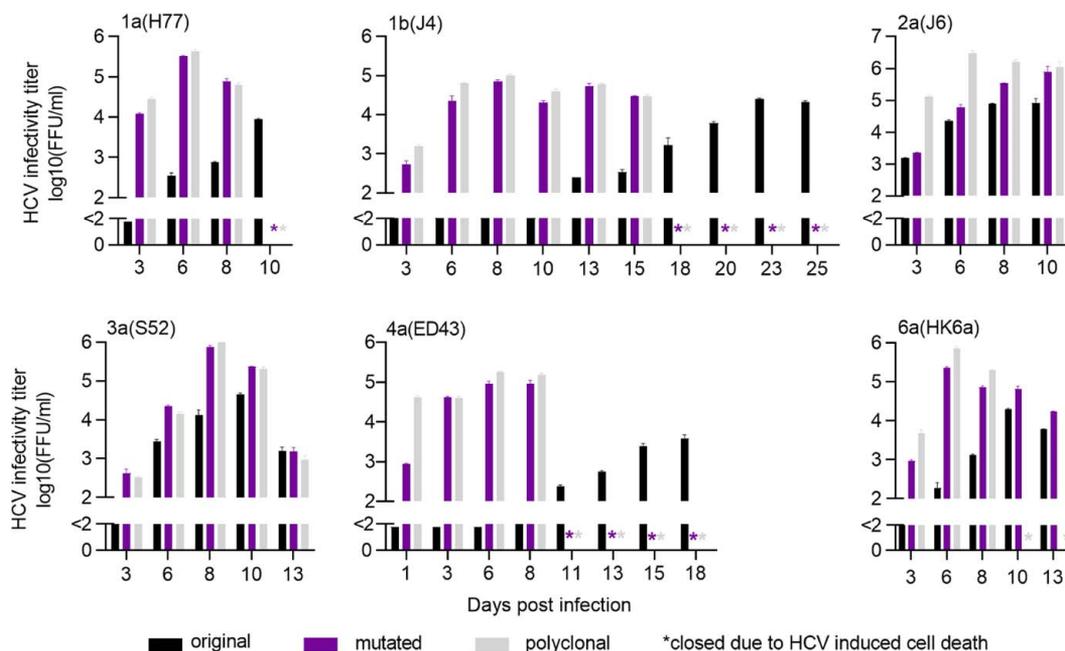
FIGURE 2 Substitutions selected during reverse evolution in the absence of nAb mediated increased viral fitness. Comparison of fitness of viruses with original envelope proteins (original), engineered viruses with all substitutions identified during *in vitro* passage in >70% of viral genomes (mutated), and polyclonal viruses resulting from *in vitro* passage (polyclonal), all tested in a first viral passage kinetic experiment inoculated at multiplicity of infection (MOI) of 0.003. HCV infectivity titers are means of three replicate determinations with SEM. *Cell culture was closed due to HCV-induced cell death. NGS of passaged viruses did not reveal additional mutations in the open reading frame (ORF) present in >20% of genomes.

polyclonal passaged viruses according to viral spread kinetics and peak infectivity titers (Figure 2) and specific infectivities (Table S8). Increased fitness appeared to be due to the engineered substitutions, as mutated viruses were genetically stable following transfection and first viral passage, as NGS analysis did not reveal additional mutations in the ORF with >20% prevalence.

Compared with the original genotype 1–4 viruses, the respective mutated and polyclonal viruses showed a similar increase in sensitivity to neutralization by human monoclonal bnAb AR3A[23] and AR4A,[24,25] targeting conformational neutralizing epitopes AR3 and AR4 associated with protection against chronic HCV infection[21,22] (Figure 3). This increase was most pronounced for 2a(J6) (up to 4250-fold) and 3a(S52) (up to >35,700-fold), followed by 1a(H77) (up to 165-fold). For 6a(HK6a), the mutated and polyclonal virus did not show increased neutralization sensitivity compared with the original virus already showing high neutralization sensitivity.[34]

Thus, reverse genetics showed that evolution without nAb pressure resulted in HCV variants with increased fitness and sensitivity to bnAb.

HCV envelope protein substitutions increased neutralization sensitivity

Further reverse genetic analysis focused on envelope protein substitutions in mutated 1a(H77), 2a(J6) and 3a(S52), showing the highest change in neutralization sensitivity. Based on recombinants with original envelope proteins, we engineered viruses harboring the identified envelope protein substitutions individually and in combination. AR3A neutralization assays revealed that the increased neutralization sensitivity of the mutated 1a(H77), 2a(J6), and 3a(S52) (Figure 3) was mediated by the envelope protein substitutions (Figure 4A, Figure S3A). For 1a(H77), none of the three investigated substitutions (Y361H in E1 and N417S and N532D in E2) mediated increased neutralization sensitivity individually. For 3a(S52), of the three investigated substitutions, G418S mediated the strongest increase in neutralization sensitivity (> 3800-fold). G418S localizes to the conserved N1 glycosylation motif in E2 (aa 417–419), although it is not predicted to influence glycosylation in accordance with the canonical glycosylation signal sequence N-X(except P)-S/T. For 2a(J6), of the four investigated substitutions, N532K mediated the strongest increase in neutralization sensitivity (1600-fold), and reversion mutants based on the mutated 2a(J6) virus confirmed importance of N532K for increased neutralization sensitivity. Interestingly, 2a(J6)N532K and mutated 2a(J6) showed similar neutralization sensitivity as 2a(J6) without HVR1, reported to have exceptionally high neutralization sensitivity.[44] N532K localizes to the conserved N6 glycosylation motif (aa 532–534) in E2 and is predicted to abrogate glycosylation.
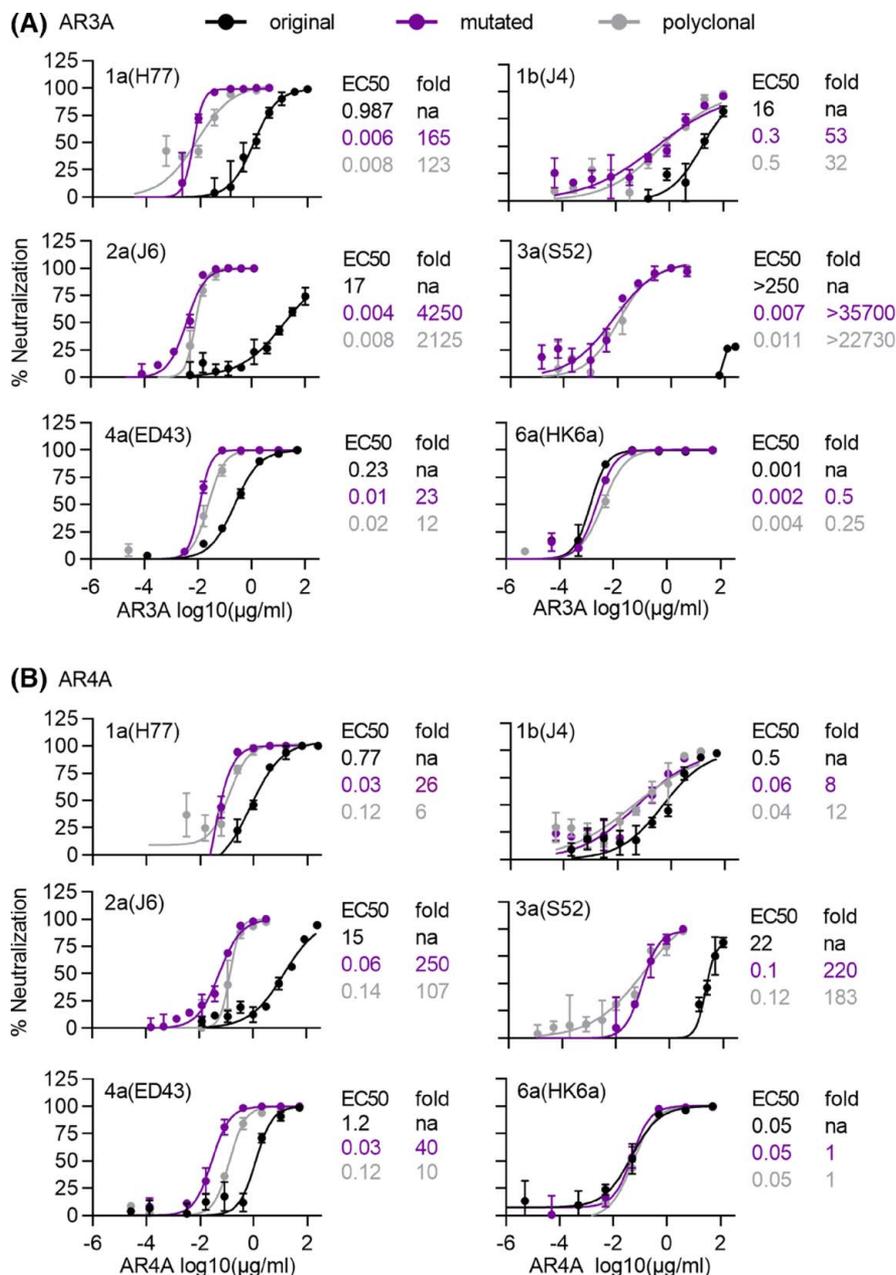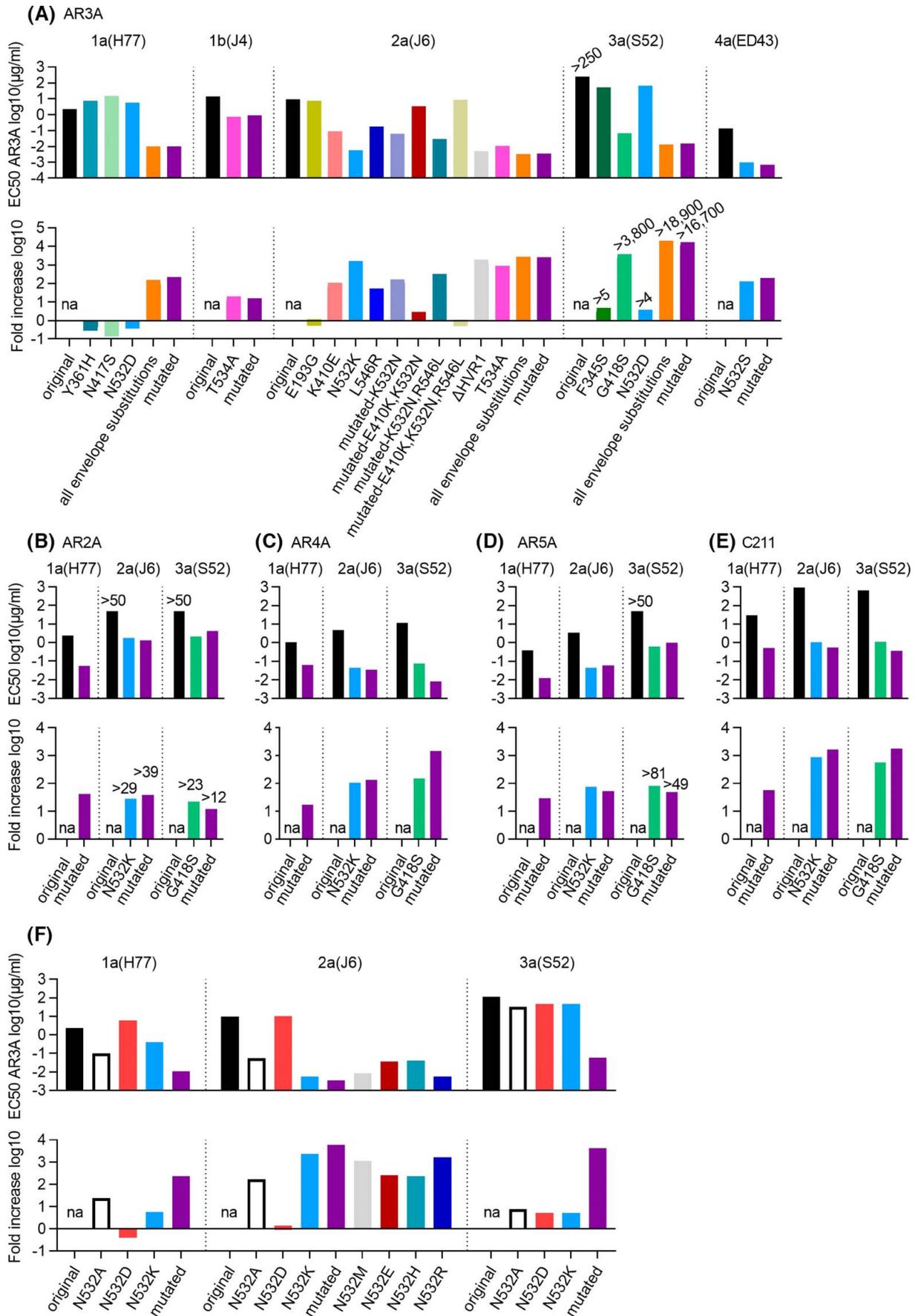
FIGURE 3 Substitutions selected during reverse evolution in the absence of nAb resulted in increased sensitivity to nAb. *In vitro* neutralization assays were carried out using original, mutated, and polyclonal genotype 1, 2, 3, 4, and 6 HCV and human monoclonal antibodies (mAb) AR3A (A) and AR4A (B). Data points are means of three replicate determinations with SD; concentration-response curves were fitted and half maximal effective concentration (EC50) values were calculated using top and bottom constraints of 0% and 100%, respectively, and the formula $y = \text{top}/(1 + 10^{[\log 10 \text{EC50} - X] \times \text{hillslope}})$ with GraphPad Prism (version 9). Fold increase values indicate increase in neutralization sensitivity and were calculated as (EC50 of original virus)/(EC50 of mutated virus [or polyclonal virus]). The envelope protein sequence of the used virus stocks was confirmed by Sanger sequencing.

Substitutions in the N6 glycosylation motif had been selected for genotype 1a, 2a, and 3a viruses during serial passage. Therefore, we also studied T534A, selected in 1b(J4), and N532S, selected in 4a(ED43), both predicted to abrogate glycosylation. 1b(J4)T534A and 4a(ED43)N532S showed a similar increase in neutralization sensitivity (20-fold and 130-fold, respectively) as the 1b and 4a mutated viruses containing all

polyprotein substitutions. In addition, we studied T534A in 2a(J6), not selected *in vitro* but also predicted to abrogate glycosylation, mediating a 900-fold increase in neutralization sensitivity (Figure 4A).

Finally, we revealed that mutated 1a(H77), 2a(J6), and 3a(S52) as well as 2a(J6)N532K and 3a(S52) G418S had highly increased sensitivity to neutralization by AR2A (Figure 4B, Figure S3B), AR4A (Figure 4C,

(A) AR3A
(B) AR2A
(C) AR4A
(D) AR5A
(E) C211
(F)

Figure S3C), AR5A (Figure 4D, Figure S3D),[23,24] and human polyclonal C211 antibodies (Figure 4E, Figure S3E),[32] compared with the respective original viruses. This suggested a broad effect of the studied substitutions on neutralization sensitivity.

Thus, E2 substitutions selected in the absence of nAb resulted in broadly increased neutralization sensitivity. For genotype 1a, increased neutralization sensitivity was mediated by a combination of three envelope protein substitutions; for genotype 2a, mostly by an individual E2 substitution in the N6 glycosylation motif; and for genotype 3a, mostly by an individual E2 substitution in the N1 glycosylation motif.

Amino acid–specific effect of position-532 substitutions in the N6 glycosylation motif on neutralization sensitivity argues against changes in glycan occupancy as the underlying mechanism

To further study the effect of different substitutions observed at the conserved N6 glycosylation site across HCV genotypes, we compared the effect of different amino acid changes at position 532, all predicted to abrogate N-linked glycosylation, on genotype 1a, 2a, and 3a viruses. We changed N (polar) at position 532 to A (nonpolar), not selected in any of the viruses during reverse evolution, to D (negatively charged), selected in 1a(H77) and 3a(S52), or K (positively charged), selected in 2a(J6). Moreover, for 2a(J6), we investigated the effect of nonpolar (M), negatively charged (E), or positively charged (H, R) amino acids at position 532. Even though all substitutions are expected to abrogate N6-linked glycosylation, at least for 1a(H77) and 2a(J6), we observed a substitution-specific effect on AR3A neutralization sensitivity, without clear dependence on amino acid characteristics (Figure 4F, Figure S4). The most striking example in this head to head comparison was that for 2a(J6), N532D did not influence neutralization sensitivity, as reported previously,[32] whereas N532K mediated 1700-fold increased neutralization sensitivity. These findings suggested that increased neutralization sensitivity was not dependent on glycan occupancy.
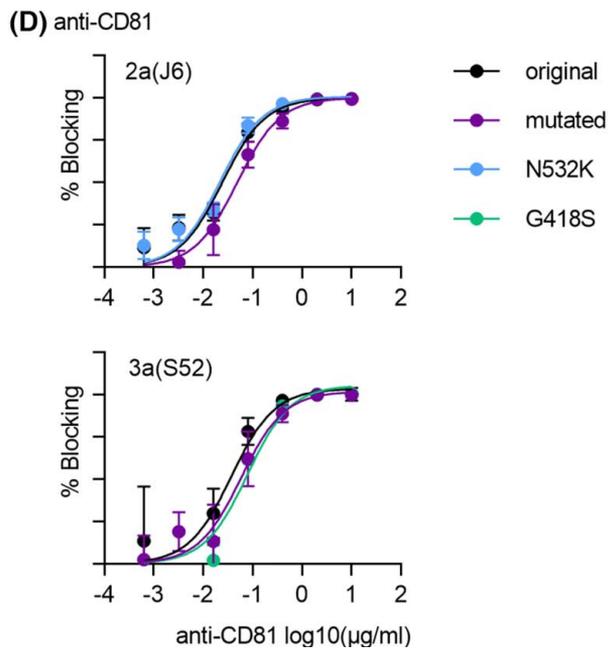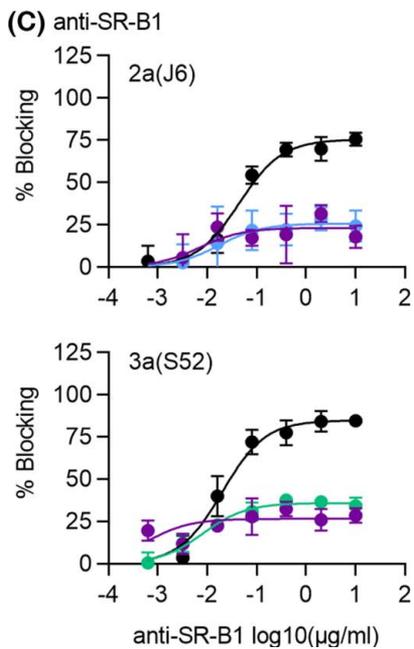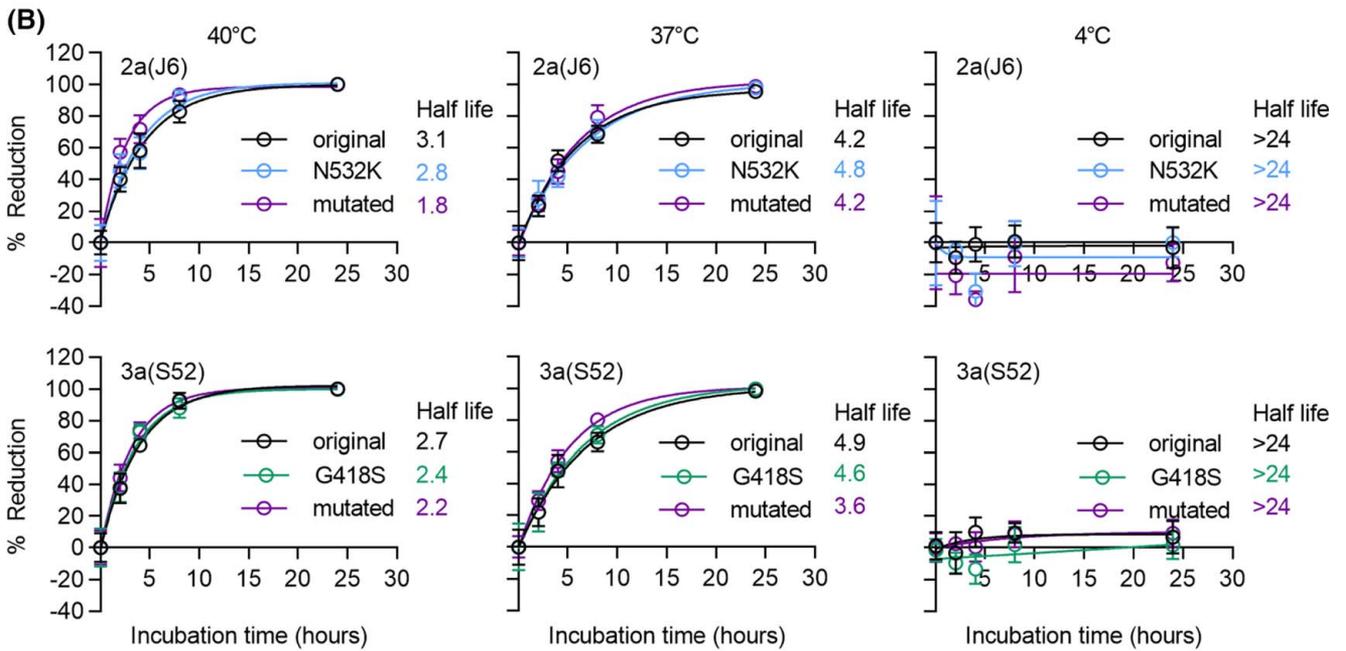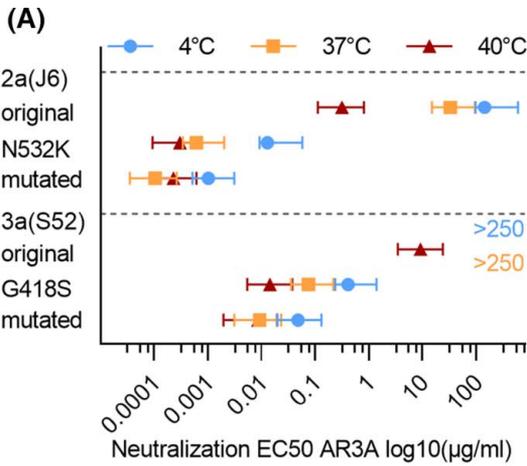
An open HCV envelope protein conformational state primarily induced by position 418 and 532 substitutions in N1 and N6 glycosylation motifs likely caused increased neutralization sensitivity

Viral envelope proteins are suggested to be in a dynamic equilibrium between open and closed conformational states with high and low neutralizing epitope accessibility, called "viral breathing."[32,45-47] This equilibrium is influenced by intrinsic factors such as the envelope protein sequence and extrinsic factors such as temperature, with temperature increase mediating a shift to an open conformation.

To investigate whether 2a(J6) and 3a(S52) envelope protein substitutions shifted this equilibrium toward an open E1/E2 state with increased neutralizing epitope accessibility, we investigated viral breathing capacities carrying out AR3A neutralization assays at different temperatures (Figure 5A, Figure S5). The original viruses showed the highest breathing capacity, suggesting a closed E1/E2 state: For 2a(J6), median effective concentration (EC50) at 40°C was 448-fold lower than EC50 at 4°C, and for 3a(S52) there was a > 27-fold difference in EC50 at 40°C and 4°C. In contrast, the mutated viruses showed the lowest breathing capacity with 4-fold and 6-fold differences in EC50 at 40°C and 4°C for 2a(J6) and 3a(S52), respectively, suggesting an intrinsically open E1/E2 state. 2a(J6)N532K and 3a(S52)G418S showed intermediate (43-fold and 28-fold) differences in EC50 at 40°C and 4°C.

Further, open E1/E2 states were previously associated with decreased temperature stability.[32,48] We observed a minor trend toward decreased temperature stability of 2a(J6) and 3a(S52) with envelope protein substitutions compared with the original viruses (Figure 5B).

Finally, open-envelope conformational states were associated with decreased dependency on HCV entry coreceptor SR-BI.[47,49] Accordingly, mutated 2a(J6) and 3a(S52), as well as 2a(J6)N532K and 3a(S52)G418S, showed strongly decreased dependency on SR-BI compared with the original viruses, with maximum inhibition being about 25% compared with about 80%

(A)

(B) 40°C / 37°C / 4°C

(C) anti-SR-B1

(D) anti-CD81

FIGURE 5 Specific envelope protein substitutions localizing to conserved glycosylation motifs and inducing increased neutralization sensitivity mediated switching from a closed to an open envelope protein conformational state. (A) Breathing capacity for specified 2a(J6) and 3a(S52) viruses, determined in *in vitro* neutralization assays using human mAb AR3A at 4°C, 37°C and 40°C, as described in the "Experimental procedures" section. EC50 values and 95% CI were calculated in GraphPad Prism (version 9) based on concentration-response curves shown in Figure S5. (B) Temperature stability of the specified 2a(J6) and 3a(S52) viruses evaluated by incubation at 40°C, 37°C, and 4°C. Data points are means of nine values derived from three replicate experiments, evaluated by FFU determinations in triplicates with SD. One-phase decay curves were fitted and half-lives were calculated using the formula $y = (Y0-Plateau)*exp(-K*X) + Plateau$ with GraphPad Prism (version 9). Scavenger receptor class B type 1 (SR-BI) (C) and cluster of differentiation 81 (CD81) (D) dependency of specified 2a(J6) and 3a(S52) viruses, determined in receptor-blocking assays using specific antibodies. Data points are means of three replicate determinations with SD; concentration-response curves were fitted and EC50 were calculated with GraphPad Prism using top and bottom constraints of 0% and 100%. The envelope protein sequence of all used virus stocks was confirmed by Sanger sequencing.

(Figure 5C). In contrast, these viruses showed similar dependency on the HCV main receptor CD81 as the original viruses (Figure 5D).

In summary, N532K in the N6 and G418S in the N1 glycosylation motif induced an open E1/E2 state. Thus, specific envelope protein substitutions localizing to conserved glycosylation motifs mediated transition from a closed to an open E1/E2 state, independent of glycan occupancy.

Envelope protein substitutions conferring increased neutralization sensitivity conferred increased viral fitness, highlighting the genetic link between E1/E2 states and viral fitness

We finally investigated whether 2a(J6) and 3a(S52) envelope protein substitutions mediating increased neutralization sensitivity (Figures 3 and 4) also mediated increased fitness of mutated viruses (Figure 2). Indeed, fitness of 2a(J6) and 3a(S52) with all envelope protein substitutions matched the fitness of the respective mutated viruses (Figure 6). For 2a(J6), this fitness increase was mediated by N532K, whereas for 3a(S52), none of the three studied envelope protein substitutions individually mediated increased fitness. 2a(J6) and 3a(S52) with all envelope protein substitutions, and to a lesser extend 2a(J6)N532K, showed increased specific infectivity, matching that of the mutated viruses (Table S9). As the engineered viruses were genetically stable, the observed phenotypes were determined by the engineered substitutions. Thus, for 2a(J6) and 3a(S52), the identified envelope protein substitutions mediating increased neutralization sensitivity were key drivers of viral fitness, likely due to exposure of the CD81 binding site overlapping with AR3, highlighting a genetic link between neutralization sensitivity and viral fitness.

DISCUSSION

We revealed mechanisms of neutralizing epitope protection by tracking reverse evolution of HCV envelope protein sequences in the absence of immune selection. Reverse genetic studies identified two N-linked glycosylation motifs as trigger sites for switching of the E1/E2 conformational state. In contrast to current perceptions, this effect appeared to be independent of N-linked glycan occupancy. Our results also suggest that viral epitope protection is associated with fitness costs.

This study has implications for rational vaccine design. Envelope protein–based HCV vaccine candidates show low immunogenicity with a lack of efficient induction of bnAb, likely due to conformational flexibility and occlusion of conserved neutralizing epitopes overlapping with the viral CD81 receptor binding site.[12,50] Thus, it is desirable to stabilize the envelope protein conformation to make these important vaccine targets accessible to nAb. Induction of nAb targeting conserved epitopes could overcome viral genetic heterogeneity. Genetic modifications inducing an open E1/E2 state could be implemented in the context of different vaccine platforms.

This study revealed that genetic changes in N-linked glycosylation motifs, apparently without altering glycan occupancy, can mediate major changes in the E1/E2 conformational state and exposure of neutralizing epitopes. This might be facilitated by strategic location of glycosylation motifs at trigger sites for conformational changes,[12] allowing strong effects of different biochemical modifications on envelope protein conformation. Previous studies only showed that abrogation of N-linked glycosylation by mutation of N-linked glycosylation sites increased neutralization sensitivity to a different extent.[29,32,51] Furthermore, engineering of specific point mutations in the E2 front layer and AS412 not associated with glycosylation motifs mediated mostly minor increases in neutralization sensitivity.[17,18,20,47] Finally, deletion of HVR1, located at the N-terminus of E2, increased HCV neutralization sensitivity by three orders of magnitude; this effect could not be increased by mutagenic removal of N-linked glycosylation sites, suggesting that HVR1 deletion induced maximal neutralization sensitivity.[32,44] Recently, increased neutralization sensitivity was linked to an open E1/E2 state.[32,47] Thus, the effect of G418S and N532K in N1 and N6 glycosylation motifs, respectively, on the E1/
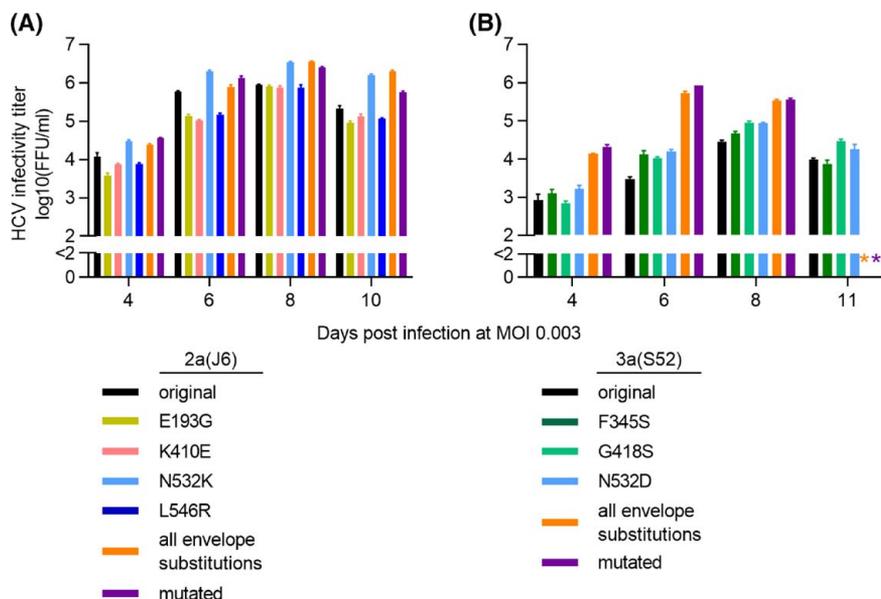
FIGURE 6 Envelope protein substitutions contributing to increased neutralization sensitivity mediated increased viral fitness. Comparison of fitness of the specified 2a(J6) and 3a(S52) viruses in first passage kinetic experiments inoculated at MOI 0.003. HCV infectivity titers for 2a(J6) (A) and 3a(S52) (B) variants with original envelope proteins, all substitutions (mutated), all envelope substitutions, or addition of single substitutions identified following serial passage. HCV infectivity titers are means of three replicate determinations with SEM. *Cell culture was closed due to HCV-induced cell death. The passaged viruses were genetically stable, revealed by Sanger sequencing or NGS of the ORF.

E2 state matched the effect of HVR1 deletion and exceeded the effect of most other previously described envelope protein substitutions.[17,18,20,29,32,44,47,51]

The E2 N1 and N6 glycosylation motifs localize to AS412 and the CD81 binding loop, respectively. Both regions show high conformational flexibility of functional importance during E2-CD81 receptor binding and likely contribute to protection of conserved neutralizing epitopes and the CD81 receptor binding site.[12,50] While these regions were either truncated or disordered in E2 core structures,[9,10] recently solved E2 ectodomain structures revealed localization of N1 and N6 glycosylation motifs to adjacent E2 areas,[11,12] possibly explaining their similar impact on E1/E2 state switching (Figure S1). The complete N1 glycosylation motif is highly conserved, including position 418, which could theoretically vary without abrogating glycosylation of N1 at position 417. This supports an important role of 418G for neutralizing epitope protection. Previously studied changes in the N1 glycosylation motif mostly localized to position 417 and mediated escape from mAb targeting AS412[17–20,32,34,51]; selected changes conferred a mostly minor increase in sensitivity to conformational nAb[17,18,51] or were associated with increased viral fitness.[17,20,34] Amino acid–specific effects of position-532 substitutions revealed in this study suggest that not glycan occupancy but specific amino acid residues at N6 govern the E1/E2 state. Even though changes at N6 showed some isolate specificity, N1 and N6 glycosylation motifs changed for different isolates. Thus, N1 and N6 glycosylation motifs are likely key regulators of E1/E2 state switching across HCV

variants. In this study, different viruses showed relatively similar high neutralization sensitivity following reverse evolution, whereas the original viruses showed largely different neutralization sensitivity. These observations support the hypothesis that different viral variants share conserved neutralizing epitopes that are protected to different extents by determinants inducing a closed E1/E2 state.[44]

The strong correlation between viral fitness and neutralizing epitope exposure observed in this study strengthens the hypothesis that protection of neutralizing epitopes implies a fitness cost.[14] As conserved neutralizing epitopes overlap with the binding site of the main HCV receptor CD81,[12] protection of these epitopes might hinder direct interaction of the CD81 binding site with its receptor. We found that HCV with closed E1/E2 state showed stronger dependency on coreceptor SR-BI. Emerging evidence suggests that this receptor induces an open E1/E2 state before HCV binding to CD81.[17,47,49] Thus, it is likely that position 418 and 532 substitutions triggered conformational changes, increasing accessibility of the CD81 binding site.

Viruses developed in this study could be highly relevant for the development of whole-virus HCV vaccines, as they grow to infectivity titers supporting vaccine development, in contrast to the originally developed viruses or viruses without HVR1.[52] Future immunogenicity studies should investigate whether vaccine antigens with increased neutralizing epitope exposure show increased immunogenicity. In a recent study, we reported high immunogenicity of HCV with

increased neutralizing epitope exposure conferred by alternative envelope protein substitutions.[53] However, in the whole virus vaccine platform, comparisons of immunogenicity are hampered by low yields of the original viruses. Similarly, the effect of HVR1 deletion on immunogenicity of a whole virus vaccine was difficult to evaluate, as the original virus already showed high neutralizing epitope exposure.[54] In recombinant envelope protein vaccine platforms, deletion of HVR1 and modification of glycosylation did not have strong effects on immunogenicity.[26,51,55-57] It should be noted that the shelf-live of whole-virus vaccines could be increased by addition of stabilizers, especially if high-yield HCV showed decreased thermostability.

JFH1-based HCV recombinants with original envelope proteins subjected to reverse evolution in this study are used widely for HCV *in vitro* studies. A high-yield genotype 5 virus had previously been developed.[36] A high-yield genotype 1–6 virus panel is relevant for different research applications in which large amounts of viruses are required, including ultrastructural studies.

We identified glycosylation motifs localizing to adjacent HCV envelope protein E2 areas as key regulators for switching of the envelope protein conformational state, governing sensitivity to nAb. Such switching was apparently independent of glycan occupancy. This mechanism might be relevant for different viruses for which evolution in humans results in protection of neutralizing epitopes, leading to escape from nAb and challenges for vaccine efficacy. This study contributes to a better understanding of viral evasion from nAb with implications for viral vaccine development.

AUTHOR CONTRIBUTIONS

Study design: Garazi P. Alzua, Anne F. Pihl, and Judith M. Gottwein. *Data acquisition*: Garazi P. Alzua, Anne F. Pihl, Anna Offersgaard, Rodrigo Velázquez-Moctezuma, Carlos R. Duarte Hernandez, Elias H. Augestad, Christian K. Mathiesen, and Henrik Krarup. *Analysis and data interpretation*: Garazi P. Alzua, Anne F. Pihl, Anna Offersgaard, Rodrigo Velázquez-Moctezuma, Carlos R. Duarte Hernandez, Elias H. Augestad, Ulrik Fahnøe, Christian K. Mathiesen, Henrik Krarup, Mansun Law, Jannick Prentoe, Jens Bukh, and Judith M. Gottwein. *Manuscript draft*: Garazi P. Alzua, Anne F. Pihl, and Judith M. Gottwein. *Study supervision*: Judith M. Gottwein. All authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supporting Information.

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