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TOPIC HIGHLIGHT

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Hepatitis C virus proteins

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Abstract

Hepatitis C virus (HCV) encodes a single polyprotein, which is processed by cellular and viral proteases to generate 10 polypeptides. The HCV genome also contains an overlapping +1 reading frame that may lead to the synthesis of an additional protein. Until recently, studies of HCV have been hampered by the lack of a productive cell culture system. Since the identification of HCV genome approximately 17 years ago, structural, biochemical and biological information on HCV proteins has mainly been obtained with proteins produced by heterologous expression systems. In addition, some functional studies have also been confirmed with replicon systems or with retroviral particles pseudotyped with HCV envelope glycoproteins. The data that have accumulated on HCV proteins begin to provide a framework for understanding the molecular mechanisms involved in the major steps of HCV life cycle. Moreover, the knowledge accumulated on HCV proteins is also leading to the development of antiviral drugs among which some are showing promising results in early-phase clinical trials. This review summarizes the current knowledge on the functions and biochemical features of HCV proteins.

Key words: Hepatitis C virus; Viral hepatitis; Viral proteins; Molecular virology

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INTRODUCTION

As for the other members of the *Flaviviridae* family the genome of Hepatitis C virus (HCV) encodes a single polyprotein. This 3010 amino acid polyprotein is processed by cellular and viral proteases to generate 10 polypeptides[1] (Figure 1). The nonstructural proteins are released from the polyprotein after cleavage by HCV proteases NS2-3 and NS3-4A, whereas the structural proteins are released by host endoplasmic reticulum (ER) signal peptidase(s)[2]. Further processing mediated by a signal peptide peptidase also occurs at the C-terminus of the capsid protein[3]. In addition to the large open reading frame encoding the polyprotein, the HCV genome contains an overlapping +1 reading frame that may lead to the synthesis of an additional protein[4]. Despite the difficulties in propagating the virus in cell culture, a large body of data has accumulated on HCV proteins since the identification of HCV genome 17 years ago. A detailed knowledge of the functions of HCV proteins is important for the development of new antiviral drugs. This review summarizes the current knowledge of the functions and biochemical features of HCV proteins. A brief summary of the functions of HCV proteins is presented in Table 1.

CORE PROTEIN

The core protein is an RNA-binding protein that is supposed to form the viral nucleocapsid. It is removed from the polyprotein by a host signal peptidase cleavage at the C-terminus, yielding the immature form of the protein[5], and the signal peptide present at the C-terminus of the core is processed further by a host signal peptide peptidase, yielding the mature form of the protein[3] (Figure 1). It has been shown that the mature form of core is a dimeric alpha-helical protein, which behaves as a membrane protein[6]. This protein can be separated into two domains: an N-terminal two-thirds hydrophilic domain (D1) and a C-terminal one-third hydrophobic domain (D2)[7]. The D1 domain includes numerous positively charged amino acids and has similar characteristics to the capsid proteins of related pestiviruses and flaviviruses[6,7]. The D2 domain is required for proper folding of domain D1 and is critical for the membrane characteristics of the core[6,8]. It is worth noting that this domain is absent in the pestiviruses and flaviviruses but is found in GB virus B[6,9].

Little is known about the mechanisms of HCV nucleocapsid assembly. *In vitro* nucleocapsid reconstitution experiments with core segments have thus far yielded irregular particles larger than those isolated from infected subjects[10]. Full-length core protein has also been shown to assemble into nucleocapsid-like particles upon de novo

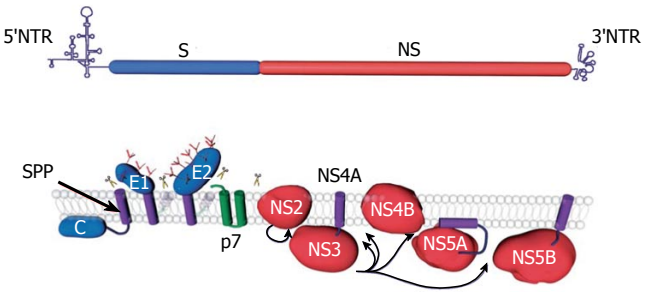

Figure 1 HCV genome organization (top) and polyprotein processing (bottom). HCV encodes a single polyprotein with the structural proteins (S) and the nonstructural proteins (NS) present in the N-terminal one-third and the C-terminal two-third of the polyprotein, respectively. The polyprotein processing and the location of the 10 proteins relative to the endoplasmic reticulum membrane are schematically represented. Scissors indicate cleavages by a host signal peptidase. Arrows indicate NS2-3 and NS3-4A cleavages. The intramembrane arrow indicates cleavage by a host signal peptide peptidase (SPP). The transmembrane domains of E1 and E2 are shown after signal-peptidase cleavage and reorientation of their C-terminus. In addition, the precleavage topology of the transmembrane domains of E1 and E2 is shown in light grey.

synthesis in cell-free systems made of rabbit reticulocyte lysate or wheat germ extracts[11]. It has also been suggested that the attachment of a core protein to a phospholipid layer is required as a template for proper assembly of the nucleocapsid[6]. Although, little is known on the assembly of the nucleocapsid, developing small molecules that block the signal peptide peptidase cleavage might be a way of inhibiting HCV assembly.

When expressed in the context of heterologous expression systems or HCV replicons, core is found both attached to the ER and at the surface of lipid droplets[7,12]. In some conditions, a minor proportion of the core protein has also been found to be located in the nucleus[13]. More recently, the core protein has also been found to colocalize with mitochondrial markers in Huh-7 cells containing a full-length HCV replicon[14]. However, in the context of an infectious virus, the core protein was only found in association with lipid droplets[15]. It has been reported that the traffic between rough ER membranes, the site of capsid protein synthesis, and lipid droplets is regulated by signal peptide peptidase cleavage in the C-terminal region of the core protein[3]. It is therefore likely that in the context of HCV-infected cells, transport of the C protein to the site of lipid droplet assembly is rapid due to rapid cleavage by the signal peptide peptidase.

The core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions[7,16,17]. It has indeed been proposed to be involved in cell signaling, apoptosis, carcinogenesis and lipid metabolism. However, in most cases, it is unclear if these interactions occur in the course of a normal infection or are artifacts of ectopic expression or protein over-expression. Further studies with the recently developed cell culture system for HCV[18-20] should help clarify whether all the functions identified for HCV core protein can be observed in the context of infected cells.

E1 AND E2 GLYCOPROTEINS

HCV glycoproteins, E1 and E2, are released from the polyprotein by a host signal peptidase cleavage[12] (Figure 1). They are type-I transmembrane proteins with a large N-terminal ectodomain and a C-terminal transmembrane domain, and they assemble as noncovalent heterodimers[21]. The ectodomains of HCV envelope glycoproteins E1 and E2 are highly modified by N-linked glycans. Indeed, E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively, and most of them are well conserved[22,23]. It is worth noting that some glycans have been shown to play a role in HCV glycoprotein folding or in virus entry[24]. Because they are essential for virus entry, HCV envelope glycoproteins are a good target for the development of antiviral molecules that block HCV entry[25].

Table 1 Viral proteins and their functions in HCV life cycle

Protein	Molecular Mass[1]	Function
Core	21 kDa	RNA binding; nucleocapsid
E1	31-35 kDa	Envelope glycoprotein; associate with E2
E2	70 kDa	Envelope glycoprotein; receptor binding; associate with E1
p7	7 kDa	Ion channel
NS2	21 kDa	Component of NS2-3 proteinase
NS3	69 kDa	N-terminal proteinase domain; C-terminal NTPase/helicase domain
NS4A	6 kDa	NS3-4A proteinase cofactor
NS4B	27 kDa	Induces membrane alterations
NS5A	56-58 kDa	Phosphoprotein
NS5B	68 kDa	RNA-dependent RNA polymerase

[1]Estimated by SDS-PAGE.

Hypervariable regions (HVR) have been identified in the E2 envelope glycoprotein sequence[26]. The first 27 amino acids of the E2 ectodomain form HVR1. The apparent variability of this region seems to be driven by antibody selection of immune-escape variants. An HCV clone lacking HVR1 was found to be infectious but strongly attenuated in chimpanzees[27], supporting a functional role of this domain, likely in virus entry[28,29]. Despite the sequence variability of HVR1, the physico-chemical properties of the residues at each position and the conformation of HVR1 are highly conserved among the various genotypes[30]. In addition, HVR1 is a globally basic region and basic residues of HVR1 have been shown to play a role in modulating virus entry[29]. Another hypervariable region, HVR2, has also been described in E2[26], and this region has been proposed to modulate E2 receptor binding[31].

Although HCV glycoproteins can be detected at the plasma membrane when they are over-expressed[32-34], the E1E2 heterodimer is mainly retained in the ER[15,35]. The determinants for ER retention of HCV envelope glycoproteins have been mapped in the transmembrane domains of E1 and E2[36,37]. In addition to a membrane-proximal heptad repeat sequence in E2[38], these domains have also been shown to be essential for heterodimerization[39]. The transmembrane domains of HCV envelope glycoproteins are not canonical transmembrane domains[40], and dynamic changes have been shown to occur in these domains after cleavage by the signal peptidase[41]. Indeed, before cleavage by a host signal peptidase, the transmembrane domains of E1 and E2 adopt a hairpin structure, and after cleavage, the signal-like sequence is reoriented toward the cytosol,

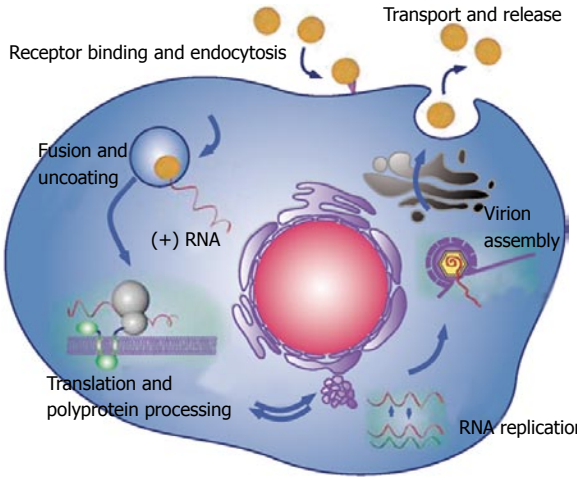

Figure 2 Schematic representation of the major steps of HCV life cycle. The virus binds to a receptor at the cell surface, which leads to endocytosis of the particle. Fusion between the viral envelope and an endosomal membrane leads to the release of HCV genome into the cytosol. HCV genome is a positive strand RNA, which is directly translated and all the viral proteins are simultaneously produced. Expression of HCV proteins induces intracellular membrane alterations (the membranous web), which is the site of RNA replication. The nonstructural proteins NS3 to NS5B assemble in association with cellular factors to form a replication complex, which is responsible for RNA replication. Accumulation of HCV genomic RNA and the structural proteins leads to the assembly of a nucleocapsid, which acquires its envelope within an intracellular compartment. The viral particle is then secreted by following the classical secretory spathway.

leading to a single transmembrane passage.

The two envelope glycoproteins, E1 and E2, play major roles at different steps of the HCV life cycle (Figure 2). They are essential for virus entry[42,43], and they participate in the assembly of infectious particles[19]. The E1 E2 heterodimer is the viral component present at the surface of HCV particles and it is therefore the obvious candidate ligand for cellular receptors. As a first approach to identify potential HCV receptor(s), a soluble form of HCV glycoprotein E2 has been used. This led to the identification of a series of putative receptors for HCV: CD81 tetraspanin[44], scavenger receptor class B type I (SR-BI)[45], heparan sulfate[46] and the mannose binding lectins DC-SIGN and L-SIGN[47-49]. An approach using virus-like particles produced in insect cells has also led to the identification of the asialoglycoprotein receptor as another candidate receptor for HCV[50]. In addition, because of the physical association of HCV with low- or very-low-density lipoproteins (LDL or VLDL) in serum, the LDL receptor has also been proposed as another candidate receptor for HCV[51,52]. Among these molecules, only CD81 and SR-BI have been shown to play a role in HCV entry[43]. However, co-expression of CD81 and SR-BI in non-hepatic cell lines does not lead to virus entry, indicating that other molecule(s) expressed only in hepatic cells, are necessary for HCV entry.

Interactions between viral envelope glycoproteins and potential receptors can have other consequences than a direct effect on virus entry. For instance, L-SIGN and DC-SIGN are not expressed on hepatocytes, and HCV interactions with these molecules may contribute to establishment or persistence of infection both by the capture and delivery of virus to the liver and by modulating dendritic cell functions as recently suggested[53,54]. It has also been shown that intracellular interaction between HCV envelope glycoproteins and CD81 can lead to secretion of exosomes containing E1 and E2 glycoproteins[55]. A soluble form of E2 is also able to bind CD81 at the surface of natural killer cells, and this interaction inhibits cytotoxicity and cytokine production by these cells[56,57]. Binding of a soluble form of E2 to CD81 can also provide a co-stimulatory signal for T cells[58,59], activate B lymphocytes[58] and up-regulate matrix metalloproteinase-2 in human hepatic stellate cells[60]. It remains however to be determined whether HCV glycoprotein expressed in the context of native particles will also have the same effects on cell functions.

Because they are exposed at the surface of the virion, the envelope proteins are targets of neutralizing antibodies. The recent development of retroviral particles pseudotyped by unmodified HCV E1 and E2 envelope glycoproteins (HCVpp)[32-34] has allowed to initiate studies on neutralizing antibodies. As determined with HCVpp, it seems that the majority of chronically infected patients have cross-reactive neutralizing antibodies[61,62]. In contrast, neutralizing antibodies have not been detected in several cases of acute resolving infection[61,62], and the detection of neutralizing antibodies in acutely infected individuals did not seem to be associated with viral clearance[61]. However, another study has shown in some patients a progressive emergence of a relatively strong neutralizing response in correlation with a decrease in viremia[63]. Further investigations on a large number of acutely infected patients will be necessary to determine the role of neutralizing antibodies in controlling HCV infections. Importantly, the majority of neutralizing anti-HCV monoclonal antibodies that have been described recognize E2[32,34,64-66]. In addition, some of the epitopes recognized by these antibodies have been mapped in the CD81 binding region of E2 and in the C-terminus of HVR1[34]. Studies with these neutralizing monoclonal antibodies will be essential to understand the mechanisms leading to HCV neutralization.

p7

The p7 polypeptide is located within the HCV polyprotein at the junction between the structural and nonstructural proteins[67,68]. It is released from the polyprotein by a host signal peptidase cleavage[12] (Figure 1). The p7 polypeptide is a small polytopic membrane protein composed of two transmembrane domains with both its N- and C-termini oriented toward the lumen of the ER[69]. The C-terminus of p7 contains a sequence for reinitiation of translocation, and when fused to a reporter protein, this sequence functions as a signal peptide[69,70]. The double membrane spanning topology of p7 with few residues accessible at one or the other side of the membrane suggests that p7 likely exerts its function(s) on membrane structures. When expressed by heterologous expression systems, p7 can be found in association with ER and/or mitochondrial membranes[69,71,72]. In addition, a small proportion of p7 can also be detected at the plasma membrane[69]. However, further investigations in the context of an infectious virus will be necessary to confirm these subcellular localizations. The

p7 polypeptide is not required for RNA replication, and it is uncertain whether it is a virion component. Interestingly, the p7 polypeptide has been shown to have an ion channel activity in artificial lipid membranes[72-75]. In addition, it has been shown to be essential for infectivity of HCV in chimpanzees[76]. These observations suggest that screening for small molecules that block the ion channel activity of p7 might be an approach to develop new anti-HCV molecules.

NS2

NS2 is an integral membrane protein that is not essential for the formation of the replication complex[77,78]. The function of NS2 in its mature form is unknown; however, before cleavage from the polyprotein, NS2 participates in a protease activity responsible for the cleavage at the NS2/NS3 junction[79] (Figure 1). The first 180 residues of NS3 are also required for this cleavage. In addition, the NS2-3 enzyme has been described as a cysteine proteinase[80]. The structure of NS2 reveals a dimeric cysteine protease with two composite active sites[81]. Surprisingly, for each active site, the catalytic histidine and glutamate residues are contributed by one monomer, and the nucleophilic cysteine by the other. The host-cell chaperone Hsp90 seems to be required to activate the NS2-3 proteinase[82]. Cleavage of the NS2 N-terminus from p7 is mediated by a signal peptidase within the ER[69,70]. When expressed alone, NS2 is found located in association with ER membranes[83]. NS2 contains several stretches of hydrophobic amino acids and is predicted to be a polytopic membrane protein[84,85]. The membrane topology of NS2 is unclear, but the presence of two internal signal-like sequences points to the existence of four transmembrane segments[85]. However, since the processing at the NS2/NS3 junction has to take place in the cytosolic space, the presence of the C-terminus of NS2 in the ER lumen suggests that a reorientation of this region would have to occur after cleavage between NS2 and NS3. Interestingly, crossover sites for natural or infectious artificial inter-genotypic HCV chimeras have been mapped in NS2[18,86,87]. These data suggest that in addition to its role in the processing at the NS2/NS3 cleavage site, NS2 is also involved in virus assembly and release. It remains however to be determined by which mechanism NS2 contributes to the latter process. Due to its involvement in NS2-3 protease activity, NS2 is an interesting target for the development of anti-HCV molecules.

NS2 has been shown to be a short-lived protein whose degradation by the proteasome is regulated in a phosphorylation-dependent manner through the protein kinase CK2[83]. In addition, it has been shown to interact with the liver-specific pro-apoptotic CIDE-B protein and to be an inhibitor of CIDE-B-induced apoptosis[88]. NS2 might also potentially affect cellular gene transcription[89]. However, all these properties need to be further investigated in the context of the newly developed cell culture system for HCV[18-20].

NS3 AND NS4A

NS3 is a multifunctional protein with an N-terminal serine-type protease domain and a C-terminal RNA helicase/NTPase domain. The NS3 protease domain has a typical chymotrypsin-like fold and is composed of two beta-barrel domains[90,91]. The protease activity of NS3 is enhanced by the NS4A cofactor. Indeed, NS4A contributes one beta-strand to the N-terminal protease domain and thereby allows its complete folding[90]. In addition, it induces a conformational change that leads to a repositioning of the catalytic triad. NS3 by itself has no transmembrane domain, but it associates non-covalently with the central domain of NS4A, which is a membrane protein. When co-expressed with NS4A, NS3 is found in association with ER or ER-like membranes whereas it is diffusely distributed in the cytoplasm and nucleus when expressed alone[92]. Deletion analyses have revealed that the hydrophobic N-terminal domain of NS4A is required for ER targeting of NS3. Interestingly, NS4A also stabilizes the protease against proteolytic degradation. The NS3-4A protease has an unusually shallow substrate-binding pocket and therefore requires rather long interaction surfaces with the substrate (reviewed in[1,93]). This made the design of efficient inhibitors of this protease challenging[94]. The NS3-4A protease is responsible for the polyprotein cleavage in the region downstream of NS3 (Figure 1), and this activity is essential for the generation of components of the viral RNA replication complex[95] (Figure 2). It is therefore not surprising that this protease has been the first target for the development of new anti-HCV molecules[94].

In addition to its role in the processing of the polyprotein, the NS3-4A protease activity is also involved in blocking the ability of the host cell to mount an innate antiviral response[96]. The NS3-4A has indeed been shown to interfere with double-stranded RNA signaling pathways. It disrupts the cellular RNA helicase retinoic acid-inducible gene I (RIG- I) pathway through proteolysis of a newly discovered essential adaptor protein of interferon regulatory factor-3 (IRF-3) activation[97]. Due to its recent simultaneous discovery by four different groups, this adaptor protein has received four different names: IPS-1, Cardif, VISA and MAVS[98]. NS3-4A cleavage of MAVS/IPS-1/VISA/Cardif results in its dissociation from the mitochondrial membrane and disruption of signaling to the antiviral immune response[99]. NS3-4A also cleaves the TRIF (also called TICAM-1) adaptor protein to ablate Toll-like receptor-3 (TLR-3) signaling of IRF-3 activation by extracellular double-stranded RNA[100]. However, this pathway has a minimal role in triggering the interferon antiviral response[101].

The C terminus of NS3 encodes a DexH/D-box RNA helicase[102]. Enzymes of this superfamily are capable of unwinding RNA-RNA duplexes in an ATP-dependent manner. The crystal structure of the HCV helicase shows a Y-shaped molecule composed of 3 nearly equally sized subdomains[103,104]. Although monomeric NS3 can bind RNA with high affinity, RNA unwinding requires an NS3 dimer[105]. Kinetic analyses indicate that this enzyme undergoes highly coordinated cycles of fast double-stranded RNA unwinding[105-107]. More recently, it has been reported that the cyclic movement of NS3 helicase is coordinated by ATP in discrete steps of 11 base pairs, and that actual unwinding occurs in rapid smaller sub-steps of 2 to 5 base pairs, also triggered by ATP binding, indicating that NS3 might move like an inchworm[108]. The NS3 helicase

activity can be modulated by interactions between the serine protease and helicase domains. Indeed the kinetics of duplex RNA unwinding is slower for the isolated helicase domain as compared with the full-length NS3 protein[109]. In addition, the presence of NS4A enhances productive RNA binding of a full-length NS3-4A complex[107]. The function of the NS3 helicase in the HCV life cycle is not known. It may be involved in initiation of RNA replication by unwinding stable stem-loop structures at the termini of positive and/or negative strand of HCV RNA. It may also contribute to the process of the replicase complex by removing stable RNA secondary structures and/or by displacing bound proteins that might interfere with RNA synthesis. Finally, it may also be required for dissociation of the replicative form. Due to its enzymatic activity, the helicase domain of NS3 is another potential target for the development of anti-HCV molecules.

The NS3 protein has been reported to interact with several cellular proteins[17], and it has been proposed to be involved in carcinogenesis[110]. However, the relevance of these interactions needs to be confirmed in the context of the recently developed cell culture system for HCV[18-20].

NS4B

The NS4B protein is a highly hydrophobic nonstructural protein, which is predicted to contain four transmembrane domains[111,112]. It has recently been shown that NS4B is palmitoylated in the C-terminal region of the protein[113]. The N- and C-termini of NS4B are localized in the cytosol; however, a fraction of the N-terminus can also be found in the ER lumen[112]. A putative amphipathic helix in the N-terminus of NS4B has been proposed to mediate membrane association[114]. The NS4B protein is detected in association with ER membranes[111,112,115]. In addition, NS4B also induces intracellular membrane alterations, suggesting that one of its functions is to induce the formation of membranous structures supporting RNA replication[116]. However, the structure of NS4B-induced membranes appears to be slightly distinct from the membranous web observed when all the HCV proteins are expressed, suggesting that other component(s) contribute to these membrane alterations. A nucleotide binding motif has been found in NS4B[117]. This structural motif binds and hydrolyzes GTP. Interestingly, mutation of this nucleotide binding motif affects HCV RNA replication[117]. The potential presence of NS4B domains on both sides of the ER membrane suggests that this protein plays a role in crosstalk between the ER lumen and the cytosol. Although a function can be attributed to this protein, it remains challenging to develop a high-throughput screening for small molecules targeting NS4B.

NS5A

NS5A is a membrane-associated protein containing a unique amphipathic alpha-helix at its N-terminus, which serves as an in-plane membrane anchor[118,119] (Figure 1). Like most HCV proteins, NS5A is detected in association with ER or ER-derived membranes[118]. Besides its membrane anchor sequence, NS5A contains three distinct domains that are separated by low complexity sequences (LCs) I and II [120]. Recently, the x-ray crystal structure of domain I was solved[121]. It is composed of a basic N-terminal subdomain IA and a predominantly acidic C-terminal subdomain IB. In subdomain IA a zinc ion is coordinated by a unique motif of 4 fully conserved cysteine residues, which are absolutely essential for RNA replication[120,121]. In subdomain IB an unusual disulfide bond linking 2 cysteine residues near the C-terminal subdomain border was found. However, this disulfide bond does not seem to be essential for HCV RNA replication. Domain I forms homodimers *via* contacts near the N-terminal end of the molecules. This dimerization results in the formation of a basic groove facing the cytosol at the surface of the membrane. This 'claw like' structure is believed to provide an RNA binding site that might be involved in regulated genome targeting within the replication complex[121]. In line with this observation, NS5A has been shown to bind to HCV RNA and more specifically to the 3'-ends of HCV plus and minus strand RNAs, with a preference for the polypyrimidine tract in the 3' non-translated region of positive strand RNA[122]. Therefore, the structure of domain I of NS5A provides a framework for the rational design of small antiviral molecules. The other two domains of NS5A are less characterized. Domain II has been proposed to be involved in inhibition of the interferon-induced double stranded RNA activated protein kinase PKR[123], and domain III is a less conserved region, which can tolerate insertions or partial deletions[124,125].

NS5A is a protein which is essential for genome replication[126,127]. Indeed, mutations that enhance RNA replication in cell culture map to the NS5A-coding sequence. In addition, NS5A has been shown to interact with NS5B, and this interaction is essential for maintenance of subgenomic replicons in Huh-7 cells[128,129]. NS5A is expressed as a basally phosphorylated and a hyperphosphorylated forms[93]. The functional relevance of the different phosphorylated forms is unknown. However, mutations that reduce NS5A hyperphosphorylation can lead to a dramatic enhancement of HCV genomic replication[124,130]. Furthermore, treatment of cells carrying non-adapted replicons with an inhibitor of the cellular kinase(s) responsible for NS5A hyperphosphorylation leads to an increase in HCV genomic replication[131]. In addition to its role in HCV genomic replication, NS5A has initially attracted considerable interest because of its potential role in modulating the interferon response[132]. NS5A has also been shown to interact with components of numerous cellular signaling pathways[17,133,134]. Among the potential cellular partners identified for NS5A, human vesicle-associated membrane protein-associated protein A (hVAP-A) is of particular interest because it is regulated by NS5A phos phorylation[130,135]. Indeed, NS5A hyperphosphorylation disrupts interaction with hVAP-A and negatively regulates viral RNA replication. VAP-A is a protein found on ER and Golgi membranes, which is involved in intracellular vesicle trafficking. It remains however to be determined why NS5A hijacks hVAP-A at some step of its life cycle. Another potentially important host cell factor interacting with NS5A is the geranylgeranylated protein FBL-2[136]. In

line with this observation, it has been shown that inhibition of geranylgeranylation in cells abolishes HCV RNA replication[137].

NS5B

NS5B is a membrane-associated protein containing a C-terminal transmembrane domain[138], which is essential for RNA replication in cell culture[139] (Figure 1). Like most HCV proteins, NS5B is detected in association with ER or ER-derived membranes[140]. NS5B is an RNA-dependent RNA polymerase, which is the catalytic component of the HCV RNA replication machinery. This enzyme synthesizes RNA using an RNA template. NS5B can initiate RNA synthesis de novo, at least *in vitro*, and it is assumed that de novo initiation is also operating *in vivo*[93]. The crystal structure of the NS5B catalytic domain shows a structural fold comparable with other polymerases with palm, finger, and thumb subdomains[141,142]. The palm domain contains the active site of the enzyme, whereas the fingers and the thumb modulate the interaction with the RNA chain. One structural peculiarity of the enzyme is the fully encircled active site, which is due to multiple interactions between the finger and thumb subdomains creating a tunnel in which a single-stranded RNA molecule is directly guided to the active site. NTPs enter the active site *via* another positively charged tunnel. Binding of the RNA template and initiation of RNA synthesis are supposed to be regulated by a highly flexible beta-hairpin loop located in the thumb domain and pointing toward the active site[126]. As observed for other viral polymerases, NS5B is an interesting and promising target for the development of new antiviral molecules targeting HCV[94].

The RNA-dependent RNA polymerase activity appears to be modulated by interaction with some other viral proteins (NS3 and NS5A)[93]. It has been shown that cyclophilin B, a peptidyl-prolyl cis-trans-isomerase, interacts with the C-terminal region of NS5B and appears to stimulate its RNA binding activity[143]. In addition, cyclosporin A, an inhibitor of cyclophilin B, inhibits HCV replication in cell culture[144]. However, how cyclophilin B activates replication remains to be determined. Furthermore, cyclophilin B does not seem to stimulate the RNA binding activity of NS5B in all genotypes[145]. NS5B has also been shown to interact with other cellular proteins[146-148].

ALTERNATIVE READING FRAME PROTEIN

In addition to the large open reading frame encoding the polyprotein, HCV genome contains an overlapping +1 reading frame that overlaps the sequence of the core protein[4]. This alternative reading frame (ARF) lacks an in-frame AUG start codon, suggesting that its expression involves unusual translation-level events. *In vitro* studies indicate that ribosomal frameshifting may the process leading to translation of the ARF. Frameshifting yields chimeric proteins that have segments encoded in the core sequence covalently attached to amino acids encoded in the ARF. Based on experiments with reporter gene constructs, the frameshift efficiency is in the range of 1% to 2%. The development of an immune response against the ARF protein in HCV infected patients indicates that this protein is expressed during natural HCV infections and stimulates specific immune responses[149]. The role of ARF protein in the HCV life cycle and/or pathogenesis is not yet known. However, the ARF protein is not required for HCV RNA replication. One cannot exclude that the ARF protein may be responsible for some of the effects attributed to the core protein. Indeed, most studies seeking to define the function of the core protein have used sequences likely to contain a combination of the core protein and ARF protein. Due to the lack of knowledge of its function, the ARF protein is not currently considered as a target for the development of new antiviral molecules.

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