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Functional characterization of p7 viroporin from hepatitis C virus produced in a cell-free expression system

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ABSTRACT

Using a cell-free expression system we produced the p7 viroporin embedded into a lipid bilayer in a single-step manner. The protein quality was assessed using different methods. We examined the channel forming activity of p7 and verified its inhibition by 5-(N,N-Hexamethylene) amiloride (HMA). Fourier transformed infrared spectroscopy (FTIR) experiments further showed that when p7 was inserted into synthetic liposomes, the protein displayed a native-like conformation similar to p7 obtained from other sources. Photoactivable amino acid analogs used for p7 protein synthesis enabled oligomerization state analysis in liposomes by cross-linking. Therefore, these findings emphasize the quality of the cell-free produced p7 proteoliposomes which can benefit the field of the hepatitis C virus (HCV) protein production and characterization and also provide tools for the development of new inhibitors to reinforce our therapeutic arsenal against HCV.

1. Introduction

The hepatitis C virus (HCV) is a small enveloped, positive-sense single-stranded RNA virus of the family *Flaviviridae*. It is considered to be a major public health problem as 150 million people are infected currently resulting in more than 300,000 deaths annually (World Health Organization, 2013). The virus causes those chronically infected to develop liver diseases that may progress into fibrosis, cirrhosis or hepatocellular carcinoma. While there are vaccines to prevent hepatitis A and B; no vaccine for hepatitis C is yet available to prevent the spread of infection [1,2]. The current treatments' effectiveness against HCV is genotype dependent, including therapy with pegylated interferon or recently launched interferon-free treatments using nucleoside/nucleotide NS5B polymerase inhibitors and proteases inhibitors. The HCV replication is highly error prone due to the lack of correcting mechanisms of the HCV RNA-dependent RNA polymerase [3]. As a result, this high mutation rate is consistent with the high degree of HCV diversity found across the population of infected individuals. Within genotypes and subtypes, mutations of the NS3, NS5A and NS5B proteins induce resistance to newly developed direct antiviral agents (DAAs) [4,5]. With the apparition of resistance associated variants, the high cost of the new DAAs and their scarce global availability, the discovery of complementary treatments to fight against HCV is still needed.

The viroporin p7 is located between the structural proteins: core, E1, E2 and the nonstructural protein NS2, resulting in the production of a precursor E2-p7-NS2. Activation of host signal peptidases induces cleavages in the precursor releasing p7 from the viral polyprotein [6]. The HCV p7 protein is involved in the viral life cycle including virus assembly and infectivity which makes it a promising drug target [7]. It is a 63-amino acid integral membrane protein and has different potential topologies and conformations as indicated by diverse studies including NMR [8–12]. Other studies also revealed an N-terminal α-helix and two transmembrane segments connected by a short hydrophilic cytosolic segment of 7 amino acids [13–15] (Fig. 1A). A recent review on p7 displays the different reported topologies of the protein [16]. The p7 protomer is

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able to oligomerize to an hexameric [15,17] and heptameric state [12] forming an ion channel. This viroporin is cation selective and a list of small molecule drugs such as amantadine, rimantadine, BIT225, hexamethylene amiloride (HMA) and long-alkyl-chain iminosugars, including N-nonyl deoxynojirimycin (*N*N-DNJ), have been identified as potential negative regulators of the p7 channel [17–20]. *In cellulo*, amantadine, *N*N-DNJ, and rimantadine have been shown to inhibit the release of infectious HCV and virus entry in a genotype-depent manner [21] while *N*N-DNJ, BIT225 and rimantadine have been reported to inhibit cell-free virus transmission [22].

Expression of the p7 membrane protein in classical overexpression systems is difficult due to its high content in hydrophobic amino acids and its small size. Several studies have been performed in order to obtain sufficient amounts of p7 for functional and structural studies, including peptide synthesis [9,18,19,23], expression in *Escherichia coli* (*E. coli*) either in inclusion bodies [12,24] or under a soluble form (i.e. when p7 is fused to the maltose binding protein (MBP) and an histidine tag [25]). However, these methods for protein production require tremendous work and display some limiting features on numerous aspects. To recover a sufficient quantity of active p7 protein embedded into a lipid bilayer, these production strategies involve several critical steps including; resolubilization, refolding and protein insertion into liposome in the presence of detergents. An attractive alternative for producing such difficult to express proteins is the use of cell-free expression systems [26,27]. Membrane proteins have been expressed using both eukaryotic and prokaryotic cell-free systems [28–30]. One of the interesting features of the cell-free expression systems resides in their capacities to be easily adapted to the production of the protein of interest because they are completely open systems, in which each reaction parameter can be modified. Production of membrane proteins in cell-free expression systems can be achieved either in a precipitated form (P-CF) without any additives or in the presence of lipids (L-CF) or detergents (D-CF) [31–35]. The detergents will shield the hydrophobic domains of the protein and help its folding and solubilization. The use of detergents can be detrimental and some of them can alter the protein structure and thus impact their activities [36,37]. They can also be incompatible with cell-free expression systems, as some can inhibit the transcription/translation machinery [38]. Addition of lipids directly in the expression reaction mix represents a more suitable alternative for producing active membrane proteins in a native-like conformation. Several studies have demonstrated that membrane proteins expressed in cell-free systems with natural or synthetic liposomes can be directly inserted into the lipid bilayer in order to form recombinant proteoliposomes [39–42]. This insertion renders the production of proteoliposomes simpler by avoiding the purification and the resolubilization of the membrane proteins using detergents before relipidation. Different lipid compositions of the vesicles mimicking the natural lipid environment of the membrane protein can be optimized for best yield and protein activity. In addition to direct addition of liposomes, small parts of lipid bilayers wrapped in a lipoprotein can form lipid disks which are able to accept membrane proteins during synthesis [29,43].

In this study, we used an *E. coli* cell-free expression system containing synthetic liposomes to express HCV p7 protein and obtain p7 proteoliposomes in a one step process. The liposomes enabled the correct folding of the viroporin into a native-like conformation as shown by Fourier transformed infrared spectroscopy (FTIR). The most commonly used method to analyze ion channel activity is the measurement of currents caused by the ion flow. In this context, we have demonstrated the functionality of the recombinant p7 protein by patch-clamp recordings. We show that the channel activity is inhibited by HMA as predicted. We also investigated the oligomerization states of the p7 protein embedded into a lipid bilayer by using photoactivatable amino acid analogs and a chemical agent for cross-linking studies. Our results emphasize that the use of cell-free protein synthesis in presence of liposomes is a reliable and efficient system for membrane protein expression. The obtained proteoliposomes represent a great tool for the study of the HCV p7 viroporin and the development of new treatments.

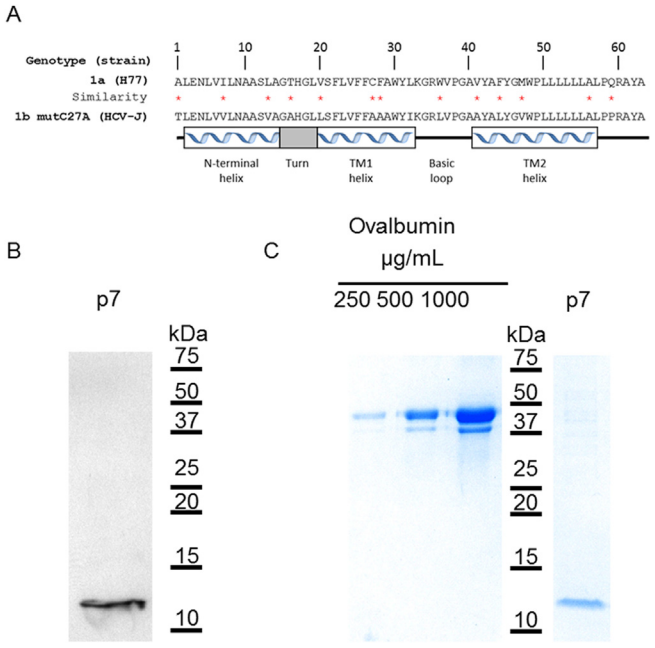

Fig. 1. Sequence, structure and expression analysis of p7. (A) The amino acid sequence of the expressed p7 from genotype 1a strain H77 is displayed and aligned with the sequence of the genotype 1b strain HCV-J. The corresponding schematic representation of helical, turn, and loop regions deduced from the NMR structure analysis of p7 genotype 1b strain HCV-J is also displayed [9] as no NMR data is available for p7 from genotype 1a strain H77. TM = transmembrane. (B) Western immunoblotting of p7 proteoliposomes; (C) Coomassie brilliant blue staining: 5 µl of ovalbumin (different concentrations were used as standards) and 1 µl of p7 proteoliposomes after sucrose gradient purification were loaded on SDS-PAGE.

2. Materials and methods

2.1. Cell-free synthesis

p7 protein sequence from HCV strain H77 genotype 1a was synthesized by DNA2.0. The gene was then cloned directly into a pIVEX2.4d (Roche Applied Science) using NdeI/XhoI restriction sites.

Cell-free reaction was carried out at 30 °C for 16 h with gentle agitation at 400 rpm by using an *E. coli* extract and energy mix provided by Synthelis SAS. Energy mix composition was prepared according to Sitaraman et al. [44] without cAMP, betaine, trehalose and with 210 mM sodium oxalate and 33 mM NAD. DNA was added at 15 µg/ml. Mg^{2+} and K^{+} ion concentrations were screened for best yield: a range from 8 mM to 30 mM Mg^{2+} was assayed using magnesium acetate in correlation with a range of 250 mM–370 mM of K^{+} using potassium acetate.

Liposomes were prepared using a 10 mg/ml lipid mixture of (1,2-dioleoyl-sn-glycero-3-phosphocholine:1,2-dioleoyl-sn-glycero-3-phosphoethanolamine:1,2-dimyristoyl-sn-glycero-3-phosphate:cholesterol; 40:20:20:20 volume ratio) in chloroform. This composition has been previously shown to enable efficient cell-free