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Structural and functional characterization of the single-chain Fv fragment from a unique HCV E1E2-specific monoclonal antibody

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ABSTRACT

The nucleotide sequence of the unique neutralizing monoclonal antibody D32.10 raised against a conserved conformational epitope shared between E1 and E2 on the serum-derived hepatitis C virus (HCV) envelope was determined. Subsequently, the recombinant single-chain Fv fragment (scFv) was cloned and expressed in *Escherichia coli*, and its molecular characterization was assessed using multi-angle laser light scattering. The scFv mimicked the antibody in binding to the native serum-derived HCV particles from patients, as well as to envelope E1E2 complexes and E1, E2 glycoproteins carrying the viral epitope. The scFv D32.10 competed with the parental IgG for binding to antigen, and therefore could be a promising candidate for therapeutics and diagnostics.

1. Introduction

Hepatitis C virus (HCV) infects an estimated 2–3% of the world population and is a major cause of chronic liver disease. The majority (80%) of infected individuals progress to chronic hepatitis that increases their risk for developing cirrhosis and hepatocellular carcinoma [1]. The standard of care (SOC) therapy for chronic infection uses a combination of pegylated interferon-α (PEG-IFN) and ribavirin (RBV), which is effective in only 50% of treated patients infected and has many side effects. Two new direct-acting antivirals (DAAs) targeting the virus protease NS3 have recently been approved for triple therapy with PEG-IFN and RBV to improve success rates and to shorten treatment [2]. This approach to treatment still suffers a number of drawbacks: regimen restricted to patients with genotype 1, and increased rate of adverse effects. There is therefore a pressing need to develop alternative anti-HCV therapies, particularly in the arena of prophylactic or therapeutic vaccines. The observation that some HCV-infected individuals (20%) can resolve spontaneously infection with virus-specific immune responses [3] has spurred interest in the potential of HCV vaccines, but as yet no such vaccine exists. Progress toward this goal has been hampered by a number of factors, in particular the extreme genetic diversity of HCV (six major genotypes and more than 50 subtypes) [4]. Therefore, identification of protective conserved immune epitopes of the virus is essential for understanding the role of neutralizing responses in disease pathogenesis, and for developing

Abbreviations: CDR, complementarity-determining region; DAAs, direct-acting antivirals; ELISA, enzyme-linked immunoabsorbent assay; FR, framework region; GT, genotype; HCV, hepatitis C virus; HCVsp, serum-derived HCV particles; HRP, horseradish peroxidase; IgG, immunoglobulin G; IMAC, immobilized metal affinity chromatography; IPTG, isopropylthio-β-galactoside; LB medium, Leibovitz medium; mAb, monoclonal antibody; MALLS, multi-angle laser light scattering; NDSB, 3-(1-pyridino)-1-propanesulfonates; NR, non-reducing; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon-α; PVDF, polyvinylidene difluoride; RBV, ribavirin; R, reducing; scFv, single chain antibody fragment; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; SOC, standard of care; TBS, Tris buffer saline; V_H, heavy chain variable region; V_L, light chain variable region

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vaccines and antibody-based therapies. We reported previously that the mouse monoclonal antibody (mAb) D32.10 recognizes a unique discontinuous antigenic determinant encompassing one sequence in the E1 glycoprotein (aa 297–306), and two sequences in the E2 glycoprotein: E2A (aa 480–494) and E2B (aa 608–62) juxtaposed on the surface of native circulating enveloped HCV particles, designated HCVsp, in chronic hepatitis C patients [5,6]. This epitope is highly conserved between the different genotypes of HCV (1a, 1b, 2a, 2b, 3a, 4, 5 and 6). Furthermore, the mAb D32.10 is so far the only antibody able to efficiently inhibit the interactions between HCVsp and hepatocytes [7] as well as in vitro HCV infection [8]. The relevance of this mAb in vivo was proven by using the D32.10 epitope as a probe to look for the presence of anti-E1E2A,B D32.10 epitope-binding antibodies in the serum of HCV-infected patients. We demonstrated that unique anti-E1E2 neutralizing antibodies were associated with spontaneous recovery or predictive of sustained virological response (SVR) in patients with chronic hepatitis C [9].

Recombinant antibody (rAb) technologies involving the handling of key antibody domains constitute an option and have been increasingly used as alternatives to mAbs in medical diagnostic and therapeutic applications. One of the most popular types of rAbs is single-chain variable fragment (scFv) as it has been successfully modified into a number of different antibody formats and is easily expressed by several expression systems [10]. We reported here the nucleotide sequence of the gene segments encoding the variable domains of D32.10, and described the cloning and expression of a scFv construction of the antibody. The antigen-binding properties of scFv D32.10 with those of the native antibody were compared.

2. Materials and methods

2.1. mAb D32.10 purification and N-terminal sequencing

Secreted IgGs (isotype IgG1, κ) were purified from the D32.10 hybridoma culture by precipitation with 75% ammonium sulfate, then by affinity chromatography on a Protein A-Sepharose column. After elution, a gel filtration on Sephadex-200 was carried out. The resulting protein was loaded on 12% SDS gels and transferred to a PVDF membrane. N-terminal sequencing of the heavy and light bands on the membrane was performed by standard Edman degradation using a model 492 sequencing system (Applied Biosystems) followed by identification using a model 140C HPLC (Applied Biosystems) and analysis using the Model 610A (V2.1) software (Applied Biosystems). The following N-terminal protein sequences were found: (i) for the light chain: **D-V-V-M-T-Q-T** (ii) for the heavy chain: **E-V-K-L-V-E-S**.

2.2. Bioinformatics analysis and primers design

The above-described protein sequences were compared to the whole murine repertoire of variable domains available (www.imgt.org). The cDNA sequences of all the variable domains starting with these protein sequences were aligned and compared, and oligonucleotide primers matching this alignment were designed. The alignment showed only one different cDNA sequence for each chain, so one oligonucleotide was designed for each chain: (i) for the first light chain (**L1**): cgg a att cat GAT GTT GTG ATG ACC CAG ACT CCA; (ii) for the first heavy chain (**H1**): cgg a att cat GAG GTG AAG CTG GTG GAG TCT GGG GGA, wherein the sections in lower case letters correspond to restriction enzyme sites introduced in the oligonucleotides for cloning purposes, and the sections in capital letters correspond to the cDNA derived sequences corresponding to the protein sequence determined by N-terminal sequencing. In addition to these forward primers, a common reverse primer based on a polyT sequence was also designed, including enzyme sequences and ordered (polyT30 ctc gag aag cttTTT TTT TTT TTT TTT TTT TTT TTT TTT –) (Table 1).

2.3. RNA Isolation, cDNA Amplification and Sequencing

Total RNA was extracted from 10^6 fresh hybridoma cells using the RNeasy mini kit (Qiagen) and cDNA was generated by reverse transcription using SuperScript® III First-Strand Synthesis SuperMix kit (Invitrogen) and the polyT primer. The cDNA was amplified by PCR using the specific and polyT primers described above and the Phusion High-Fidelity PCR master mix (Finnzymes). The PCR was performed on a MasterCycler (Eppendorf), and resulting PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced by Eurofins MWG biotech. Finally PCR products were cloned into the pPROEX HTb vector (Life Technologies) and sequenced by using L1 and H1 primers (Table 1).

2.4. Cloning and expression of the scFv D32.10

A set of new primers were designed to amplify the light chain variable region (V_L) and the heavy chain variable region (V_H) genes and to add special tags as restriction enzymes, $(Gly_4Ser)_3$ linker and a stop codon [11]. The V_L gene was amplified with the primers L1forlink and L1revXho (Table 1) and the V_H gene with the primers H1 and H1revlink (Table 1) to generate D32.10 scFv synthetic gene by splice overlapped extension. A clone pPROEX HTb-scFv D32.10 has been used to transform competent *Escherichia coli* BL21-Gold (Stratagene) and cultivated in LB medium supplemented with 100 μg/ml ampicillin at 37 °C to an optical density (OD) of 0.4–0.6 at 600 nm. After 4 h of 1 mM IPTG induction at 37 °C, the cells were harvested by centrifugation and suspended in 20 mM Tris–HCl at pH 8 and 150 mM NaCl (Tris–NaCl buffer) before sonication for 5 min (60% amplification). The resulting bacterial extract was centrifuged (20,000 rpm for 30 min at 4 °C), the pellet resuspended in 20 mM Tris–HCl at pH 8 and 2 M NaCl and centrifuged again. The resulting pellet was solubilized in Tris–NaCl buffer containing 0.1% Triton-X100, centrifuged, washed by resuspension/centrifugation and solubilized in denaturation buffer (100 mM Tris–HCl at pH 8, 500 mM NaCl, 8 M urea). The final extract was centrifuged again and the supernatant subjected to immobilized metal affinity chromatography (IMAC) using Ni-NTA resin (Qiagen) for purification of the scFv (Fig. 1). The purified scFv was diluted 50 times in refolding buffer (100 mM Tris pH 9, 500 mM NaCl, 5% glycerol, 250 mM

Table 1
List of primers used to sequence D32.10 V_H, V_L and to generate synthetic genes encoding scFv D32.10. The restriction sites *Xho*I and *Eco*RI are in dotted and solid lines, respectively. The linker serine-glycine $(Gly_4Serine)_3$ is in bold letters.

Primers	Sequences (5′–>3′)
H1	CG**GAATTC**ATGAGGTGAAGCTGGTGGAGTCTGGGGGA
L1	CG**GAATTC**ATGATGTTGTGATGACCCAGACTCCA
polyT30	C**TCGAGAAGC**TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
H1revlink	ACCACCGGATCCGCCTCCGCCAGAGACAGTGACCAGCGTCCC
L1forlink	GGCGGAGGCGGATCCGGTGGTGGCGGATCTGGAGGTGGCGGAGATGTTGTGATGACCCAGACT
L1revXho	ATGCCTCGAGCTAAGCCCGTTTTATTTCCAACTT

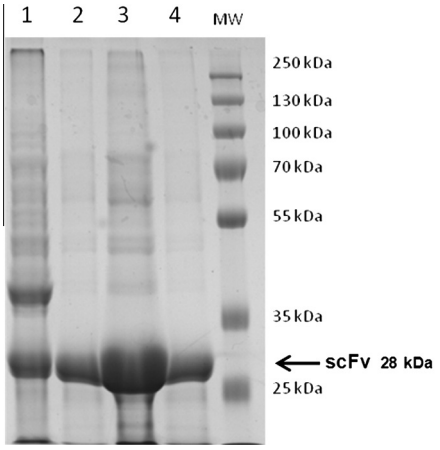

Fig. 1. scFv D32.10 purification (using a Ni2+-charged column). Control of the purified His-tagged scFv D32.10 by 10% SDS–PAGE after Coomassie staining. Lane 1, crude cell extract; Lanes 2, 3 and 4, eluates with 300 mM imidazole. MW, Molecular Weight markers in kilodaltons (kDa).

L-arginine, 100 mM NDSB (3-(1-pyridino)-1-propanesulfonates), 2 mM reduced glutathione, 1 mM oxidized glutathione) for 30 min. A FLPC (Biologic DuoFlow system, Biorad) was performed using a commercially packed high resolution 10/30 Superdex 75 column equilibrated with 100 mM Tris at pH 9 and 500 mM NaCl.

2.5. Antigen-binding and competitive binding of scFv

Two different HCV RNA-positive enriched pellet preparations or HCVsp (HCV-*Fan*, genotype *1a/2a*; HCV-*Lat*, genotype *3*) prepared as previously described from two different HCV chronically-infected patients [6,7] were used as antigenic probes in dot blot and western blot experiments for testing the scFv D32.10 recognition to natural complete HCV particles, to E1E2 envelope complexes and to E1 and E2 proteins. For dot blot assay, the HCV preparations (HCVsp-*Fan* and HCVsp-*Lat*, 5 μg of protein) were spotted onto nitrocellulose paper. The immunoblotting was performed by incubation overnight at 4 °C with either 5 μg/ml of mouse IgGs D32.10 or 10 μg/ml of scFv D32.10 as the primary antibodies. Mouse IgGs or scFv bound were detected by incubation for 1 h with peroxydase-conjugated anti-mouse immunoglobulins (diluted 1/10 000) or anti-His-tag (diluted 1/5000) as the secondary antibodies (Sigma–Aldrich). For competitive binding experiments, a mixture of a constant amount of IgG D32.10 (5 μg/ml) and increasing amounts of scFv D32.10 (10, 20 or 50 μg/ml) diluted in PBS, supplemented with 0.02% BSA and 0.05% Tween 20, were added for 1 h of incubation. Conversely, a mixture of a constant amount of scFv D32.10 (10 μg/ml) and increasing amounts of IgG D32.10 (5, 10, 20, 50 μg/ml) diluted in the same conditions were added for 1 h of incubation. Spots were then visualized by chemiluminescence detection using SuperSignal West Pico Luminol/Enhancer solution (Thermo Scientific). Densitometric measurement was performed by using ImageJ 1.43u software (National Institute of Health, USA).

For western blotting assay, the preparations (HCVsp-*Fan* and HCVsp-*Lat*, 5 μg of protein) were subjected to SDS–PAGE under reducing or non-reducing conditions (2% SDS ± 5% 2-ME). After transfer, the mouse IgGs D32.10 (10 μg/ml) or scFv (20 μg/ml) diluted in TBS-Tween-5% milk were added as primary antibodies. The revelation phase was performed using peroxydase-conjugated anti-mouse or anti-His-tag antibodies.

To assess the specificity of the antibody staining (IgG and scFv D32.10), normal human serum (NHS) was used as control probe in both dot and western blot experiments. His-tag protein was used as positive control for revelation using anti-His tag antibodies.

2.6. Biophysics characterization and structure determination

Online MALLS detection was performed with a DAWN-HELEOS II detector (Wyatt Technology, Santa Barbara, CA, United States). Data were analyzed, and weight-averaged molecular masses (M_w) were calculated using the ASTRA software (Wyatt Technology, Santa Barbara, CA, United States). The S75 Superdex column (GE Healthcare) was equilibrated with 100 mM Tris at pH 9 containing 500 mM NaCl.

3. Results

3.1. Production, purification and structure of the scFv D32.10

The nucleotide sequence obtained from the rearranged V region genes of D32.10 is shown in Fig. S1 (Supplementary data) and was deposited at EMBL (European Bioinformatics Institute, United Kingdom) with WEBIN ID number Hx2000032426. The scFv was constructed by introducing the 15-residue linker, $(Gly_4Ser)_3$, connecting the C-terminus of V_H to the N-terminus of V_L (Fig. 2). The recombinant protein contains a hexahistidine tail to facilitate purification and identification. An induction of 4 h at 37 °C with 1 mM IPTG was performed. The final crude bacterial extract (Fig. 1, lane 1) was subjected to purification by denaturing IMAC chromatography, and SDS–PAGE analysis of fractions showed that elution of the protein was obtained at 300 mM imidazole in quite pure form (Fig. 1, lanes 2, 3 and 4). The yield of recombinant scFv was 10 mg/L of culture medium corresponding to the scFv present in the starting material. Although the molecular weight (MW) was estimated from the sequence to be 29.6 kDa, SDS–PAGE analysis gave an apparent MW of approximately 28 kDa.

In order to determine the oligomeric state in solution of the scFv, a western blotting assay (Fig. 3B) and a multiangle laser light scattering study (MALLS) (Fig. 3C) were performed on renatured and gel filtration purified products (Fig. 3A). Fig. 3A showed one large shoulder (numbered as 1 & 2) and two peaks (numbered 3 & 4). The corresponding fractions were subjected to SDS–PAGE and western blotting in reducing (R) and non-reducing (NR) conditions (Fig. 3B). Under NR conditions, Fig. 3B, lanes 1 and 2 showed

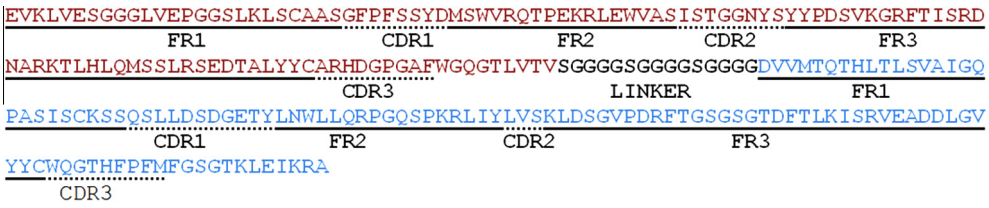

Fig. 2. Encoded amino acid sequence of the scFv D32.10. The red color show the V_H domain of D32.10, the black color the linker $(Gly_4Ser)_3$, and the blue color the V_L domain of D32.10. Framework (FR) and CDR were in solid and dotted lines, respectively.

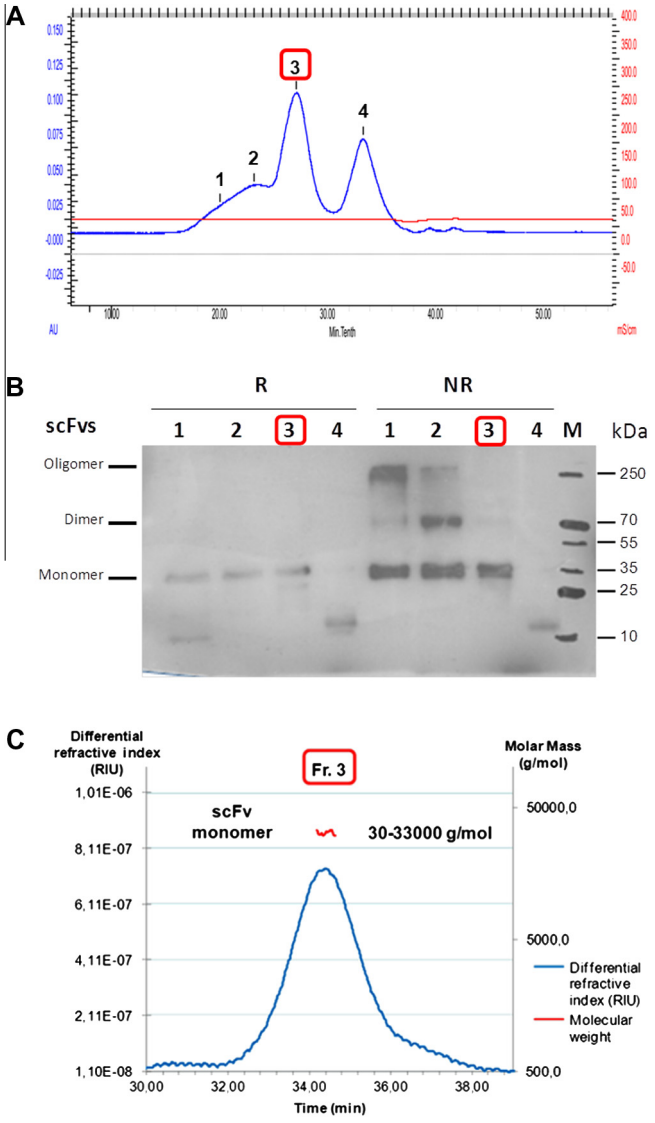

Fig. 3. Analysis of the purified scFv D32.10 by using MALLS methodology. After purification of the scFv on high resolution 10/30 Superdex 75 column (A), the fractions 1, 2, 3 and 4 were subjected to SDS–PAGE (B) under reducing (R) and non-reducing (NR) conditions, and the fraction 3 was analyzed by MALLS (C).

the presence of misfolded oligomeric and dimeric scFv, in the peaks 1 and 2, respectively. Only scFv monomers were detected in the fractions 3 under both NR and R conditions (Fig. 3B). Possibly, the fraction 2 may also contain functional non-covalent dimers or diabodies [10]. The results achieved by MALLS confirmed that the fraction 3 is pure and consists of a homogenous monomeric scFv D32.10 species with a MW of 30–33 kDa (Fig. 3C).

3.2. The scFv specifically recognizes serum-derived HCV particles (HCVsp), E1E2 envelope complexes and E1, E2 glycoproteins and competes with the full IgG

Two HCVsp isolates, from patient 1 with genotype (GT) 3, and from patient 2 with two distinct GT1a and 2a [12], were used in dot blot experiments for their recognition by IgG D32.10 as positive controls in comparison with the scFv. As shown in Fig. 4, IgGs and scFv D32.10 specifically and strongly reacted with both HCVsp isolates, whereas did not react with NHS used as control probe (Fig. 4A and B, lanes 0). As the native antibody, the scFv showed a higher reactivity with the HCVsp isolate 1 (HCV-*Lat*, GT3) than with the HCV isolate 2 (HCV-*Fan*, GT1a/1b), independently of the genotype but dependent on surface pattern of HCV particles [6,7]. It is worth noticing that the scFv significantly inhibited in a concentration-dependent manner the binding of IgG D32.10 to both HCVsp isolates (>50% for the isolate 1 and >70% for the isolate 2 at a concentration of 50 μg/ml corresponding to a molecular ratio IgG/scFv = 2) (Fig. 4A). Conversely, IgG D32.10 inhibited the binding of scFv to both HCVsp isolates in a concentration-dependent manner with a complete (100%) inhibition at a concentration of 50 μg/ml for the isolate 1 and at a concentration of 20 μg/ml for the isolate 2 (Fig. 4B). This supports that the scFv and full IgG molecule of D32.10 recognize the same epitope and exhibit similar binding affinities to antigen.

The monoclonal antibody D32.10 has been shown to also react with large E1E2 complexes and E1 and E2 glycoproteins [5]. Therefore, using the same HCVsp isolates as antigenic probes, the HCV polypeptide specificity of scFv D32.10 was tested by western blot analysis (Fig. 5) under non-reducing (NR) and reducing (R) conditions. When the two GT3 and GT1a/2a isolates (corresponding to lanes 1 and 2, respectively) were analyzed under NR conditions (Fig. 5A), scFv D32.10 recognized disulfide-linked complexes recovered in the upper part of the gel (⩾250 kDa) as IgG D32.10. These high MW bands likely correspond to hetero-oligomeric E1E2 complexes [6,13]. Under R conditions (Fig. 5B), scFv D32.10 recognized two (HCVsp GT1a/2a, lane 2) or three (HCVsp GT3, lane 1) bands at 31 kDa, 62 kDa and around 100 kDa, corresponding to E1, E2, and heterodimer E1E2, respectively. Same patterns were observed with IgGs D32.10. No reactivity was detected with NHS (lanes 3) as control antigenic probe. As positive control, His tag protein (lane 4) was detected by anti-His tag antibodies.

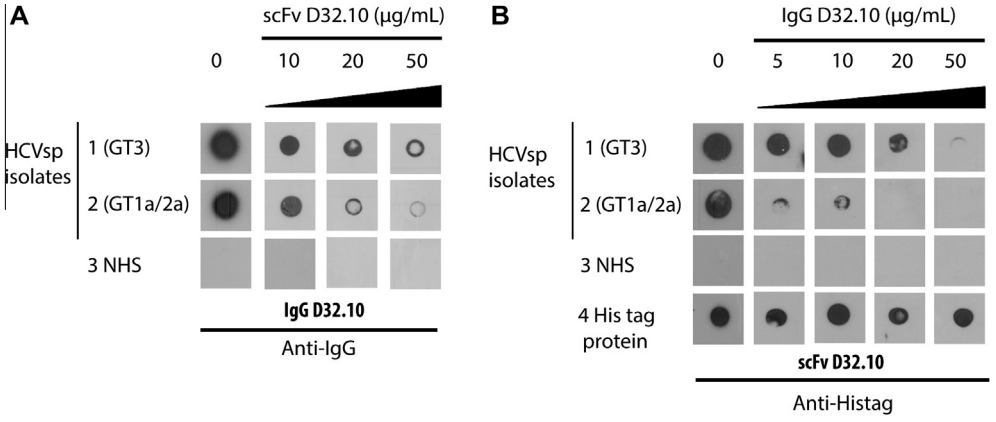

Fig. 4. Cross-competition of scFv with the parental IgG D32.10. Dot blot experiments with two HCVsp isolates 1 (GT3) and 2 (GT1a/2a), NHS as control antigenic probe (3) and His tag protein (4) as positive control for anti-Histag antibodies. (A) Competition with increasing amounts of scFv. (B) Competition with increasing amounts of parental IgG.

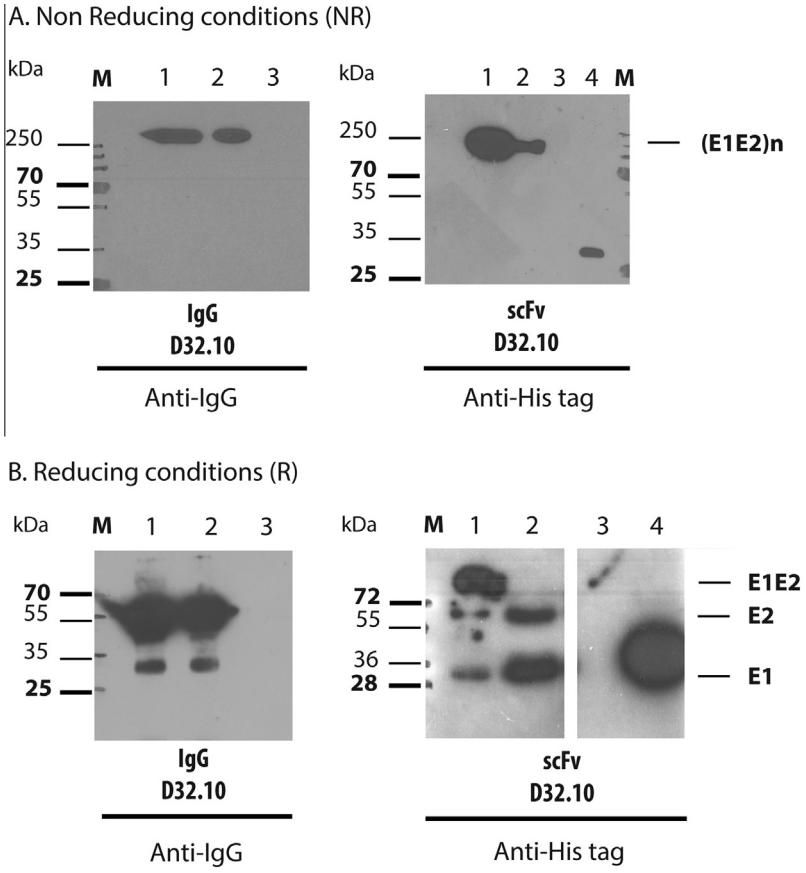

Fig. 5. scFv D32.10 reactivity with serum-derived HCV E1E2 proteins. Western blot with two HCVsp isolates (lanes 1, GT3 & lanes 2, GT1a/2a), NHS (lanes 3) and His tag protein (lanes 4) as control probes, tested under NR (A) and R (B) conditions. Immunoblotting was performed using IgG or scFv D32.10. The molecular masses of markers are indicated in kilodaltons (kDa).

Altogether, these results confirm that the scFv D32.10 exhibits the same HCV E1E2 antigenic specificity as the native antibody.

4. Discussion

The scFv construction of anti-HCV E1E2 D32.10 mAb is functional since it recognizes serum-derived HCV 1a/2a and HCV 3a particles, as well as large E1E2 complexes and E1 and E2 glycoproteins [5–7]. Moreover, it competes with D32.10 IgG for binding to antigen. By using size-exclusion chromatography associated with light-scattering, we show that the scFv D32.10 forms misfolded dimers and oligomers as frequently observed with scFv constructions, but functional well-refolded monomers are in great majority.

HCV envelope glycoproteins, which are involved in HCV entry into host cells, represent the major targets of neutralizing antibodies (nAbs). To date the majority of neutralizing mAbs against HCV infection target conserved regions on E2 [14]. Mapping of neutralization epitopes on the HCV E2 protein sequence showed that nAbs to linear epitopes targeted a segment adjacent to HVR1, encompassing aa 410–425 as the mouse mAb AP33 having contact residues within aa 412–423 [15,16], and to conformational epitopes targeted mainly two discontinuous regions, aa 424–443 and aa 523–540 [15]. However, the 412-423 epitope appeared to be weakly immunogenic and AP33-like antibodies are less prevalent in HCV-infected patients suggesting that such nAbs do not play a major role in natural clearance of HCV infection [17]. Moreover, AP33-escape mutants arose under selection pressure and are resistant to AP33-mediated neutralization [18].

The anti-HCV E1E2 mAb D32.10 is the unique antibody obtained by immunization with HCV particles isolated from infected patients. Epitope mapping identified the D32.10 epitope being formed by three well-conserved discontinuous E1 (aa 297–306) and E2 segments (aa 480–494 and aa 613–621), obviously highly immunogenic and juxtaposed on the surface of native circulating enveloped HCV particles (HCVsp) in chronic hepatitis C patients [5,6]. Unlike well-characterized anti-HCV E2 human mAbs with potent cross-neutralizing activity, D32.10-like antibodies have been shown to be associated with spontaneous virus clearance and predictive for complete viral response in chronically-infected patients under SOC antiviral therapy [9]. The E1E2 epitope targeted by the D32.10 mAb has been shown to be preferentially involved in interactions between the virus and polarized hepatocytes [7]. In the recently proposed 3D-model of HCV E2 ectodomain (E2e) [19], the E2 segment, aa 613–621, recognized by D32.10 encompassed residues 613–618 important for CD81 binding. The E2 segment, aa 480–494, also recognized by D32.10 encompasses a highly conserved stretch of residues stabilized by disulphide bridge 3 (Cys486–Cys494). This segment was close to the putative highly conserved fusion peptide, which may also form a contact with E1 glycoprotein [19]. Obviously, these three segments which composed the discontinuous antigenic determinant identified by the D32.10 mAb were exposed and physically very close in the quaternary structure of the E1E2 envelope glycoprotein complexes expressed on the surface of natural HCVsp of all genotypes.

The unique properties of mAb D32.10 highlighted the relevance of developing D32.10-based anti-HCV therapy. Therefore, this study was conducted to generate a scFv more convenient to use

than the full-length mAb, for medical applications. Here, we demonstrate that the scFv D32.10 fragment with a V_H-linker-V_L orientation is expressed mainly as functional forms. Indeed, it binds native HCVsp as well as E1 and E2 antigens and significantly inhibits the binding of the IgG D32.10 to HCVsp. Therefore, it efficiently recognizes the same epitope than the original parental mAb. Future studies should include investigations in in vitro neutralization assay and in vivo animal models to enhance the therapeutic applicability of this scFv, especially in the issue of HCV reinfection after liver transplantation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.057.

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