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The elusive function of the hepatitis C virus p7 protein

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ABSTRACT

Hepatitis C virus (HCV) is a major global health burden with 2–3% of the world's population being chronically infected. Persistent infection can lead to cirrhosis and hepatocellular carcinoma. Recently available treatment options show enhanced efficacy of virus clearance, but are associated with resistance and significant side effects. This warrants further research into the basic understanding of viral proteins and their pathophysiology. The p7 protein of HCV is an integral membrane protein that forms an ion-channel. The role of p7 in the HCV life cycle is presently uncertain, but most of the research performed to date highlights its role in the virus assembly process. The aim of this review is to provide an overview of the literature investigating p7, its structural and functional details, and to summarize the developments to date regarding potential anti-p7 compounds. A better understanding of this protein may lead to development of a new and effective therapy.

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Contents

Introduction

Hepatitis C virus (HCV) infection is a global health problem with an estimated 130–170 million people infected worldwide (Levrero, 2006). In chronically infected individuals development of cirrhosis and hepatocellular carcinoma are common clinical outcomes (Thomas, 2013). A combination of pegylated interferon alpha (peg-IFN-α) and ribavirin is the current standard of care, but is associated with poor tolerance and variable efficacies. Recently, an increase in the sustained virological response to HCV treatment was achieved through the use of direct-acting antiviral compounds in combination with peg-IFN-α and ribavirin. Unfortunately, these protease inhibitors are susceptible to the development of resistance and are associated with adverse side effects in addition to those already observed with the canonical treatment. Fortunately, additional promising antiviral compounds are in line for further analysis and development, and some, including novel classes of inhibitors, will be available in the clinic in the very near future (Feld and Hoofnagle, 2005). We believe that a greater understanding of the intricacies of protein function in the viral life cycle will provide insightful information plausibly leading to a broader range of better treatment options.

HCV belongs to the *Hepacivirus* genus within the *Flaviviridae* family. It has a single-stranded RNA genome of positive polarity. Classification of HCV has expanded to include seven discernibly different genotypes and multiple subtypes (Kuiken and Simmonds, 2009). HCV mainly infects hepatocytes through a sequential binding to multiple host receptors (CD81, scavenger receptor class B type 1 (SR-B1), Low density lipoprotein (LDL), claudin-1 and occludin), which synergistically promote tight binding and internalization of the virion.

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Next, a poorly understood pH-dependent fusion and uncoating step takes place, releasing the viral genome into the cytoplasm (HCV entry reviewed in (Zeisel et al., 2013)). The viral RNA genome is then recognized by host ribosomes via the internal ribosomal entry site (IRES) sequence located at the 5′ end of the untranslated region (UTR) in order to mediate viral genome translation. The translated polypeptide chain is approximately 3000 amino acids (aa) long and is co- and post-translationally processed by host- and virus-encoded proteases in association with the endoplasmic reticulum (ER) to generate the individual mature forms of the viral proteins (Fig. 1) (Hoffman and Liu, 2011). The envelope glycoproteins E1 and E2, as well as the core protein comprise the physical structure of the virion. The non-structural (NS) proteins (NS3, NS4A, NS4B, NS5A and NS5B) form a complex that performs viral RNA amplification (HCV RNA replication reviewed in (Moradpour et al., 2007)). Many of these proteins also interact in some way to support viral assembly (Appel et al., 2008; Ma et al., 2008; Masaki et al., 2008; Jones et al., 2009; Kohlway et al., 2013; Han et al., 2013; Hughes et al., 2009, 2009). The viral proteins p7 and NS2 are not involved in viral RNA replication (Jones et al., 2007; Steinmann et al., 2007,; Jirasko et al., 2008, 2010; Popescu et al., 2011; Stapleford and Lindenbach, 2011). NS2 is a transmembrane autoprotease that is composed of two domains: a N-terminal membrane anchor and a C-terminal cysteine protease, which mediates cleavage from the adjacent NS3 protein after translation (Hijikata et al., 1993; Lorenz et al., 2006). NS2 is required for HCV infectivity in vivo and virus production in HCV cell culture system (Pietschmann et al., 2006; Kolykhalov et al., 2000). It has been shown that NS2 homodimerizes and can interact with other viral proteins including p7. These interactions have led to the suggestion that NS2 has a major role in the HCV assembly process (Jirasko et al., 2008, 2010; Stapleford and Lindenbach, 2011; Dimitrova et al., 2003; Yi et al., 2009).

In the past, HCV research has been hindered by the inability to study the virus in the context of a complete life cycle. The replicon system was a systematic breakthrough that harnessed a neomycin selection gene and an ECMV IRES to mediate the translation of HCV NS proteins and enable RNA replication in vitro (Lohmann et al., 1999). Considering the replicon system failed to yield infectious

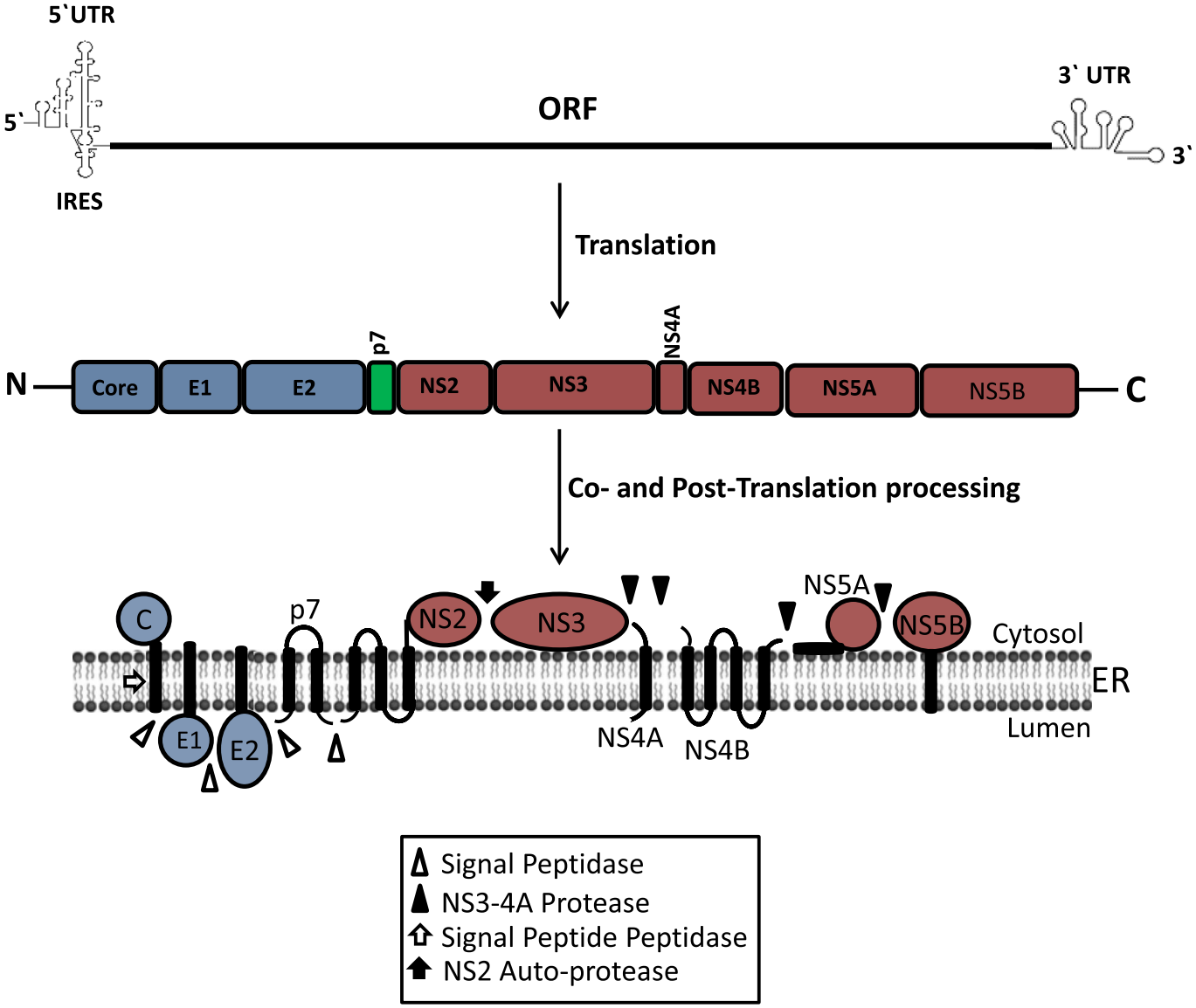

Fig. 1. HCV genome translation and processing. The HCV genome (top panel) contains an approximately 9.6 kb open reading frame (ORF) flanked by 5′ and 3′ un-translated regions (UTR). Translation is initiated at the IRES (internal ribosome entry site) sequence within the 5′ UTR to generate a 3000aa polypeptide chain comprised of unprocessed structural proteins core and envelope glycoproteins 1 and 2 (blue boxes; middle panel), as well as the non-structural proteins 2-5B (red boxes). The viral protein p7 is neither a structural or non-structural protein (green box). The polypeptide undergoes co- and post-translational processing in the ER to generate the mature form of the viral proteins (enzymatic digestion sites are indicated by symbols (bottom panel) and are defined in the inset).

virus and had no structural protein expression, the development of the pseudoparticle system (HCVpp) partially filled this gap. The HCVpp system exploited retrovirus biology to create chimeric viruses containing HCV glycoproteins on the surface. In this system 293 T cells were used for generation of particles containing E1 and E2, these particles could then be used to infect permissive hepatocyte cell lines and entry could be analyzed through the use of a reporter gene (luciferase or GFP) in the recombinant construct (Bartosch et al., 2003; Hsu et al., 2003). The discovery of the hepatitis C virus cell culture system (HCVcc) has provided the investigative capacity to study the whole HCV life cycle, including assembly and release, and thereby opened the door for mutagenesis studies of viral proteins in order to identify their individual functions and interactions within the HCV life cycle (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCVcc system revolves around the replicative power of the JFH-1 (Genotype 2a) or the H77 (Genotype 1a) construct (Wakita et al., 2005; Yi et al., 2006). The system provides insightful capabilities through the implementation of adaptive mutations and chimeric constructs that increase viral titers facilitating the assessment of genotypic variability (Pietschmann et al., 2006; Lindenbach et al., 2005; Russell et al., 2008).

The virus assembly process is initiated by recruitment of core to the surface of lipid droplets (LD) (an ER-associated organelle primarily functioning to store neutral lipids and cholesterol esters), which are believed to provide a platform for nucleocapsid formation (Boulant et al., 2006; McLauchlan et al., 2002). Viral RNA packaging and downstream envelopment steps are still poorly understood, but NS2 and p7 are believed to support the communication between replication complex and maturing virions (Jirasko et al., 2010; Stapleford and Lindenbach, 2011; Phan et al., 2009; Boson et al., 2011). The assembly process is hypothesized to coincide with the host VLDL pathway, and newly-formed virions are released as lipo-viral particles (LVPs) (HCV assembly reviewed in (Bartenschlager et al., 2011)).

The HCV p7 protein is located between E2 and NS2 and in its mature form is 63aa in length (Lin et al., 1994). In artificial lipid membranes p7 performs ion-channel activity, and is therefore included in the group of viral proteins termed viroporins (Griffin et al., 2003). These proteins are known to modulate membrane permeability to support virus entry, assembly or release (viroporins reviewed in (Nieva et al., 2012)). Depending on the genotype, p7 activity can be hindered by multiple ion-channel blockers, such as amantadine, rimantadine and iminosugar derivatives (Griffin et al., 2003, 2008; Pavlovic et al., 2003; Steinmann et al., 2007; Foster et al., 2011). In addition, p7 is dispensable for viral RNA replication, but is important for in vivo infectivity in the chimpanzee model, and for production of infectious HCVcc (Jones et al., 2007; Steinmann et al., 2007; Sakai et al., 2003; Brohm et al., 2009; Atoom et al., 2013). The function of p7 in the HCV life cycle remains a matter of debate, but multiple studies have placed its role to be at a late stage of the virus assembly process (Atoom et al., 2013; Gentzsch et al., 2013). In this review we aim to summarize the functional and structural comprehension achieved to date in the p7 field. We firmly believe that p7 represents an attractive target for novel HCV treatment, and a better understanding of its function is of the utmost importance for development of a comprehensive therapeutic regimen.

HCV p7 processing, topology, and localization

The p7 protein was identified through expression of a series of C-terminally truncated HCV polyproteins fused to a human c-myc epitope tag. This analysis located p7 between the envelope glycoprotein E2 and the NS2 protein (Fig. 1) (Lin et al., 1994). Homologous proteins have been identified in the *Pestivirus* genus, e.g., bovine viral diarrhea virus (BVDV), classical swine fever virus, and border disease virus, all containing such a characteristic protein between E2 and NS2 (Elbers et al., 1996). However, Flaviviruses yellow fever virus, dengue virus, and West Nile virus do not encode a protein homologous to p7 (Murray et al., 2008).

Upon HCV genome translation, p7 processing is mediated by host-encoded proteases (Fig. 1) (McLauchlan et al., 2002; Reed and Rice, 2000), but scission at the E2-p7 and p7-NS2 junctions is delayed, resulting in the presence of precursor E2-p7-NS2 polyproteins. The cleavage at the E2-p7 junction is incomplete and performed by a unique mammalian signal peptidase leading to the presence of E2 and p7 mature forms, as well as some E2-p7 species (Lin et al., 1994; Dubuisson et al., 1994; Mizushima et al., 1994). Partial cleavage was attributed to a structural determinant located N-terminal to the signal peptides. This is supported by the finding that fusion of reporter proteins N-terminal to the signal peptides improves cleavage efficiency (Carrere-Kremer et al., 2004). The function of E2-p7 or p7-NS2 precursors is yet unidentified, but hypotheses have been developed: (i) the precursor itself plays a role in the HCV life cycle, (ii) the precursor could be important for regulating the kinetics and/or levels of final product expression to prevent premature virus assembly (Carrere-Kremer et al., 2004; Shanmugam and Yi, 2013) (such a case was illustrated in the coordinated cleavage of C-preM in the Murray Valley encephalitis virus (Stocks and Lobigs, 1998)), and (iii) the E2-p7 precursor might be important for pulling p7 into the physical composition of the virion by retaining a pool in the ER as E2-p7 form (Griffin et al., 2005). However, it's likely that cleavage of E2-p7 precursors play a regulatory role because complete separation of E2 and p7 by insertion of an EMCV IRES between these two proteins moderately reduced the level of virus production in the HCVcc system. This suggests the E2-p7 precursor serves no functional purpose on its own, but that a temporal dependence on E2-p7 processing is critical for virus production (Jones et al., 2007; Shanmugam and Yi, 2013). In trans-complementation studies, it was found that whether p7 alone or p7 in the context of cleavage intermediates were sufficient for restoration of virus production depended on the extent of the original p7 deletion and the degree of indirect effects such deletions had on polyprotein processing. Brohm et al. (2009). In summary, it is clear that the p7-including processing intermediates in some way impact virus production, but whether these intermediates have specific functions within the viral life cycle requires further investigation.

The p7 protein is a polytopic membrane protein that crosses the endoplasmic membrane twice, forming trans-membrane domain 1 (TM1) and trans-membrane domain 2 (TM2) connected by a short segment (Fig. 2A and B), with its N- and C- termini oriented toward the cytosol (Lin et al., 1994). The subcellular localization of p7 was identified by fusion of p7 to CD4 or Myc. This fusion in HepG2 cells identified a large fraction of p7 in an early compartment of the secretory pathway suggesting the presence of a retention signal maintaining localization of p7 in the ER (Carrere-Kremer et al., 2002). Conversely, intracellular staining of GFP- or Flag-tagged p7 in 293 T cells showed that p7 partially co-localized with mitochondria and adjacent membrane structures (Griffin et al., 2004). Interestingly, staining of native and tagged p7 demonstrated that untagged p7 was exclusively detected on the ER, while N-terminally tagged p7 was detected on ER or mitochondrial adjacent membranes. This work is indicative of complex trafficking of p7 that could be regulated by the cleavage from its upstream signal peptide and targeting signals present within the protein sequence (Griffin et al., 2005). Immunofluorescence (IF) and electron microscopy (EM) in the context of a full-length, RNA replication-competent HCV genome derived

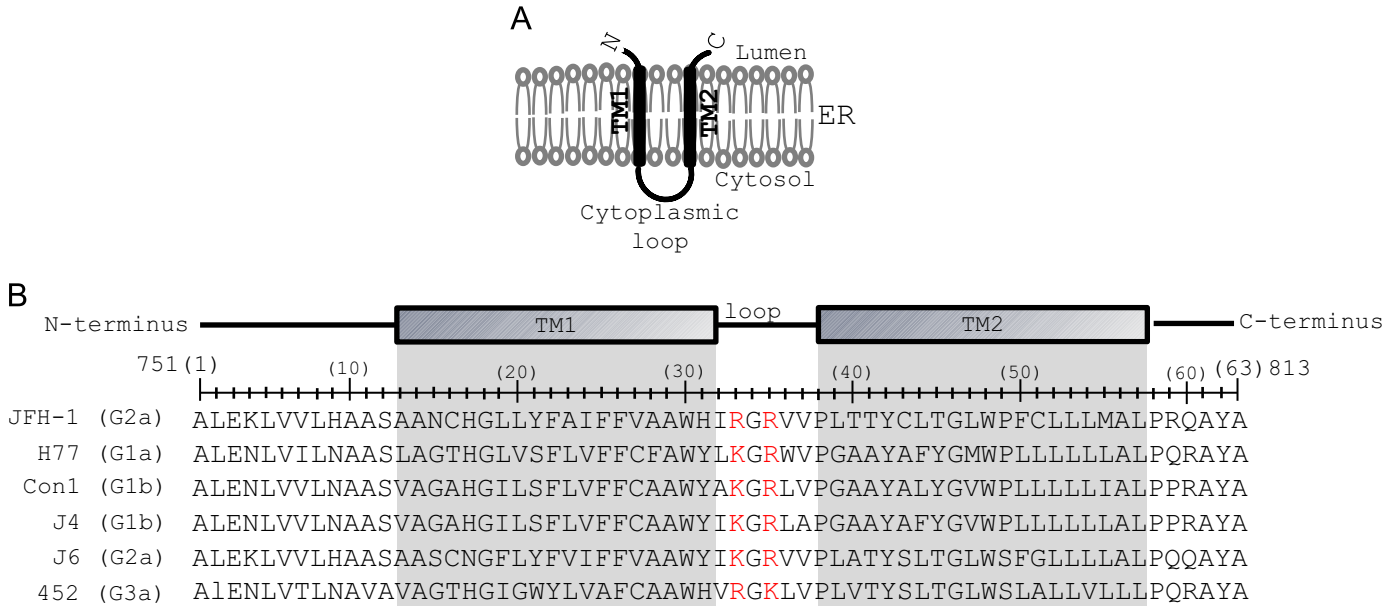

Fig. 2. p7 topology and sequence analysis. (A) Representative diagram of p7 topology within the endoplasmic reticulum (ER) showing the two transmembrane domains (TM1) and (TM2) connected by a cytoplasmic loop. Both N- and C-termini are oriented toward the ER lumen. (B) Examples of commonly used isolates with genotype/subtype sequence variability and domain locations shown. TM1 (aa13-32) and TM2 (aa38-57) are indicated by dark gray boxes. The two conserved basic residues in the cytoplasmic loop are shown in red.

from the JFH-1 sequence, showed that eGFP-p7 or p7 tagged with HA 4aa downstream of the potential E2-p7 cleavage site were localized only at the ER in Huh7 cells. However, it is important to note that these tags likely disrupted virus production (Haqshenas et al., 2007). Consequently, recent studies have double-HA-tagged p7 at the N-terminus in Jc1 (a genotype 2a chimeric construct containing J6 and JFH-1 genes with a junction point within NS2) creating a viable virus. This replicating virus showed significant co-localization of p7 with ER markers and a lesser extent of association with mitochondrial or lipid droplet markers. p7 showed a reticular staining pattern that mainly co-localized with HCV E2 and partially with NS2, NS3, and NS5A (Vieyres et al., 2013). Hypothetically, p7 localization with multiple organelles may be indicative of this protein's dual role in HCV assembly and trafficking of nascent virions through multiple cellular pathways.

The relatively small size of p7 and its membrane integration topology have made analysis of localization and function laborious and sometimes inconclusive. It is possible that the accessibility of antibodies or the duration of infection chosen in the above-mentioned literature inaccurately identified localization of p7 in multiple cellular compartments. Therefore, more sensitive proteomic analysis for p7 tracking and localization on multiple separated cellular organelles at various times during HCV infection in cell culture is needed in order to gain more reliable insights into the role(s) of p7 in the HCV life cycle.

Ion-channel function and potential anti-p7 compounds

The p7 proteins of BVDV and HCV were originally proposed to oligomerize and form ion-channels by Harada et al. (2000) and Carrere-Kremer et al. (2002), respectively. Oligimerization was observed when it was shown that p7 formed a hexamer in artificial membranes and functioned as a calcium ion-channel in black lipid membranes. The ion-channel activity of p7 in this assay could be abrogated by the drug Amantadine (Griffin et al., 2003). The ion-channel activity of a cross-linked p7 lead to inclusion of this protein in the viroporin family, which consists of small hydrophobic proteins with the ability to permeabilize membranes for ion and small molecule movement (examples are listed in Table 1 and reviewed in (Nieva et al., 2012; Gonzalez and Carrasco, 2003; Wang et al., 2011; Liang and Li, 2010; Ruch and Machamer, 2012)). An important example is the viral protein M2 of influenza virus, which forms proton ion-channels and is activated in acidic environments. M2 triggers viral uncoating during entry within acidic endosomes and protects the HA glycoprotein from premature maturation in the trans-golgi network during egress, indicating that a multitude of functions can be performed by virally encoded ion-channels. The channel activity of M2 is blocked by amantadine and rimantadine, suggesting the two could be used to treat influenza infection (reviewed in (Kelly et al., 2003; Schnell and Chou, 2008)). p7 conductance across artificial lipid membranes was confirmed when pentamer formation was shown to be necessary for accommodation of ion transport. This study also reported a concentration-dependent inhibition of p7 channel activity after treatment with long-alkyl-chain iminosugar derivatives such as *N*-nonyl deoxygalactonojirimycin (*N*N-DGJ), *N*-nonyl deoxynojirimycin (*N*N-DNJ) and *N*-7-oxanonyl-6-deoxy-DGJ (Pavlovic et al., 2003). These compounds were tested because of their potent anti-viral activity against BVDV (Durantel et al., 2001).

In cell-based assays, Griffin et al. demonstrated that expression of p7 in mammalian cells can substitute for influenza virus M2 channel activity to maintain infectivity of influenza, and that this activity also could be blocked by the antiviral drug amantadine. In addition, it also was shown that mutation of the two basic residues within the p7 cytoplasmic loop abrogated ion-channel activity, highlighting the importance of this loop for p7 function (Griffin et al., 2004). A subsequent study confirmed the ion-channel function of p7 in planar lipid bilayer membranes and showed that p7 channels were permeable to potassium and sodium ions, with limited permeability to calcium ions (Premkumar et al., 2004). Later, fluorescence-based liposome assays were developed to more conveniently assay for p7 channel activity and therapeutic targets within p7. Employment of such assays using p7 from genotype 1b showed a dose-dependent release of fluorescent indicator when mixed with liposomes. The release activity was blocked by the drugs amantadine, rimantadine and several related compounds

Table 1
Examples of known viroporins. Ion-channel names and their respective virus and family names are shown (1st and 2nd columns). The known or expected functions are listed (3rd column). The number of monomers needed to form the channel upon oligomerization and the classification are shown (4th column). Potential inhibitors (5th column). Abbreviations: HA: Hemagglutinin, HIV-1: Human immunodeficiency virus type 1, Vpu: viral protein U, CoV: Coronavirus. (Table information is gathered from references (Nieva et al., 2012; Gonzalez and Carrasco, 2003; Wang et al., 2011; Liang and Li, 2010; Ruch and Machamer, 2012)).

Viroporin name	Virus/family	Main function	Oligomerized state/Class*	Inhibitors
Influenza A M2	*Orthomyxoviridae*	1. Viral genome uncoating. 2. Assembly of functional HA conformation	Tetramer/Class IA	Amantadine and Rimantadine
HIV-1 Vpu	*Retroviridae*	1. Facilitate budding of newly formed virion. 2. Enhance degradation of CD4.	Pentamer/Class IA	Hexamethylene amiloride, BIT225
Picornavirus P2B	*Picornaviridae*	1. Modulate virus release and host cell apoptosis (suggested)	Dimer or tetramer/Class IIB	DIDS (classic anion exchanger inhibitor) tested only in Enterovirus
CoV-E	*Coronaviridae*	1. Induce assembly by enhancing membrane scission. 2. Induce virion release.	Dimer, tetramer, or pentamer/ Class1A	Hexamethylene amiloride

Class I – single TM domain.
Subtype A: N-terminus oriented toward membrane lumen.
Subtype B: N-terminus oriented toward cytosolic face.
Class II – forms helix-turn-helix hairpin motifs.
Subtype A: both termini oriented toward membrane lumen.
Subtype B: both termini oriented toward the cytosolic space.
* Classification.

(StGelais et al., 2007). Taken together, these similarities with M2 protein function and inhibition provide strong evidence that p7 is also a viroporin.

Investigation of sequence determinants in p7 critical for ion-channel activity or drug sensitivity led to a number of insightful outcomes concerning the role of the putative channels. One report showed that the leucine-rich motif at the C-terminal end of TM2 partially contributed to drug sensitivity. Mutation of the two basic residues within the cytoplasmic loop (K33/R35 in G1b) was found to affect channel activity and caused disruption of the ion-channel insertion into membranes. The histidine at residue 17 in TM1 also showed significant effects on ion-channel function (StGelais et al., 2009). H17 is part of a HXXX(Y/W)-like motif that is also found in influenza M2 and is integral for ion-channel opening (Meshkat et al., 2009). H17 was shown to be in a critical position in the ion pore, demonstrating its key role in ion-channel conductance (Chew et al., 2009). Additionally, the L20F mutation and F25A polymorphism in HCV p7 were found to confer adamantane and iminosugar resistance, respectively (Foster et al., 2011). These findings highlight the importance of mutational analyses of the p7 sequence and the associated ion-channel activity, with major implications on drug binding and potential identification of novel inhibitors.

p7 channel activity had never been tested in a cell culture-based assay recapitulating the entire HCV life cycle because such a system (HCVcc) was not established until 2005 (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). In contrast with earlier studies, discrepancy was observed when the HCVcc system was employed to characterize the potential antiviral effects of amantadine and iminosugar derivatives (*N*N-DGJ, *N*N-DNJ and *N*B-DNJ). It was found that amantadine did not affect virus release, infectivity or ion-channel activity of JFH-1 (G2a) or chimeric genomes (H77 (G1a), Con1 (G1b), and J6 (G2a)). Contrarily, the iminosugar derivatives reduced virus titers in a dose-dependent manner in the context of multiple genotypes, primarily at virus assembly or release steps (Steinmann et al., 2007). The iminosugar derivatives *N*N-DGJ, *N*N-DNJ and *N*B-DNJ have been shown also to target cellular α-glucosidase I and II, and their inhibition leads to misfolding of various host (Bergeron et al., 1994) and viral glycoproteins such as BVDV E2 (Branza-Nichita et al., 2001) and gp120 of HIV (Fischer et al., 1995). With this in mind, the above-mentioned effect of iminosugar derivatives on virus production could be attributed to alteration in HCV envelope proteins, as shown previously (Steinmann et al., 2007) or to a manipulation of a host cellular glycoprotein crucial for virus propagation in cell culture. Similar discrepancy was also observed in a related protein called p13-c of GBV-B, whereby amantadine inhibited the function of p13-c ion-channel activity but failed to inhibit replication and secretion of GBV-B from virally-infected marmoset hepatocytes (Premkumar et al., 2006).

The development of the HCVcc system introduced greater opportunities for p7 characterization. Griffin et al. extensively differentiated the genotype- and subtype-dependent sensitivity of p7 to multiple inhibitors using both HCVcc and artificial membrane assays. It was found that p7 from the JFH-1 isolate was not blocked by amantadine in HCVcc or liposome-based assays, whereas amantadine successfully inhibited G3a in both systems. Amantadine inhibits J4 (G1b) while rimantadine was able to inhibit both JFH-1 and J4 channel activity and virus production. In addition, the iminosugar derivatives *N*N-DGJ and *N*N-DNJ reduced infectious virus production for genotype 2a and 1b in a dose-dependent manner, with no observed effect on viral protein synthesis, processing, HCV RNA replication or cellular cytotoxicity (Griffin et al., 2008). Until now, differing results have been obtained with various inhibitors in the context of different genotypes. These contrasting results suggest that p7 inhibitors may need to be developed in a genotype-specific manner.

Interestingly, p7 ion-channel activity was identified in native cell membrane vesicles and infected cells by measuring H^+ proton conductance and fluorescent detection of lysosomal pH indicators. Similarly, expression of p7 in HEK-293FT cells equilibrated H^+ conductance. HCV infection increased lysosomal pH in infected cells, but defective ion-channel mutants yielded no such increase. Furthermore, amantadine, rimantadine and hexamethylene amiloride block H^+ conductance through native vesicular membranes (Wozniak et al., 2010). In a very recent article, Atkins et al.

provided new evidence suggesting that p7 directly influences pH maturation via an as yet undefined mechanism (Atkins et al., 2014). This strongly advocates the importance of ion-channel activity during virus propagation.

A small compound termed BIT225 was shown to inhibit HIV production at a late stage in the viral life cycle by blocking Vpu ion-channel activity and impeding virus release from monocyte-derived macrophages (Khoury et al., 2010). BIT225 was also shown to bind p7 using a computational model of a p7 monomer, along with amantadine, rimantadine and *N*N-DNJ (Wang et al., 2013). BIT225 was shown to inhibit p7 ion-channel activity in lipid membranes and demonstrated antiviral activity in a BVDV infection assay (Luscombe et al., 2010).

A small number of clinical trials that included potential p7 inhibitors have been performed to date. Combination therapy of amantadine and IFN-α exhibited an insignificant correlation between amino acid variation within p7 and response to treatment in G1a/b-infected individuals. However, a L20F mutation was observed more often in non-responder patients infected with G1b receiving this combination therapy, which gives weight to the theory that amantadine targets p7 (Mihm et al., 2006). Subsequently, an increase in early virological response was observed in G1a/b patients treated with IFN-α, ribavirin and amantadine, yet sustained virological response rates remained the same as the arm receiving standard of care (Castelain et al., 2007). Such early inhibition was observed previously when a combination of amantadine, ribavirin and IFN-α was shown to reduce plasma viral RNA levels in patients in a phase 1 trial (Lake-Bakaar et al., 2003). These data indicate that amantadine could be useful in the case of new infections or to promote viral decay before the establishment of persistent infection.

It is still premature to include an ion-channel inhibitor with IFN-α and ribavirin. The discrepancies observed among the inhibition studies performed to date could be attributed to system inconsistencies, e.g., artificial membrane, liposome assay or HCVcc, and/or use of dissimilar p7 genotypes studied in native form versus tagged versions of the protein. Clinical trial data is still confined to response rates and lacks drug delivery analysis, plausibly accountable for minimal therapeutic outcomes. Completion of extensive mutational analyses on p7 could further our structural and functional understanding of the ion-channel and identify specific regions within p7 that could represent novel therapeutic targets. Subsequent or concomitant analyses of patient genomes from clinical trials could provide penetrative information concerning resistance and susceptibility in the p7 region.

NMR analyses of p7

NMR structural analysis was performed on p7 from G1b and showed that the N-terminal portion formed an α-helix (aa1-14) and TM1 (aa15-18) and TM2 (aa19-32) formed α-helixes connected by a short loop (aa33-39) (Montserret et al., 2010). However, the structural features and the helix lengths observed through NMR were contrastive to the molecular model of p7 (Carrere-Kremer et al., 2002; StGelais et al., 2009; Clarke et al., 2006; Patargias et al., 2006). The monomeric NMR structure of p7 unveiled a dynamic nature, and the structure identified predicted a multifunctional protein rather than one strictly acting as an ion-channel (Cook et al., 2013). Also, monomeric NMR structural analysis performed on Flag-p7 from G1b identified a relevant allosteric cavity compatible with drug binding. These findings highlight the importance of p7 as a potential target for the development of HCV inhibitors (Foster et al., 2014).

NMR data obtained on oligomerized p7 indicated that the protein forms a hexameric channel complex in the presence of detergent, and EM analysis identified a flower-like shape with six distinctive petals with the p7 helices oriented toward the lumen of the ER. The identified structure also showed an accessible area facing the interior of the ER that could provide an interaction face with other viral or host proteins (Luik et al., 2009). Additionally, the G1b p7 sequence can assemble sequentially to form an oligomerized structure of four to seven subunits with the most putatively functional oligomer being the hexamer in which the cylindrical structure accommodates the flower-like shape. The sequential formation of the channel was suggested to regulate the function of p7, whereby p7 would be retained in an inactive state that allows p7 to bind to other viral proteins early in the assembly process. Subsequently, pore formation could take place to accommodate channel activity required at a later stage in the life cycle (Chandler et al., 2012). A more recent NMR structural identification of the p7 ion-channel showed that the G5a sequence forms an unusual hexameric complex with a funnel-like shape see Fig. 3A and B. This study also suggested that amantadine and rimantadine bind to six equivalent hydrophobic pockets between the pore-forming and peripheral helices, defining a possible mechanism of inhibitive action. OuYang et al., (2013).

The diversity of structural predictions for p7 based on NMR analyses may be attributed to a number of variables, such as the different genotypic p7 amino acid sequences, the purification method used, or the solvent and environmental conditions used in the preparation of the protein for NMR structural analyses. However, structural agreement in NMR studies will prove to be important for identification of structural features of monomeric and oligomeric p7 structures, as well as drug binding determinants within p7.

Functional analyses of p7

In 2003 deletion or mutations at the two conserved positively charged residues in the cytoplasmic loop of p7 within infectious clones of G1a failed to cause viremia after intrahepatic transfection in the chimpanzee model. Furthermore, substitution G1a p7 with p7 from an infectious G2a clone was also not viable. These results clearly demonstrated the importance of p7 for the virus life cycle in vivo, as well as the restrictive genotypic context of p7 function (Sakai et al., 2003). Given the accumulating evidence suggesting that p7 forms an ion-channel and acts as a viroporin, it is conceivable that such an activity might be required for the virus to facilitate entry, assembly or release in a manner similar to that of other viroporins (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004).

After the development of the HCVcc system, mutagenesis studies on various viral proteins for functional analysis became possible (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Early mutational analyses demonstrated the importance of p7 for virus production (Jones et al., 2007; Steinmann et al., 2007). Steinmann et al. showed that p7 is involved at a late stage of the replication cycle, since mutants in p7 reduced both total infectivity and the ratio of intracellular to released infectious particles. Successively, p7 was shown to be less likely involved in the viral entry process due to the maintenance of specific infectivity observed in released virions from genomes mutated in p7 (Steinmann et al., 2007). A subsequent study illustrated that p7 mutant genomes generated lower levels of intracellular virus, released core protein and infectious particles in the culture supernatant, suggesting involvement of p7 at an early stage of the viral morphogenesis cycle (Jones et al., 2007). In our lab we have created an extensive set of triple alanine mutations covering TM1 and the cytoplasmic loop of p7 (Atoom et al., 2013). These mutants were subjected to transfection in a single-cycle virus

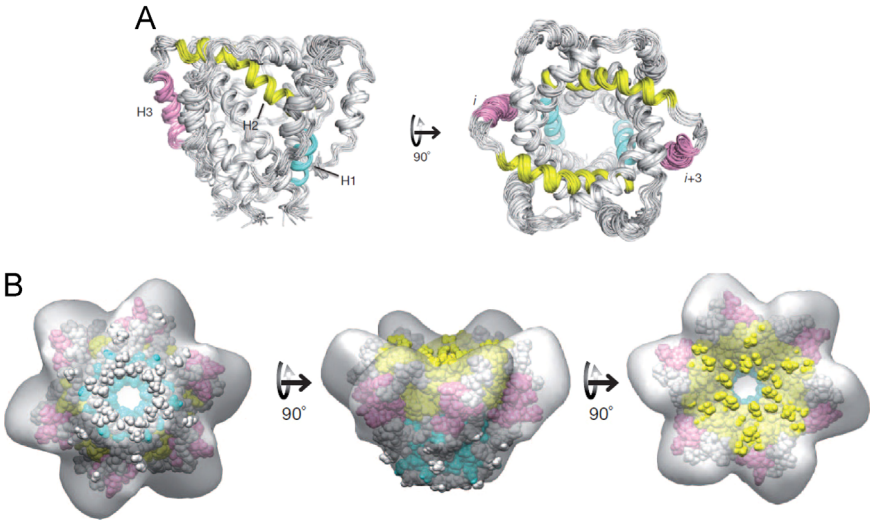

Fig. 3. Monomeric and hexameric structures of p7. (A) Hexameric structure representation of p7 after calculations of 1.5 low-energy structures ensemble using NMR restraints identified by the authors. (B) The Fitted model of p7 hexameric structure (the lowest energy structure of the ensemble fitted on the 16 Å EM map with a fitting correlation equal to 0.94 as calculated by the authors' program chimera). (A) and (B) showing the H1: helix 1 (N terminal helix aa5–16)(blue), H2: middle helical segment, aa 20–41 (yellow) and H3: the third C-terminal helix, aa 48–58 (pink). Reprinted with permission from (OuYang et al., 2013).

production assay that employs a subclone of Huh-7 cells, termed S29 cells (Russell et al., 2008). These cells express significantly lower levels of CD81 (an HCV entry receptor) and permit RNA replication and virus production, but do not support entry, thereby allowing generation of infectious virus without the confounding effects of multiple infection cycles. Using this assay we illustrated the importance of each domain in the maintenance of infectious virus production (Atoom et al., 2013).

Currently, a number of convincing pieces of evidence exist suggesting that p7 is not involved in the entry steps of the viral life cycle: (I) HCV pseudoparticles (HCVpp) produced from 293 T cells lack p7 but remain infectious to hepatocyte-derived cell lines (Bartosch et al., 2003). (II) cis-expression of p7 during HCVpp production did not increase infectivity (Flint et al., 2004). (III) HCV virions are acid-resistant, suggesting that ion-channel activity during entry is not needed (Tscherne et al., 2006). (IV) p7 from the related virus BVDV was not found to be a component of the virion structure (Elbers et al., 1996). (V) We have characterized a p7 adaptive mutation and found it to increase the levels of extracellular virus particles even in a virus entry-null system (Atoom et al., manuscript in preparation). (VI) More importantly, using an infectious construct containing a double HA-tagged p7, it was found that p7 was not contained within the virion, even in concentrated, affinity-purified or Flag-tagged preparations of virus (Vieyres et al., 2013). All things considered, a viroporin-like activity of p7 might be required at a step downstream of virus entry that resembles the influenza A M2 activity during viral egress and HIV-1 Vpu (see Table 1).

Regarding steps subsequent to viral entry, current evidence indicates that p7 has no role in RNA replication. (I) Replicons lacking p7 replicate efficiently in Huh-7 cells (Lohmann et al., 1999; Blight et al., 2002) (II) BVDV containing mutations in p7 was able to replicate in cell culture, but failed to produce infectious particles (Harada et al., 2000). (III) Mutations or deletions within p7 generated in the context of HCVcc replicate RNA efficiently with impaired infectious virus production (Jones et al., 2007; Steinmann et al., 2007).

The establishment of the HCVcc system facilitated many studies that could not have been performed in the past, and as a result, the HCV assembly process became intensely investigated. One intriguing aspect of the assembly mechanism is the accumulation of core protein around a cellular organelle termed the lipid droplet (LD), which is proposed to function as a platform for virus assembly (Miyanari et al., 2007). We have found that efficiently-assembled adaptive strains of JFH-1 as well as genomes mutated in TM1 and the cytoplasmic loop of p7 show no differences in their abilities to associate core with LDs (Atoom et al., 2013). However, others have suggested that core does not accumulate on LD in the context of an efficiently-assembling virus, suggesting that core/LD accumulation is an indication of an assembly defect (Boson et al., 2011). It is worth mentioning that an NS2-mutated, assembly-deficient HCV strain could be rescued by compensatory mutations in p7 that restored virus production and colocalization of NS2, E2 and NS3 to a site in close proximity to LDs (Jirasko et al., 2010). Correspondingly, other reports visualized that p7 can manipulate the intracellular distribution of NS2 and disrupt its binding pattern with other viral proteins (Ma et al., 2011). This assumption was confirmed recently by showing that NS2 distribution is affected by the presence of p7 and its N-terminal signal peptide (Tedbury et al., 2011). Presenting a different perspective, the efficiency of core accumulation on LDs due to p7 mutations was quantified and it was found that mutations in the p7 cytoplasmic loop affected the unloading of core protein from LDs, resulting in a retention of core around LDs (Gentzsch et al., 2013). The preceding observations could indicate that p7 is important for colocalization of NS2 with LDs, a process that is crucial for NS2 function. Interestingly, p7 has been also shown important for unloading of newly-formed capsids toward further assembly, maturation and envelopment (Popescu et al., 2011; Gentzsch et al., 2013).

Subsequent to core protein recruitment to LDs, core oligomerizes to form capsid structures; this presumably facilitates the formation of multi-order forms during the morphogenesis cycle that would include monomeric core, partial oligomers, fully oligomerized capsids and virions associated with triglycerides and β-lipoproteins, all of which would show different densities upon intracellular lysate fractionation (Andre et al., 2002; Gastaminza et al., 2006; Jones et al., 2011). We and others have shown that p7 mutations in TM1 and the cytoplasmic loop did not prevent the assembly of core-containing intracellular particles after iodixanol equilibrium gradient sedimentation (Atoom et al.,

2013; Bentham et al., 2013). Compellingly, another report performed core quantitation on the fractions obtained after sucrose-based rate zonal fractionation and observed an increased proportion of incompletely assembled capsids. Further analyses on these fractions indicated that p7 mutations or deletions in the cytoplasmic loop led to accumulation of non-enveloped capsids in transfected cells (Gentzsch et al., 2013). These data reflect the necessity of p7 downstream of capsid formation.

The postulation that p7 functions as an ion-channel gained momentum when it was shown that HCV infection equilibrates intracellular acidic vesicles. This loss of acidity was not observed with HCV genomes harboring p7 ion-channel defective mutations (KR33/35AA in the J4 sequence or RR33/35AA in JFH-1). It was found that intracellular HCV virions were acid-sensitive, whereas extracellular virions were acid-stable. In confirmation of this finding, the acidification inhibitor Bafilomycin A1 was able to rescue virus production of ion-channel defective mutants (Wozniak et al., 2010). Moreover, we found that triple alanine substitutions at amino acid residues 33–35 (RGR to AAA) reduced the amount of E2 present in HCV-transfected cells at 72 h post-transfection (Atoom et al., 2013). Furthermore, HCV replication-defective viruses containing mutations in TM1 of p7 or the cytoplasmic loop showed a restoration of infectivity by *in trans* expression of the M2 proton channel of influenza A, indicative of an ion-channel requirement for HCV propagation in cell culture (Bentham et al., 2013). Interestingly, proteinase K digestion of p7 mutant viruses showed significant reductions in core protein, indicative of nucleocapsids lacking envelopes, which demonstrated that p7 affects virus production at a step prior to envelopment (Gentzsch et al., 2013). p7 ion-channel activity could be an important step to protect the immature virion from an acidic environment during the late stage of assembly, particularly at the envelopment step.

In summation, it is clear that p7 is an important factor in virus production, specifically at a late stage in viral assembly, and it might be that p7 is a dual function protein. In one aspect, p7, presumably in its monomeric form, assists NS2 in gathering newly-formed capsids at LDs and glycoprotein complexes on the ER lumen for proper envelopment. Whereas, in its oligomeric state, p7 protects glycoproteins from immature degradation during trafficking and release through an ion-channel-like activity. A third possibility, which has received little attention to date, is that p7 may play some as yet undetermined function that involves an interaction with a cellular factor. In this regard, p7 has been suggested to modulate cell death signaling and may be targeted by cellular kinases (Aweya et al., 2013; Lee et al., 2013). It would be interesting to know if p7 interacts with cellular proteins in order to promote virus production.

p7 interactions with other viral proteins

The binding pattern of p7 with other viral proteins is continually being revealed through genetics-based investigations and protein–protein interaction analyses. The first clue concerning the necessity for p7 to interact with other proteins came through intrahepatic RNA injection of chimpanzees with a chimeric virus generated by replacing the p7 sequence from G1a with that of G2a. The resulting chimera was not viable after injection, illustrating p7s genotype-specific interactions (Sakai et al., 2003). The JFH-1 infectious clone was used in multiple studies to generate infectious chimeras by replacing the core-NS2 (or in some cases core to part of NS2) fragment of the JFH-1 sequence (G2a) with that of other genotypes. In this case it was observed that chimeric viruses were only viable when there was genotypic homology between p7 and substituted structural genes. This characteristic of chimera construction suggested there is an important interplay between p7 and one or more structural proteins (Pietschmann et al., 2006; Gottwein et al., 2009; Yi et al., 2007). To successfully create a viable chimeric construct, H77 (G1a) was fused to JFH-1 (2a) at a site within NS2 and passaged to allow accumulation of adaptive/compensatory mutations. The resulting chimera acquired mutations within p7, NS2, E1 and NS3. The combination of p7 and NS2 mutations increased specific infectivity, suggesting an interaction between p7 and NS2. This interaction was proposed to anchor p7 to NS2 within the membrane, thereby enhancing the ability of p7 to protect the nascent virion from premature inactivation (Yi et al., 2007). Furthermore, compensatory mutations in p7 (F776L/S) significantly enhanced the fitness of defective core mutants in the context of a J6/JFH-1 chimera, providing genetic evidence for an interaction between p7 and core (Murray et al., 2007). Taken together, it remains unlikely that a protein the size of p7 interacts with multiple viral proteins. That being said, it is possible that p7 interacts with a single viral protein in order to stabilize other downstream interactions and promote viral propagation.

Biochemical and proteomic assays have also been used to identify p7 binding partners. One report that utilized tagged NS2 showed efficient pull-down with p7, E2, NS3 and to a lower extent, NS5A (Jirasko et al., 2010). Other work has shown NS2 can be co-immunoprecipitated with E1 as well (Ma et al., 2011). Importantly, mutagenesis studies have demonstrated that p7 affects NS2 colocalization with E2 and NS3 around LDs, and p7 basic loop mutations significantly reduced NS2 interactions with E1, E2 and NS3 (Jirasko et al., 2010; Ma et al., 2011). It is noteworthy that mutations in the two basic residues of the cytoplasmic loop of p7 caused considerable alteration of intracellular distribution of NS2 and E2 (Ma et al., 2011). However, it is difficult to conclude whether these results indicate a direct interaction with the loop, or if the removal of the charged residues changes the topology of p7 to indirectly affect the localization of other proteins. Recently, NS2 was confirmed to interact with p7 and E2; mutations in p7 changed subcellular localization of NS2 and reduced viral assembly (Popescu et al., 2011). p7 interaction with NS2 was shown to be independent of p7 ion-channel activity and NS2 localization was affected primarily by p7 and its signal peptide at the N-terminus, suggesting an alternative function of p7 during the assembly process (Tedbury et al., 2011). More recently, a co-immunoprecipitation study using a replication-competent virus containing a double HA-tagged p7 confirmed the specific interaction between p7 and NS2, which was proposed to be critical for virus production in cell culture (Vieyres et al., 2013). Despite the discrepancies observed for NS2 as a p7 binding partner we can conclude that NS2 maintains the organization of the viral assembly process and that p7 is a mandatory interaction partner. It is also plausible that p7 binds NS2 in a manner that regulates its function in virus assembly.

The size and topology of p7 have made it poorly immunogenic for antibody production. Consequently, the determination of p7 binding partners by standard methods is problematic and difficult to interpret. The topology of p7 in membrane structures and data generated from purified or tagged proteins might not reflect the actual picture. Considering p7 exists as multiple forms: unprocessed E2-p7, p7-NS2, monomeric p7 or oligomers, reproducibility of data becomes further complicated. Accordingly, more accurate experimental approaches need to be investigated or identified to better define the protein–protein interaction activities of p7, both viral and potentially cellular.

Conclusion

p7 is required for in vivo infectivity, and important for infectious virus production in cell culture. For unknown reasons,

the cleavage of p7 from adjacent viral proteins is delayed, leading to the presence of precursor polypeptides (E2-p7, p7-NS2 or E2-p7-NS2) proposed to have regulatory roles in virus production. Recent structural and topological studies have confirmed that p7 mainly localizes to the ER and contains two trans-membrane domains connected by a short cytoplasmic loop. By oligomerizing in the membrane p7 is believed to form an ion-channel that facilitates proton permeabilization in order to equilibrate intracellular vesicle pH to promote infectious virus production. The ion-channel activity can be inhibited by several compounds with BIT225 and iminosugar derivatives showing the most promising results. Moreover, p7 interacts with NS2 and supports a late step in viral assembly and envelopment. The interaction pattern of p7 with other viral and host factors, and its exact contribution to infectious virus production remains ambiguous. In our opinion, the contrasting hypotheses in the field regarding the function of p7 partly stem from the fact that numerous systems are being employed to analyze this protein (artificial membranes, single protein expression, HCVcc and HCVcc with tagged p7). Although all of these relevant systems provide critical information regarding p7, it can be difficult to reconcile the various results. Perhaps the greatest limitation we face at present is the lack of a reliable monoclonal antibody recognizing p7, and the generation of such would be a major advance that would allow relevant analysis of unmodified p7 in the context of the full viral genome.

References

Andre, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J.L., et al., 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J. Virol. 76, 6919–6928.

Appel, N., Zayas, M., Miller, S., Krijnse-Locker, J., Schaller, T., et al., 2008. Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. PLoS Pathog. 4, e1000035.

Atkins, E., Tatineni, R., Li, H., Gretch, D., Harris, M., et al., 2014. The stability of secreted, acid-labile H77/JFH-1 hepatitis C virus (HCV) particles is altered by patient isolate genotype 1a p7 sequences. Virology 448, 117–124.

Atoom, A.M., Jones, D.M., Russell, R.S., 2013. Evidence suggesting that HCV p7 protects E2 glycoprotein from premature degradation during virus production. Virus Res. 176, 199–210.

Aweya, J.J., Mak, T.M., Lim, S.G., Tan, Y.J., 2013. The p7 protein of the hepatitis C virus induces cell death differently from the influenza A virus viroporin M2. Virus Res. 172, 24–34.

Bartenschlager, R., Penin, F., Lohmann, V., Andre, P., 2011. Assembly of infectious hepatitis C virus particles. Trends Microbiol. 19, 95–103.

Bartosch, B., Dubuisson, J., Cosset, F.L., 2003. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J. Exp. Med. 197, 633–642.

Bentham, M.J., Foster, T.L., McCormick, C., Griffin, S., 2013. Mutations in hepatitis C virus p7 reduce both the egress and infectivity of assembled particles via impaired proton channel function. J. Gen. Virol. 94, 2236–2248.

Bergeron, J.J., Brenner, M.B., Thomas, D.Y., Williams, D.B., 1994. Calnexin: a membrane-bound chaperone of the endoplasmic reticulum. Trends Biochem. Sci. 19, 124–128.

Blight, K.J., McKeating, J.A., Rice, C.M., 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76, 13001–13014.

Boson, B., Granio, O., Bartenschlager, R., Cosset, F.L., 2011. A concerted action of hepatitis C virus p7 and nonstructural protein 2 regulates core localization at the endoplasmic reticulum and virus assembly. PLoS Pathog. 7, e1002144.

Boulant, S., Montserret, R., Hope, R.G., Ratinier, M., Targett-Adams, P., et al., 2006. Structural determinants that target the hepatitis C virus core protein to lipid droplets. J. Biol. Chem. 281, 22236–22247.

Branza-Nichita, N., Durantel, D., Carrouee-Durantel, S., Dwek, R.A., Zitzmann, N., 2001. Antiviral effect of N-butyldeoxynojirimycin against bovine viral diarrhea virus correlates with misfolding of E2 envelope proteins and impairment of their association into E1-E2 heterodimers. J. Virol. 75, 3527–3536.

Brohm, C., Steinmann, E., Friesland, M., Lorenz, I.C., Patel, A., et al., 2009. Characterization of determinants important for hepatitis C virus p7 function in morphogenesis by using trans-complementation. J. Virol. 83, 11682–11693.

Carrere-Kremer, S., Montpellier-Pala, C., Cocquerel, L., Wychowski, C., Penin, F., et al., 2002. Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. J. Virol. 76, 3720–3730.

Carrere-Kremer, S., Montpellier, C., Lorenzo, L., Brulin, B., Cocquerel, L., et al., 2004. Regulation of hepatitis C virus polyprotein processing by signal peptidase involves structural determinants at the p7 sequence junctions. J. Biol. Chem. 279, 41384–41392.

Castelain, S., Bonte, D., Penin, F., Francois, C., Capron, D., et al., 2007. Hepatitis C Virus p7 membrane protein quasispecies variability in chronically infected patients treated with interferon and ribavirin, with or without amantadine. J. Med. Virol. 79, 144–154.

Chandler, D.E., Penin, F., Schulten, K., Chipot, C., 2012. The p7 protein of hepatitis C virus forms structurally plastic, minimalist ion channels. PLoS Comput. Biol. 8, e1002702.

Chew, C.F., Vijayan, R., Chang, J., Zitzmann, N., Biggin, P.C., 2009. Determination of pore-lining residues in the hepatitis C virus p7 protein. Biophys. J. 96, L10–12.

Clarke, D., Griffin, S., Beales, L., Gelais, C.S., Burgess, S., et al., 2006. Evidence for the formation of a heptameric ion channel complex by the hepatitis C virus p7 protein in vitro. J. Biol. Chem. 281, 37057–37068.

Cook, G.A., Dawson, L.A., Tian, Y., Opella, S.J., 2013. Three-dimensional structure and interaction studies of hepatitis C virus p7 in 1,2-dihexanoyl-sn-glycero-3-phosphocholine by solution nuclear magnetic resonance. Biochemistry.

Dimitrova, M., Imbert, I., Kieny, M.P., Schuster, C., 2003. Protein-protein interactions between hepatitis C virus nonstructural proteins. J. Virol. 77, 5401–5414.

Dubuisson, J., Hsu, H.H., Cheung, R.C., Greenberg, H.B., Russell, D.G., et al., 1994. Formation and intracellular localization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia and Sindbis viruses. J. Virol. 68, 6147–6160.

Durantel, D., Branza-Nichita, N., Carrouee-Durantel, S., Butters, T.D., Dwek, R.A., et al., 2001. Study of the mechanism of antiviral action of iminosugar derivatives against bovine viral diarrhea virus. J. Virol. 75, 8987–8998.

Elbers, K., Tautz, N., Becher, P., Stoll, D., Rumenapf, T., et al., 1996. Processing in the pestivirus E2-NS2 region: identification of proteins p7 and E2p7. J. Virol. 70, 4131–4135.

Feld, J.J., Hoofnagle, J.H., 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 436, 967–972.

Fischer, P.B., Collin, M., Karlsson, G.B., James, W., Butters, T.D., et al., 1995. The alpha-glucosidase inhibitor N-butyldeoxynojirimycin inhibits human immunodeficiency virus entry at the level of post-CD4 binding. J. Virol. 69, 5791–5797.

Flint, M., Logvinoff, C., Rice, C.M., McKeating, J.A., 2004. Characterization of infectious retroviral pseudotype particles bearing hepatitis C virus glycoproteins. J. Virol. 78, 6875–6882.

Foster, T.L., Verow, M., Wozniak, A.L., Bentham, M.J., Thompson, J., et al., 2011. Resistance mutations define specific antiviral effects for inhibitors of the hepatitis C virus p7 ion channel. Hepatology 54, 79–90.

Foster, T.L., Thompson, G.S., Kalverda, A.P., Kankanala, J., Bentham, M., et al., 2014. Structure-guided design affirms inhibitors of hepatitis C virus p7 as a viable class of antivirals targeting virion release. Hepatology 59, 408–422.

Gastaminza, P., Kapadia, S.B., Chisari, F.V., 2006. Differential biophysical properties of infectious intracellular and secreted hepatitis C virus particles. J. Virol. 80, 11074–11081.

Gentzsch, J., Brohm, C., Steinmann, E., Friesland, M., Menzel, N., et al., 2013. hepatitis c Virus p7 is critical for capsid assembly and envelopment. PLoS Pathog. 9, e1003355.

Gonzalez, M.E., Carrasco, L., 2003. Viroporins. FEBS Lett. 552, 28–34.

Gottwein, J.M., Scheel, T.K., Jensen, T.B., Lademann, J.B., Prentoe, J.C., et al., 2009. Development and characterization of hepatitis C virus genotype 1-7 cell culture systems: role of CD81 and scavenger receptor class B type I and effect of antiviral drugs. Hepatology 49, 364–377.

Griffin, S., Clarke, D., McCormick, C., Rowlands, D., Harris, M., 2005. Signal peptide cleavage and internal targeting signals direct the hepatitis C virus p7 protein to distinct intracellular membranes. J. Virol. 79, 15525–15536.

Griffin, S., Stgelais, C., Owsianka, A.M., Patel, A.H., Rowlands, D., et al., 2008. Genotype-dependent sensitivity of hepatitis C virus to inhibitors of the p7 ion channel. Hepatology 48, 1779–1790.

Griffin, S.D., Beales, L.P., Clarke, D.S., Worsfold, O., Evans, S.D., et al., 2003. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. FEBS Lett. 535, 34–38.

Griffin, S.D., Harvey, R., Clarke, D.S., Barclay, W.S., Harris, M., et al., 2004. A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. J. Gen. Virol. 85, 451–461.

Han, Q., Manna, D., Belton, K., Cole, R., Konan, K.V., 2013. Modulation of hepatitis C virus genome encapsidation by nonstructural protein 4B. J. Virol. 87, 7409–7422.

Haqshenas, G., Mackenzie, J.M., Dong, X., Gowans, E.J., 2007. Hepatitis C virus p7 protein is localized in the endoplasmic reticulum when it is encoded by a replication-competent genome. J. Gen. Virol. 88, 134–142.

Harada, T., Tautz, N., Thiel, H.J., 2000. E2-p7 region of the bovine viral diarrhea virus polyprotein: processing and functional studies. J. Virol. 74, 9498–9506.

Hijikata, M., Mizushima, H., Akagi, T., Mori, S., Kakiuchi, N., et al., 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. 67, 4665–4675.

Hoffman, B., Liu, Q., 2011. Hepatitis C viral protein translation: mechanisms and implications in developing antivirals. Liver Int. 31, 1449–1467.

Hsu, M., Zhang, J., Flint, M., Logvinoff, C., Cheng-Mayer, C., et al., 2003. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. Proc. Natl. Acad. Sci. U. S. A. 100, 7271–7276.

Hughes, M., Gretton, S., Shelton, H., Brown, D.D., McCormick, C.J., et al., 2009. A conserved proline between domains II and III of hepatitis C virus NS5A influences both RNA replication and virus assembly. J. Virol. 83, 10788–10796.

Hughes, M., Griffin, S., Harris, M., 2009. Domain III of NS5A contributes to both RNA replication and assembly of hepatitis C virus particles. J. Gen. Virol. 90, 1329–1334.
Jirasko, V., Montserret, R., Appel, N., Janvier, A., Eustachi, L., et al., 2008. Structural and functional characterization of nonstructural protein 2 for its role in hepatitis C virus assembly. J. Biol. Chem. 283, 28546–28562.
Jirasko, V., Montserret, R., Lee, J.Y., Gouttenoire, J., Moradpour, D., et al., 2010. Structural and functional studies of nonstructural protein 2 of the hepatitis C virus reveal its key role as organizer of virion assembly. PLoS Pathog. 6, e1001233.
Jones, C.T., Murray, C.L., Eastman, D.K., Tassello, J., Rice, C.M., 2007. Hepatitis C virus p7 and NS2 proteins are essential for production of infectious virus. J. Virol. 81, 8374–8383.
Jones, D.M., Patel, A.H., Targett-Adams, P., McLauchlan, J., 2009. The hepatitis C virus NS4B protein can trans-complement viral RNA replication and modulates production of infectious virus. J. Virol. 83, 2163–2177.
Jones, D.M., Atoom, A.M., Zhang, X., Kottilil, S., Russell, R.S., 2011. A genetic interaction between the core and NS3 proteins of hepatitis C virus is essential for production of infectious virus. J. Virol. 85, 12351–12361.
Kelly, M.L., Cook, J.A., Brown-Augsburger, P., Heinz, B.A., Smith, M.C., et al., 2003. Demonstrating the intrinsic ion channel activity of virally encoded proteins. FEBS Lett. 552, 61–67.
Khoury, G., Ewart, G., Luscombe, C., Miller, M., Wilkinson, J., 2010. Antiviral efficacy of the novel compound BIT225 against HIV-1 release from human macrophages. Antimicrob. Agents Chemother. 54, 835–845.
Kohlway, A., Pirakitikulr, N., Barrera, F.N., Potapova, O., Engelman, D.M., et al., 2013. Hepatitis C virus RNA replication and virus particle assembly require specific dimerization of the NS4A protein transmembrane domain. J. Virol. 88, 628–642.
Kolykhalov, A.A., Mihalik, K., Feinstone, S.M., Rice, C.M., 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo. J. Virol. 74, 2046–2051.
Kuiken, C., Simmonds, P., 2009. Nomenclature and numbering of the hepatitis C virus. Methods Mol. Biol. 510, 33–53.
Lake-Bakaar, G., Ruffini, L., Kuzmic, P., 2003. Effect of ribavirin and amantadine on early hepatitis C virus RNA rebound and clearance in serum during daily high-dose interferon. Dig. Dis. Sci. 48, 126–139.
Lee, A., Liu, S., Wang, T., 2013. Identification of novel human kinases that suppress hepatitis C virus infection. J. Viral Hepat., Nov 10.
Levrero, M., 2006. Viral hepatitis and liver cancer: the case of hepatitis C. Oncogene 25, 3834–3847.
Liang, X., Li, Z.Y., 2010. Ion channels as antivirus targets. Virol. Sin. 25, 267–280.
Lin, C., Lindenbach, B.D., Pragai, B.M., McCourt, D.W., Rice, C.M., 1994. Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. J. Virol. 68, 5063–5073.
Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., et al., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.
Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., et al., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.
Lorenz, I.C., Marcotrigiano, J., Dentzer, T.G., Rice, C.M., 2006. Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. Nature 442, 831–835.
Luik, P., Chew, C., Aittoniemi, J., Chang, J., Wentworth Jr., P., et al., 2009. The 3-dimensional structure of a hepatitis C virus p7 ion channel by electron microscopy. Proc. Natl. Acad. Sci. U. S. A. 106, 12712–12716.
Luscombe, C.A., Huang, Z., Murray, M.G., Miller, M., Wilkinson, J., et al., 2010. A novel Hepatitis C virus p7 ion channel inhibitor, BIT225, inhibits bovine viral diarrhea virus in vitro and shows synergism with recombinant interferon-alpha-2b and nucleoside analogues. Antivir. Res. 86, 144–153.
Ma, Y., Yates, J., Liang, Y., Lemon, S.M., Yi, M., 2008. NS3 helicase domains involved in infectious intracellular hepatitis C virus particle assembly. J. Virol. 82, 7624–7639.
Ma, Y., Anantpadma, M., Timpe, J.M., Shanmugam, S., Singh, S.M., et al., 2011. Hepatitis C virus NS2 protein serves as a scaffold for virus assembly by interacting with both structural and nonstructural proteins. J. Virol. 85, 86–97.
Masaki, T., Suzuki, R., Murakami, K., Aizaki, H., Ishii, K., et al., 2008. Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. J. Virol. 82, 7964–7976.
McLauchlan, J., Lemberg, M.K., Hope, G., Martoglio, B., 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. 21, 3980–3988.
Meshkat, Z., Audsley, M., Beyer, C., Gowans, E.J., Haqshenas, G., 2009. Reverse genetic analysis of a putative, influenza virus M2 HXXXW-like motif in the p7 protein of hepatitis C virus. J. Viral Hepat. 16, 187–194.
Mihm, U., Grigorian, N., Welsch, C., Herrmann, E., Kronenberger, B., et al., 2006. Amino acid variations in hepatitis C virus p7 and sensitivity to antiviral combination therapy with amantadine in chronic hepatitis C. Antivir. Ther. 11, 507–519.
Miyanari, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., et al., 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9, 1089–1097.
Mizushima, H., Hijikata, M., Asabe, S., Hirota, M., Kimura, K., et al., 1994. Two hepatitis C virus glycoprotein E2 products with different C termini. J. Virol. 68, 6215–6222.
Montserret, R., Saint, N., Vanbelle, C., Salvay, A.G., Simorre, J.P., et al., 2010. NMR structure and ion channel activity of the p7 protein from hepatitis C virus. J. Biol. Chem. 285, 31446–31461.
Moradpour, D., Penin, F., Rice, C.M., 2007. Replication of hepatitis C virus. Nat. Rev. Microbiol. 5, 453–463.
Murray, C.L., Jones, C.T., Tassello, J., Rice, C.M., 2007. Alanine scanning of the hepatitis C virus core protein reveals numerous residues essential for production of infectious virus. J. Virol. 81, 10220–10231.
Murray, C.L., Jones, C.T., Rice, C.M., 2008. Architects of assembly: roles of Flaviviridae non-structural proteins in virion morphogenesis. Nat. Rev. Microbiol. 6, 699–708.
Nieva, J.L., Madan, V., Carrasco, L., 2012. Viroporins: structure and biological functions. Nat. Rev. Microbiol. 10, 563–574.
OuYang, B., Xie, S., Berardi, M.J., Zhao, X., Dev, J., et al., 2013. Unusual architecture of the p7 channel from hepatitis C virus. Nature 498, 521–525.
Patargias, G., Zitzmann, N., Dwek, R., Fischer, W.B., 2006. Protein-protein interactions: modeling the hepatitis C virus ion channel p7. J. Med. Chem. 49, 648–655.
Pavlovic, D., Neville, D.C., Argaud, O., Blumberg, B., Dwek, R.A., et al., 2003. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. Proc. Natl. Acad. Sci. U. S. A. 100, 6104–6108.
Phan, T., Beran, R.K., Peters, C., Lorenz, I.C., Lindenbach, B.D., 2009. Hepatitis C virus NS2 protein contributes to virus particle assembly via opposing epistatic interactions with the E1-E2 glycoprotein and NS3-NS4A enzyme complexes. J. Virol. 83, 8379–8395.
Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., et al., 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Natl. Acad. Sci. U. S. A. 103, 7408–7413.
Popescu, C.I., Callens, N., Trinel, D., Roingeard, P., Moradpour, D., et al., 2011. NS2 protein of hepatitis C virus interacts with structural and non-structural proteins towards virus assembly. PLoS Pathog. 7, e1001278.
Premkumar, A., Wilson, L., Ewart, G.D., Gage, P.W., 2004. Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. FEBS Lett. 557, 99–103.
Premkumar, A., Dong, X., Haqshenas, G., Gage, P.W., Gowans, E.J., 2006. Amantadine inhibits the function of an ion channel encoded by GB virus B, but fails to inhibit virus replication. Antivir. Ther. 11, 289–295.
Reed, K.E., Rice, C.M., 2000. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr. Top. Microbiol. Immunol. 242, 55–84.
Ruch, T.R., Machamer, C.E., 2012. The coronavirus E protein: assembly and beyond. Viruses 4, 363–382.
Russell, R.S., Meunier, J.C., Takikawa, S., Faulk, K., Engle, R.E., et al., 2008. Advantages of a single-cycle production assay to study cell culture-adaptive mutations of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. 105, 4370–4375.
Sakai, A., Claire, M.S., Faulk, K., Govindarajan, S., Emerson, S.U., et al., 2003. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. Proc. Natl. Acad. Sci. U. S. A. 100, 11646–11651.
Schnell, J.R., Chou, J.J., 2008. Structure and mechanism of the M2 proton channel of influenza A virus. Nature 451, 591–595.
Shanmugam, S., Yi, M., 2013. Efficiency of E2-p7 processing modulates production of infectious hepatitis C virus. J. Virol. 87, 11255–11266.
StGelais, C., Tuthill, T.J., Clarke, D.S., Rowlands, D.J., Harris, M., et al., 2007. Inhibition of hepatitis C virus p7 membrane channels in a liposome-based assay system. Antivir. Res. 76, 48–58.
StGelais, C., Foster, T.L., Verow, M., Atkins, E., Fishwick, C.W., et al., 2009. Determinants of hepatitis C virus p7 ion channel function and drug sensitivity identified in vitro. J. Virol. 83, 7970–7981.
Stapleford, K.A., Lindenbach, B.D., 2011. Hepatitis C virus NS2 coordinates virus particle assembly through physical interactions with the E1-E2 glycoprotein and NS3-NS4A enzyme complexes. J. Virol. 85, 1706–1717.
Steinmann, E., Penin, F., Kallis, S., Patel, A.H., Bartenschlager, R., et al., 2007. Hepatitis C virus p7 protein is crucial for assembly and release of infectious virions. PLoS Pathog. 3, e103.
Steinmann, E., Whitfield, T., Kallis, S., Dwek, R.A., Zitzmann, N., et al., 2007. Antiviral effects of amantadine and iminosugar derivatives against hepatitis C virus. Hepatology 46, 330–338.
Stocks, C.E., Lobigs, M., 1998. Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J. Virol. 72, 2141–2149.
Tedbury, P., Welbourn, S., Pause, A., King, B., Griffin, S., et al., 2011. The subcellular localization of the hepatitis C virus non-structural protein NS2 is regulated by an ion channel-independent function of the p7 protein. J. Gen. Virol. 92, 819–830.
Thomas, D.L., 2013. Global control of hepatitis C: where challenge meets opportunity. Nat. Med. 19, 850–858.
Tscherne, D.M., Jones, C.T., Evans, M.J., Lindenbach, B.D., McKeating, J.A., et al., 2006. Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry. J. Virol. 80, 1734–1741.
Vieyres, G., Brohm, C., Friesland, M., Gentzsch, J., Wolk, B., et al., 2013. Subcellular localization and function of an epitope-tagged p7 viroporin in hepatitis C virus-producing cells. J. Virol. 87, 1664–1678.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., et al., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.
Wang, K., Xie, S., Sun, B., 2011. Viral proteins function as ion channels. Biochim. Biophys. Acta 1808, 510–515.

Wang, Y.T., Hsu, H.J., Fischer, W.B., 2013. Computational modeling of the p7 monomer from HCV and its interaction with small molecule drugs. Springerplus, 2; p. 324.
Wozniak, A.L., Griffin, S., Rowlands, D., Harris, M., Yi, M., et al., 2010. Intracellular proton conductance of the hepatitis C virus p7 protein and its contribution to infectious virus production. PLoS Pathog. 6, e1001087.
Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T., Lemon, S.M., 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. U. S. A. 103, 2310–2315.
Yi, M., Ma, Y., Yates, J., Lemon, S.M., 2007. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J. Virol. 81, 629–638.
Yi, M., Ma, Y., Yates, J., Lemon, S.M., 2009. Trans-complementation of an NS2 defect in a late step in hepatitis C virus (HCV) particle assembly and maturation. PLoS Pathog. 5, e1000403.
Zeisel, M.B., Felmlee, DJ, Baumert, T.F., 2013. Hepatitis C virus entry. Curr. Top. Microbiol. Immunol. 369, 87–112.
Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., et al., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102, 9294–9299.