FEBS Letters 587 (2013) 3335–3340

FEBS Letters

journal homepage: www.FEBSLetters.org

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

Catherine Fallecker [a,b,c], Nicolas Tarbouriech [a,b,c], Mohammed Habib [a,b,c], Marie-Anne Petit [d,e,*], Emmanuel Drouet [a,b,c,*]

[a] Univ. Grenoble Alpes, Unit for Virus Host-Cell Interactions, F-38000 Grenoble, France
[b] CNRS, Unit for Virus Host-Cell Interactions, F-38000 Grenoble, France
[c] Unit for Virus Host-Cell Interactions, Univ. Grenoble Alpes-EMBL-CNRS, 6 rue Jules Horowitz, 38042 Grenoble, France
[d] Centre de Recherche en Cancérologie de Lyon (CRCL) INSERM U1052/CNRS UMR5286, 68424 Lyon Cedex 03, France
[e] Université Claude Bernard Lyon 1, 69008 Lyon, France

ARTICLE INFO

Article history:
Received 4 May 2013
Revised 12 July 2013
Accepted 19 July 2013
Available online 7 September 2013

Edited by Hans-Dieter Klenk

Keywords:
Hepatitis C virus
E1E2
Antibody engineering
Single chain Fv fragment
Anti-HCV therapy

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

* Corresponding authors. Address: CRCL INSERM Unit 1052, 151, Cours Albert Thomas, 69424 Lyon Cedex 03, France. Fax: +33 (0)472 681 971 (M.-A. Petit). Address: Unit for Virus Host Cell Interactions UVHCI, UMI 3265 UJF-EMBL-CNRS, 6 rue Jules Horowitz, B.P. 181, 38042 Grenoble Cedex 9, France. Fax: +33 (0)4 76 20 94 00 (E. Drouet).

E-mail addresses: marie-anne.petit@inserm.fr (M.-A. Petit), drouet@embl.fr, emmanuel.drouet@ujf-grenoble.fr (E. Drouet).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2013.07.057

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