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HCV core protein and virus assembly: what we know without structures

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Abstract Chronic hepatitis C virus (HCV) infection results in a progressive disease that may end in cirrhosis and, eventually, in hepatocellular carcinoma. In the last several years, tremendous progress has been made in understanding the HCV life cycle and in the development of small molecule compounds for the treatment of chronic hepatitis C. Nevertheless, the complete understanding of HCV assembly and particle release as well as the detailed characterization and structure of HCV particles is still missing. One of the most important events in the HCV assembly is the nucleocapsid formation which is driven by the core protein, that can oligomerize upon interaction with viral RNA, and is orchestrated by viral and host proteins. Despite a growing number of new factors involved in HCV assembly process, we do not know the three-dimensional structure of the core protein or its topology in the nucleocapsid. Since the core protein contains a hydrophobic C-terminal domain responsible for the binding to cellular membranes, the assembly pathway of HCV virions might proceed via coassembly at endoplasmic reticulum membranes. Recently, new mechanisms involving viral proteins and host factors in HCV particle formation and egress have been described. The present review aims to summarize the advances in our understanding of HCV assembly with an emphasis on the core protein as a structural component of virus particles that possesses the ability to interact with a variety of cellular components and is potentially an attractive target for the development of a novel class of anti-HCV agents.

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Introduction

Hepatitis C virus (HCV) is a blood-born, hepatotropic virus and a major cause of chronic liver diseases, including progressive liver fibrosis, cirrhosis, and hepatocellular carcinoma [1, 2]. Since most acute HCV infections (60–70 %) are asymptomatic, the approximations are based on published prevalence data, which estimated that 130–170 million persons, or 2–3 % of the world's population, are infected with HCV [3]. Moreover, prospective studies have shown that up to 85 % of cases of acute hepatitis C progress to chronicity [4], largely due to inadequate control by the host immune response [5]. Patients with chronic hepatitis C first develop chronic inflammation leading in some to liver cirrhosis (20–40 %) and subsequently (4–6 % of patients) to hepatocellular carcinoma (10–40 years after infections) [6]. Although it still remains controversial in the pathogenesis of hepatocellular carcinoma associated with HCV as to whether the virus plays a direct or an indirect role, there are strong evidences that the HCV proteins potentiate oncogenic transformation [1, 7]. Globally, 25 % of liver cancer is attributable to HCV [8]. Since the current population with chronic HCV infection constitutes an aging group, the annual rate of HCV-related hepatocellular carcinoma is projected to triple by 2030 [9]. No prophylactic or therapeutic vaccines for HCV are in sight [10]. However, in the last several years, tremendous progress has been made in the understanding of HCV

life cycle and in the development of small molecule compounds for the treatment of chronic hepatitis C. Recent basic and clinical data indicate that the HCV therapy will change for the better in the next few years [11–14].

For almost a decade, HCV infection was treated with a weekly injection of pegylated interferon alpha and a twice-daily weight-based dose of ribavirin [13]. Unfortunately, this standard therapy has numerous side effects and is associated with less than a 50 % response rate for HCV genotype 1 [9, 13]. HCV is highly heterogeneous and can be classified into seven major genotypes, differing in their nucleotide sequence by 30–35 %, most of which have multiple subtypes (indicated as a, b, and so on) [15, 16]. Besides distinct geographic distributions and alterations in pathogenesis exhibited by different genotypes, the response to therapy also varied between them [17]. Most prevalent worldwide is genotype 1 that is also poorly responsive to interferon-based therapy [17, 18]. Two years ago, two NS3-4A protease inhibitors, telaprevir and boceprevir, were approved in combination with pegylated interferon alpha and ribavirin for the treatment of chronic HCV genotype 1 infection [12]. This first selective antiviral therapy increased substantially cure rates. However, the triple therapy still exhibits severe limitations such as a restriction to genotype 1 infections, a selection of drug-resistant viruses, and an increase in the spectrum of serious side effects [19]. Thus, current research is focusing on developing the most efficacious combination regimen. So far, only molecules that target polyprotein processing (NS3-4A protease inhibitors) and inhibitors of HCV replication through various targets and mechanisms (inhibitors of HCV RNA-dependent RNA polymerase, NS5A inhibitors, cyclophilin inhibitors, and microRNA-122 antagonist) have reached clinical development [12]. Although much has been done toward a highly efficacious, all-oral, and well-tolerated treatment, the development of alternative therapeutics targeting other viral and host proteins, which could be effective against all HCV genotypes, remains an attractive area of research [11, 20].

Hepatitis C virus is a member of the *Hepacivirus* genus within the *Flaviviridae* family, a group of small, enveloped, single-stranded RNA viruses [15, 21]. The HCV particles contain a positive polarity RNA genome with 5′ and 3′ untranslated regions (UTR) and a long open reading frame encoding a polyprotein precursor of about 3,000 amino acids. UTRs constitute highly conserved, cis-acting RNA elements regulating viral genome translation and replication [21–23]. Translation of the polyprotein is initiated by ribosome binding to an internal ribosome entry site (IRES), which spans most of the 5′-UTR and the first 24–40 nucleotides of the core coding region [21, 22, 24]. This results in the production of a single precursor polyprotein, which is processed by cellular and viral proteases into 10 structural and nonstructural proteins (core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Core protein, which forms the nucleocapsid, and the envelope glycoproteins (E1 and E2) make up the structural components of the virion. Nonstructural proteins from NS3 to NS5B are thought to assemble into a membranous-web-associated HCV RNA replicase complex that catalyzes the amplification of the viral RNA genome [21, 22, 25]. Whereas viral RNA replication is independent of the structural proteins, the assembly and egress of infectious virus particles require almost all viral proteins [26–28]. A development of the infectious HCV cell culture system (HCVcc) based on the genotype 2a strain called JFH-1 (isolated from a Japanese patient with fulminant hepatitis) allowed analysis of the essential contribution of nonstructural proteins and host cell factors to virion morphogenesis [16, 29–32]. Most studies with the infectious HCVcc system are limited to derivatives of the JFH-1 clinical isolate [33] and highly permissive Huh7 cell clones enhancing virus titers [32, 34, 35].

Properties of HCV core protein

The HCV core protein is a multifunctional protein, but with respect to the virus, its main function is to form the viral capsid to surround and protect the genomic RNA, while the virus passes from a cell to another [36]. The monomeric, mature form of HCV core is a 21-kDa protein that possesses lipid and RNA binding activities [37]. Because the three-dimensional structure of core is still unknown, insights into its overall conformation as well as biochemical and biophysical properties have been mostly provided by studies using circular dichroism, analytical centrifugation, intrinsic fluorescence measurements, and limited proteolysis [38]. It has been shown that the HCV core protein (1–169 aa) adopts an α-helical conformation for nearly 50 % of the protein and essentially assembles as dimers in the presence of mild detergents, whereas in the absence of detergent, it forms soluble aggregates [38]. Structures of core proteins from two other flaviviruses, the dengue and West Nile viruses, have been determined by nuclear magnetic resonance (NMR) and X-ray crystallography, respectively. These core proteins were shown to be mostly α-helical and dimeric in solution with no need of detergent to keep them monodispersed [39, 40]. The fact that HCV core protein is much larger and contains an additional

C-terminal hydrophobic domain makes it unique among *Flaviviridae* core proteins [41].

The mature HCV core protein consists of two distinct domains—D1 and D2—distinguished by different amino acid compositions and hydrophobicity profiles (Fig. 1) [42]. The D1 domain of core is hydrophilic, is rich in basic residues, and is located at the N-terminal two-thirds of the core (~117 aa), whereas the D2 domain encompasses the C-terminus and is more hydrophobic [38, 43]. Based on the charge distribution of amino acids [44], the D1 domain of core can be subdivided into three basic clusters: the basic domain 1 (BD1; 2–23 aa), the basic domain 2 (BD2; 38–74 aa), and the basic domain 3 (BD3; 101–121 aa). The D1 domain is mainly involved in RNA binding [23, 37, 44] and oligomerization necessary for particle formation [41, 45–49]. It was demonstrated that the N-terminal part of core (82 first amino acids) was sufficient to trigger the formation of nucleocapsid-like particles in vitro when structured RNA was added to the purified protein [50]. In the same study, mutational analysis of a truncated core (C1-82) revealed that it is the global positive charge rather than any specific basic residue that is important for the assembly process. Circular dichroism and NMR analysis indicated that core1-82 lacks secondary structure and is highly disordered [51]. It was also proposed that intrinsic disorder feature is important for RNA chaperoning functions of core, although the physiological relevance of this interaction is still not clear [52, 53]. These properties may be critical for the structural remodeling and packaging of the RNA genome into the viral particle [54]. As other intrinsically unstructured proteins, it is expected that the D1 domain of core can adopt different conformations depending on the presence of specific cellular partners. Although its major function is to encapsidate the HCV genome, the core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions such as gene transcription, lipid metabolism, apoptosis, and cell signaling [42, 55]. The intrinsic disorder of the N-terminal part of HCV core may explain why many viral and host interactions have been mapped to residues within the D1 domain [42]. Furthermore, it appeared that the folding of the highly basic domain depends on the presence of the hydrophobic domain [38]. It was shown that the D1 domain also contains immunodominant antigenic site involving a helix-loop-helix motif that spans amino acids 2–45 (PDB entry 1CWX) [56, 57]. The D2 domain is responsible for core association with endoplasmic reticulum (ER) and with lipid droplet (LDs) membranes [43]. It contains two amphipathic α-helices, Helix I (119–136 aa) and Helix II (148–164 aa), separated by a hydrophobic loop (HL). The helix-turn-helix motif in the D2 domain is essential for efficient LD localization, and probably, it mediates an in-plane membrane interaction [43]. Core mutant proteins unable to associate with LDs were rapidly degraded by the proteasome, indicating that the D2 domain is necessary for folding and stability of mature core [43]. Additionally, the binding of the core protein to membranes as well as its conformation might be regulated by palmitoylation of a conserved cysteine residue Cys-172 shown to be required for proper core localization and infectious particle production [58].

The core protein is released from the HCV precursor polyprotein by two cellular proteases, signal peptidase and signal peptide peptidase. First, cleavage by signal peptidase occurs in ER lumen separating core from E1 glycoprotein [37]. Next, the precursor core of 191 amino acids is processed within the transmembrane region by a signal peptide peptidase, giving a mature protein of 177 residues or so, which is targeted to LDs [59–61]. Although mutagenesis and trans-complementation studies demonstrated that the minimal length of core protein for infectious virus production consists of 177 residues [59], the sequence of its C-terminus is still not precisely known [62]. NMR structure of the internal signal sequence located between core and E1 (synthetic peptide spanning the region of 171–195 aa; PDB entry 2LIF), which targets the nascent polyprotein to the ER membrane, has been recently determined [63]. Overexpression systems have suggested the targeting of core to different compartments such as the nucleus and mitochondria [64]. However, studies with HCVcc system demonstrated that the core protein is solely localized at the surface of LDs and in the membranous compartment associated with LDs [65]. A visualization study of core trafficking during assembly in live virus-producing cells identified core proteins as polarized caps on immotile LDs and as small motile puncta along microtubules [66]. Mutational analysis revealed that the nucleocapsid-like particle of HCV most likely contains a dimer of core protein that is stabilized by a disulfide bond formation at Cys-128 [67]. A disulfide-bonded dimer is created probably at the ER, where the core protein is initially produced and processed. The nucleocapsid-like particles with disulfide-bonded core dimer were resistant to trypsin digestion, suggesting that the capsid is built up as a tightly packed structure of core proteins with their N-terminus being concealed inside [67].

Core and HCV assembly

While core proteins provide the essential structural elements of the virion, the assembly of infectious HCV particles is a complex process, which requires the coordinated interactions between viral structural and nonstructural proteins and host factors [26, 28–30, 68]. The major

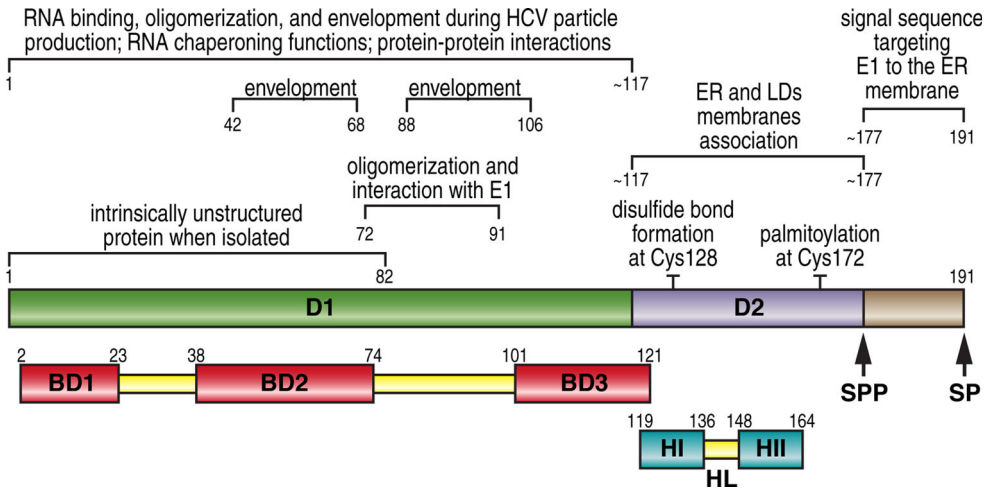

Fig. 1 The functional map of HCV core protein. The core protein is released from polyprotein by a signal peptidase (SP). Next, the precursor core of 191 amino acids is processed by a signal peptide peptidase (SPP), giving a mature protein of around 177 amino acids that is composed of two domains, D1 and D2. Based on the charge distribution, the D1 domain can be subdivided into three basic clusters: BD1 (basic domain 1), BD2 (basic domain 2), and BD3 (basic domain 3). The D2 domain contains two amphipathic α-helices, Helix I (HI) and Helix II (HII), separated by a hydrophobic loop (HL)

contributions from the host cells to virion assembly are from LDs [69], the storage sites for neutral lipids in cells, and from the pathway producing very low density lipoproteins (VLDLs) that exports cholesterol and triglyceride from hepatocytes [29]. HCV particles isolated either from patient sera, often called lipoviroparticles (LVPs) as they are associated with lipoproteins, or from the infectious cell culture system (HCVcc particles) were found to be heterogeneous in terms of their density, morphology, and composition [30]. The situation is even more complicated as HCVcc particles differ in lipoprotein composition from LVPs most probably due to an impairment of the VLDL assembly pathway in Huh7 cells [30, 70, 71]. The density of circulating HCV particles in infected individuals ranges from below 1.06 to 1.25 g/ml, and particles with the lowest density have the highest specific infectivity [30]. Current biochemical and morphological characterization of infectious HCVcc particles is mostly based on studies using affinity purification of secreted virus particles combined with electron microscopy analysis [72] or electron cryomicroscopy of particles purified by density-gradient ultracentrifugation [73]. Both approaches revealed that HCVcc particles are pleomorphic, mostly spherical, with an average diameter of about 60 [73] or 73 nm [72]. It has been assumed that the infectious HCV particle contains ~45 nm nonicosahedral capsid that is not tightly associated with the viral envelope [73]. The precise nature of the association between virus particles and lipoproteins remains unclear [26, 28].

The current model for HCV particle production (Fig. 2) implies the assembly of virions on or near the surface of LDs, envelopment at the ER, and egress via the secretory pathway in association with components of VLDL synthesis [66, 70, 71, 74]. The HCV assembly process appeared to be spatially associated with LDs [75, 76]. Many years before HCVcc system has been developed, the HCV core protein was reported to be associated with the surface of LDs [77, 78]. Mutations in the signal peptide peptidase cleavage site (ASC/180/183/184VLV) block core trafficking to LDs [61] and render the virus noninfectious [79]. Mutations in the D2 domain of core (F130E or P138A/P143A) also abolish viral assembly [75, 80]. Studies using the HCVcc system demonstrated that LDs are required for the formation of infectious virus particles and indicated a key role of core and NS5A in this process [75, 76]. Mutations of core (mentioned above) and NS5A (APK99-101AAA and PPT102-104AAA), which abolish targeting of those proteins to LDs, impair virus production [75]. It has been demonstrated that the core protein recruits NS proteins, HCV RNAs, and the replication complex to LD-associated membranes, thus suggesting that LDs can serve as a platform for virion formation [75]. It has also been proposed that core recruits NS5A to LDs through the direct interaction involving three serine residues at positions 2,428, 2,430, and 2,433 of NS5A (amino acid positions within the HCV polyprotein corresponding to the positions 452, 454, and 457 of full-length NS5A) [81]. Mutations of these serine residues reduced NS5A basal phosphorylation, caused NS5A mislocalization, impaired virion assembly, and disrupted core-NS5A interactions [81]. Thus, NS5A most likely plays a double role in both viral RNA replication and assembly process and may serve as a potential switch between the two

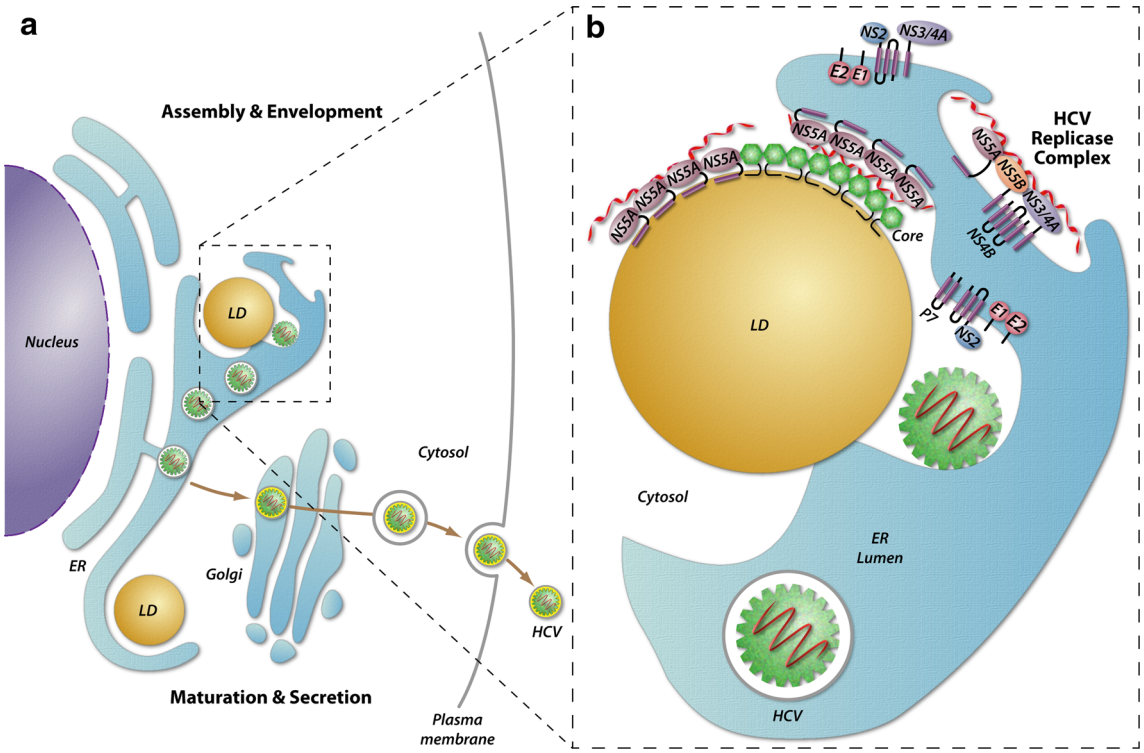

Fig. 2 Model of HCV particle production. **a** HCV particle formation starts on or near the surface of LDs, most probably concurrently with envelopment at the ER, and continues through the secretory pathway in association with components of VLDL synthesis in order to produce a mature form of lipoviroparticles coupled with the specific lipoproteins (*depicted by yellow color*). **b** Core interacts with NS5A on the surface or in the close proximity of LDs, so the viral RNA can be packed into the newly formed core oligomers. This event most probably triggers the capsid assembly. p7 recruits the core assembly intermediate to the ER membrane and is necessary for the final steps of capsid assembly and the initiation of budding. NS2 brings replication and assembly sites in the close vicinity through its interaction between glycoproteins, E1 and E2, and NS3/4A (Color figure online)

steps [82–85]. Other studies showed that the core protein of the high-titer Jc1 recombinant virus was hardly detected on LDs, but was mainly localized at ER membranes [76, 86]. Further analysis of core trafficking using visualization methods in live virus-producing cells suggested that during virus assembly, the core protein is recruited from the surface of LDs to the assembly sites and that the interaction between NS2 and NS3-4A is necessary for this recruitment [66]. Independent studies of HCV particle assembly have implied that NS2, through the interactions with structural (E1 and E2) and nonstructural proteins (NS3/4A) [87, 88], provides a close vicinity of replication and assembly sites, while p7 might directly or indirectly recruit the core assembly intermediate to the ER membrane [89]. Protein p7 has been shown to be necessary for the final steps of capsid assembly and the initiation of budding supporting a model where the virion assembly is linked with the envelopment [89]. Up to date, contributions for all of the nonstructural proteins in HCV assembly were described including p7 [86, 89], NS2 [86–88, 90], NS3 [66, 87, 88], NS4A [91], NS4B [92, 93], NS5A [81–85], and NS5B [94], although most of the mechanisms remained elusive. A study of core binding to E1 has suggested that this interaction depends on core amino acids, 72–91, promoting core oligomerization [46]. It has been shown that regions spanning the residues 42–68 and residues 88–106 in the D1 domain of core were important for core envelopment [95]. A membrane protection assay demonstrated that those two deletions significantly increased the sensitivity of core protein to proteinase K digestion, indicating that the mutant viruses were not efficiently enveloped by membranes [95].

Furthermore, the participation of host cell factors in the HCV assembly process was also investigated such as the diacylglycerol acetyltransferase-1 (DGAT1) [96]. It has been demonstrated that DGAT1, enzyme essential for LD biogenesis, binds the core protein and targets it to LDs and that the inhibition of this DGAT1-mediated LD targeting of core impairs HCV particle production [96]. Targeting of the core protein to LDs also requires the mitogen-activated protein kinase (MAPK)-regulated protein cytosolic phospholipase A2 (cPLA2) [97]. Another host factor interfering with HCV assembly and binding core is AP2M1, the μ subunit of clathrin adaptor protein complex 2, involved in recruiting cargo into clathrin-mediated endocytosis at the

plasma membrane [98]. Silencing AP2M1 expression or overexpressing a dominant negative AP2M1 mutant inhibited intra- and extracellular infectivity, but had no effect on HCV RNA replication, which is consistent with a defect in viral assembly [98]. Lately, a growing body of evidence implied that host proteins such as DGAT1, which was mentioned above, and the tail-interacting protein of 47 kDa (TIP47) are involved in targeting NS5A carrying viral RNA to the HCV assembly sites on the surface of LDs [99–101]. Recent publications described an important role of DGAT1 in HCV infection, suggesting that this host protein facilitates the binding of NS5A to core and guides both proteins onto the surface of LDs [99]. The direct interaction between TIP47 and NS5A was also revealed, and importantly, the silencing of TIP47 in cells was shown to be crucial for HCV life cycle [100]. A very recent study has presented a model where TIP47 through its interaction with NS5A plays a new role in HCV infectivity, possibly by integrating LD membranes into the membranous web [101]. In this model, a close interface between LDs and the ER of the membranous web has been established in the absence of core and other structural proteins. Since NS3, NS5B, and NS5A together with TIP47 and viral RNA were able to colocalize with LD membranes even in the absence of core, it has been suggested that LD membranes are critical parts of the membranous web, independent from viral assembly [101].

On the other hand, comprehensive mutagenesis studies of the core protein region containing amino acids 57–191 revealed numerous residues that are critical for infectivity, but not for viral RNA replication, arguing that core plays a key role in efficient HCV particle formation [102]. Moreover, it has been demonstrated that the single alanine substitution of the highly conserved core residue G33 was sufficient to severely reduce infectious virus production [103]. Another core mutagenesis study focused on the two first basic clusters of the D1 domain [104]. The alanine substitution of four residues (R50, K51, R59, and R62) within the second cluster was shown to completely abolish virus infectivity. Although the exact stage at which these mutations impacted virus assembly was not found, defects in core protein stability, colocalization with LDs and NS5A, RNA encapsidation, oligomerization, and envelopment by intracellular membranes were excluded experimentally [104]. Our recent findings suggested that the basic residues in the D1 domain of core at positions 50, 51, 59, and 62, which are critical for the formation of infectious extracellular and intracellular viral particles, also play a role in core-NS5A interactions [105]. Importantly, we demonstrated that mutating the basic residues (R50A, K51A, R59A, and R62A) in the HCVcc system impairs core-NS5A interactions and abolishes both infectivity and release of HCV particles. These data further emphasize that core-NS5A interactions could represent an attractive drug target.

HCV core as a potential target for antiviral intervention

In principle, each step of the HCV life cycle could be a target for antiviral strategy [21]. The amino acid sequence of the core protein is well conserved among different HCV isolates and genotypes compared with other HCV proteins [106]. Because core is the least variable of the ten HCV proteins in variant viruses emerging constantly in patients [107], it has been proposed as an attractive target for the development of new anti-HCV agents [108]. By screening for inhibitors of core dimerization, peptides and small molecules, which disrupt interactions between core and other HCV proteins, mainly NS3 helicase, and block HCV production, have been identified [109]. It has been shown that the N-terminal fragment of core (106 aa) was sufficient to achieve 91 % inhibition of dimerization, while the 15- and 18-residue peptides derived from the region 82–106 aa inhibited 50 and 68 % of core dimerization, respectively (IC_{50} of 21.9 μM) [110]. These peptides were also tested on HCV-infected cells and were found to block release of infectious particles, but not viral RNA replication [110]. Small molecules identified during that screening displayed IC_{50} of core dimerization and EC_{50} of HCV infectivity mostly at a nanomolar range [111]. Additionally, it has been demonstrated that the biotinylated derivative of one of these small molecule inhibitors (SL209) with an EC_{50} of 3.2 μM binds directly to HCV core [112]. This study supported the hypothesis that inhibitors of HCV nucleocapsid formation might represent potent antiviral agents [112]. Very recent study has reported for the first time a direct antiviral activity of DNA aptamers for HCV core protein in the infectious cell culture system [113]. The data suggested that DNA aptamers against core protein inhibit the production of infectious particles through disrupting the localization of core with LDs and its binding to viral RNA. Thus, the HCV core protein could be another attractive target for the development of a novel class of antiviral drugs.

Conclusions

Systemic approaches determined that for the overall cell interactome, HCV core, NS3, and NS5A proteins were the most connected viral proteins with 76, 214, and 96 host protein partners, respectively [114]. In infected cells, the HCV core protein is synthesized on the ER membrane with a larger basic domain facing the cytoplasm. The HCV core protein has been shown to form oligomers, and the self-

interaction of core proteins is a critical event in the assembly of the viral nucleocapsid. During the process of HCV particle formation, core interacts with viral RNA, LDs, and ER membranes, and with a plethora of different cellular and viral proteins in a coordinated manner in order to promote successfully the assembly of new virions. HCV core protein expression may also influence critical processes that have implications in cellular pathophysiology such as insulin, Jak/STAT, and TGFβ pathways [114], as well as apoptosis, gene transcription, and lipid metabolism [42, 55, 115]. Inhibitors of core protein oligomerization or inhibitors of its interactions with cellular proteins (i.e., DGAT1) or viral proteins (i.e., NS5A) could be useful tools to study HCV particle assembly and serve as a basis for the development of a novel class of anti-HCV agents.

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