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Recombinant Flag-tagged E1E2 glycoproteins from three hepatitis C virus genotypes are biologically functional and elicit cross-reactive neutralizing antibodies in mice

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ABSTRACT

Hepatitis C virus (HCV) is a globally disseminated human pathogen for which no vaccine is currently available. HCV is highly diverse genetically and can be classified into 7 genotypes and multiple sub-types. Due to this antigenic variation, the induction of cross-reactive and at the same time neutralizing antibodies is a challenge in vaccine production. Here we report the analysis of immunogenicity of recombinant HCV envelope glycoproteins from genotypes 1a, 1b and 2a, with a Flag tag inserted in the hypervariable region 1 of E2. This modification did not affect protein expression or conformation or its capacity to bind the crucial virus entry factor, CD81. Importantly, in immunogenicity studies on mice, the purified E2-Flag mutants elicited high-titer, cross-reactive antibodies that were able to neutralize HCV infectious particles from two genotypes tested (1a and 2a). These findings indicate that E1E2-Flag envelope glycoproteins could be important immunogen candidates for vaccine aiming to induce broad HCV-neutralizing responses.

1. Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is a globally disseminated human pathogen causing liver disease, such as cirrhosis and hepatocellular carcinoma (Alter and Seeff, 2000). Globally, in 2015, an estimated 71 million people were living with chronic HCV infection (WHO, 2017). Despite the recent development of highly effective direct-acting antivirals (DAA) (González-Grande et al., 2016), the infection remains a major health problem worldwide. This is due to the limited availability and high cost of new therapies, low infection awareness and high probability of reinfection in high-risk groups (Baumert et al., 2014). Therefore, an effective prophylactic and/or therapeutic vaccine is still needed to control the virus globally. One of the major obstacles for vaccine development is the extreme genetic variability of HCV, driven by its escape from immune pressure.

The HCV envelope glycoproteins E1 and E2 play a crucial role in the complex process of virus entry into the host cell. They are a primary target for the antiviral adaptive immune response and therefore are important immunogen candidates for the design and development of vaccines against HCV (Wang et al., 2011). The current knowledge of E1E2 structure and functions comes from numerous biochemical, molecular and immunological studies and was recently improved by obtaining the crystal structure of E2 core (Khan et al., 2014; Kong et al., 2013). However, the genetic diversity and the complex structure of the heterodimer formed by E1 and E2 makes them a very difficult research target.

Here we show the construction, purification and broad functional and immunological evaluation of E1E2-based antigens derived from three different HCV genotypes. The E1E2 recombinant proteins were tagged with the Flag tag, for the facilitation of protein isolation and purification. Numerous recombinant Flag-tagged viral proteins have been previously described and efficiently purified by various groups. These include the gp120 of simian immunodeficiency virus (SIV) (Laird and Desrosiers, 2007), ORF virus envelope proteins (Tan et al., 2009) and the VP1 protein from foot-and-mouth disease virus (FMDV) (Lawrence et al., 2013). Furthermore, the Flag tag has been successfully used in the study of HCV for the purification of cell cultured viral particles (HCVcc) (Merz et al., 2011; Prentoe and Bukh, 2011).

We previously identified a site within the hypervariable region 1 (HVR-1) of the genotype 1a HCV strain H77 glycoprotein E2 where a small insertion of 5–6 amino acids was tolerated without a negative effect on the protein structure and function (Rychlowska et al., 2011).

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Based on that data, in the present report we constructed and analyzed three E1E2 mutants with the Flag octapeptide inserted at amino acid position 409 in the HVR-1 of E2. We show that such an insertion is well tolerated in three different HCV genotypes (1a, 1b and 2a). We also demonstrate that Flag insertion in this site does not hinder protein expression, proper conformation of E2 and the activity of the glycoprotein – E1E2 dimer formation and CD81 binding. Moreover, we examined the immunogenic properties of E1E2-Flag and found that immunization of mice with affinity purified recombinant Flag-tagged proteins induced anti-E2 antibodies capable of neutralizing cell cultured HCV (HCVcc). These results establish the E1E2-Flag as potential vaccine immunogens as well as tools for molecular and antigenic studies.

2. Results

2.1. Construction and expression of E1E2-Flag glycoproteins

In this study, we have constructed Flag-tag modified E2 glycoproteins derived from the two HCV genotypes most prevalent in Europe and North America – 1a and 1b (Petruzziello et al., 2016), as well as from genotype 2a, from which the first clone replicating efficiently in cell culture was isolated (Wakita et al., 2005; Zhong et al., 2005; Kato et al., 2006) (Fig. 1. A.). The sequences used for this study were previously described by (Tarr et al., 2007), who amplified E1E2 from patient-derived sera and cloned them into the pcDNA3 expression vector, under the control of the human cytomegalovirus (CMV) promoter. The Flag DYKDDDDK octapeptide tag was introduced at position 409 in E2, immediately preceding the conserved Q410 glutamine residue.

The E1E2-Flag proteins were transiently expressed in 293T cells and the expression was examined by Western blotting and in situ immunostaining. The recombinant proteins were recognized by both the anti-E2 mAb AP33, as well as by the anti-Flag mAb M2 (Fig. 1. B.). E2 proteins were of expected molecular weight of 62 kDa and the expression level was similar to the wild type E2, indicating that the addition of the tag did not affect the efficiency of protein synthesis. Immunohistochemical staining of 293T cells after transfection was performed in parallel to Western blotting in order to visualize the expression of the E2 proteins directly in cells. The detection of immunostained E1E2 wt and Flag from genotype 1a is shown on Fig. 1. C. Similarly, when using an anti-E2 mAb positive signal was observed in cells expressing both unmodified and tagged E1E2, while anti-Flag mAb recognized exclusively the Flag tagged proteins and these results were consistent for all three genotypes. Efficient cell immunostaining with anti-Flag mAb suggested that the Flag epitope was exposed on the surface of recombinant HCV E2 glycoproteins and could be used for further purification purposes.

2.2. Affinity purification of E1E2-Flag heterodimers

It has been previously described that HCV E1 and E2 proteins assemble into non-covalent heterodimer (Castelli et al., 2017; Dubuisson et al., 1994; Freedman et al., 2017). Ideally, the insertion of an affinity tag in either E1 or E2 should not interfere in this process and should allow the efficient purification of functional E1E2 heterodimer with correct conformation.

Agarose coupled to the M2 anti-Flag antibody was used as affinity resin to purify E1E2-Flag heterodimers from the lysates of transfected 293T cells. Proteins were expressed transiently for 48 h and purified from cell lysates. To optimize the elution process, two different methods were used – a competition with the 3x Flag peptide and a low pH buffer elution. We observed significantly higher efficiency of E1E2-Flag elution using glycine buffer pH 2.7, which together with its cost effectiveness was the reason why this method was chosen for further experiments. In the optimized elution procedure, the low pH in the eluted fractions was immediately neutralized by Tris-HCl buffer pH 8.8. Consistently with other reports (Meertens et al., 2006; Tscherne et al., 2006), we have observed that short incubation in low pH followed by a return to neutral pH did not result in precipitation or denaturation of HCV glycoproteins. Following analysis of protein content and concentration, selected elution fractions were pooled and concentrated 10x by ultrafiltration.

We have estimated the purification efficiency of E1E2-Flag after transfection of 293T cells as 15 μg from 5×10^6 cells growing in stationary cultures. Protein concentration was estimated by Bradford assay and the purity was verified on a Coomassie-stained 10% SDS-PAGE gel and Western blotting. The same purification procedure was used for all three genotypes and an example for the purification of gt1a E1E2-Flag is shown on Fig. 2.

2.3. Analysis of E1E2 heterodimer formation

As mentioned before, correctly folded and functional intracellular E1 and E2 proteins form a non-covalent heterodimer. In order to investigate the ability of E2-Flag to bind its partner glycoprotein E1, radioimmunoprecipitation assay (RIPA) was performed. The anti-E2 mAb AP33 was used to precipitate the putative complexes from the lysates of transfected 293T cells. To differentiate between precipitated covalently bound protein aggregates and co-precipitated non-covalent E1E2 heterodimers the proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions (Fig. 3. A.). As seen on the non-reducing gel, monomeric E1 and E2 can be detected apart from a higher molecular weight, b-mercaptoethanol-resistant multimers. Such monomeric forms indicate that at least a part of E1 and E2-Flag are able to associate into functional non-covalent heterodimers. For glycoprotein E1 multiple bands could be detected, most probably representing different glycosylation states of the protein, as observed by others in earlier studies (Dubuisson et al., 2000). These results were further confirmed using anti-Flag antibody for IP. As expected, the M2 mAb co-precipitated E1 together with E2 from the Flag mutants exclusively (data not shown). Concluding, insertion of the Flag epitope did not influence the heterodimerisation process of E1 and E2 HCV glycoproteins expressed transiently in HEK 293T cells.

2.4. E1E2-Flag proteins bind to HCV receptor CD81

As a next step, we investigated the ability of the E1E2-Flag proteins to bind the HCV receptor CD81. CD81 is one of the essential entry receptors and blocking E1E2 interaction with CD81 neutralizes HCV infection in vitro (Fénéant et al., 2014). The regions in E2 and CD81 responsible for the interaction have been mapped (Drummer et al., 2006) and CD81 binding is often used as a functional “quality control” assay for recombinant HCV glycoproteins (Heile et al., 2000). Here, the assay was based on purified recombinant large extracellular loop (LEL) of human CD81 protein, fused to GST. Immobilized GST-hCD81-LEL was used as bait in pull-down assay to bind E1E2 proteins from cell lysates (Fig. 3. B.). As a control, GST alone was used as bait. Recombinant CD81, but not GST alone, was able to pull down detectable amounts of all the HCV E1E2 variants but with different efficiency. Results indicate that the addition of the Flag epitope did not disrupt the CD81 binding site on E2.

2.5. The addition of the Flag epitope does not affect the folding and conformational epitopes of glycoprotein E2

To analyze the influence of the insertion of Flag epitope in the HVR-1 region on E2 folding, we used a panel of human monoclonal antibodies recognizing different conformational epitopes in E2. First, we immobilized the recombinant E1E2 onto ELISA plates by GNA-capture. The lectin bound proteins were then probed with conformation-sensitive antibodies mapped to different domains in HCV E2. The load of E1E2 proteins immobilized on the ELISA plates was normalized based