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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 .......................................................................................................................... 377
HCV p7 processing, topology, and localization .............................................................. 379
Ion-channel function and potential anti-p7 compounds ........................................... 380
NMR analyses of p7 ........................................................................................................ 382
Functional analyses of p7 .............................................................................................. 382
p7 interactions with other viral proteins ...................................................................... 384
Conclusion .................................................................................................................... 384
References ..................................................................................................................... 385

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.
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 (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

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**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 3006aa 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).
HCV p7 processing, topology, and localization

The p7 protein was identified through expression of a series of C-terminally truncated HCV polypeptides 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 HCV cell 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
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 (Hashşen 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., 2007). Consequently, recent studies have double-HA-tagged p7, either 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. Oligomerization 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 blocked by the antiviral drug amantadine. In addition, it was also shown that mutation of the two basic residues within the p7 cytoplasmic loop abrogated ion-channel activity, 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-40 monononyl deoxygalactonojirimycin (NN-DGJ), N-40 monononyl deoxynojirimycin (NN-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 was also 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 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)).
Table 1
Examples of known viroporins. Ion-channel names and respective virus and family names are shown (1st and 2nd columns). The number of monomers needed to form the channel upon oligomerization and the classification are shown (3rd 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) |
| CoV-E | Coronaviridae | 1. Induce assembly by enhancing membrane scission. 2. Induce virion release. | Dimer, tetramer, or pentamer/ClassIA | 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 HXX(X/Y)-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 derivatives (Chew et al., 2009). 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 (Khouri et al., 2010). BIT225 was also shown to bind p7 using a computational model of a p7 monomer, along with amantadine, rimantadine and NN-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-responders 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, and 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-helices 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; Patargas 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 (Lindenhach et al., 2005; Wakiya 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
was found that p7 was not contained within the virion, even in using an infectious construct containing a double HA-tagged p7, it (Atoom et al., manuscript in preparation). (VI) More importantly, extracellular virus particles even in a virus entry-null system p7 adaptive mutation and found it to increase the levels of HCV assembly process became intensely investigated. One effect 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 replicates 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 (Miyamari 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., 2011; Gentzsch et al., 2013).

![Fig. 3. Monomeric and hexameric structures of p7.](Image)

(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 aa 5–16)(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).
The resulting chimera was not viable after injection, illustrating the importance of p7 in virus production. Intrahepatic RNA injection of chimpanzees with a chimeric virus containing the p7 sequence from G1a with that of G2a showed 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 (Woźniak 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 intracellular mRNA injection of chimpanzees with a chimeric virus containing the precursor sequence generated by replacing the p7 sequence from G1a with that of G2a. The resulting chimera was not viable after injection, illustrating the importance of p7 in virus production. 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 BT225 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 HCVwc 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.

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