Hepatitis C Virus Proteins: From Structure to Function

Darius Moradpour and François Penin

Abstract Great progress has been made over the past years in elucidating the structure and function of the hepatitis C virus (HCV) proteins, most of which are now actively being pursued as antiviral targets. The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins include the p7 viroporin, the NS2 protease, the NS3-4A complex harboring protease and NTPase/RNA helicase activities, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase. NS4B is a master organizer of replication complex formation while NS5A is a zinc-containing phosphoprotein involved in the regulation of HCV RNA replication versus particle production. Core to NS2 make up the assembly module while NS3 to NS5B represent the replication module (replicase). However, HCV proteins exert multiple functions during the viral life cycle, and these may be governed by different structural conformations and/or interactions with viral and/or cellular partners. Remarkably, each viral protein is anchored to intracellular membranes via specific determinants that are essential to protein function in the cell. This review summarizes current knowledge of the structure and function of the HCV proteins and highlights recent advances in the field.

Abbreviations

aa	Amino acid
AH	Amphipathic α-helix
CHV	Canine hepacivirus
CK	Casein kinase
CsA	Cyclosporin A

D. Moradpour (✉)
Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, 1011 Lausanne, Switzerland
e-mail: Darius.Moradpour@chuv.ch

F. Penin (✉)
Institut de Biologie et Chimie des Protéines, Bases Moléculaires et Structurales des Systèmes Infectieux, UMR 5086, CNRS, University of Lyon, 69367 Lyon, France
e-mail: f.penin@ibcp.fr

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CypA	Cyclophilin A
DHPC	1,2-diheptanol-sn-glycero-3-phosphocholine
E	Envelope glycoprotein
ER	Endoplasmic reticulum
GBV-B	GB virus B
HCV	Hepatitis C virus
HVR	Hypervariable region
igVR	Intergenotypic variable domain
IRES	Internal ribosome entry site
ISDR	Interferon sensitivity determining region
LCS	Low complexity sequence
LD	Lipid droplet
MD	Molecular dynamics
NCR	Noncoding region
NMR	Nuclear magnetic resonance
NS	Nonstructural protein
ORF	Open reading frame
PI4KIII	Phosphatidylinositol 4-kinase III
PI4P	Phosphatidylinositol 4-phosphate
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
RdRp	RNA-dependent RNA polymerase
SPP	Signal peptide peptidase
TMD	Transmembrane domain

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1 Introduction

Hepatitis C virus (HCV) infection is now the leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma in many parts of the world, with a peak of the disease burden expected around 2020 (Nature Outlook 2011). On this

background, great progress has been made in elucidating the structure and function of the HCV proteins, most of which are now actively being pursued as antiviral targets (Moradpour et al. 2007; Lindenbach et al. 2013).

HCV as well as GB virus B (GBV-B) and canine hepacivirus (CHV) have been classified in the *Hepacivirus* genus within the *Flaviviridae* family, which also includes the genera *Flavivirus* (e.g., yellow fever virus and dengue virus), *Pestivirus* (e.g., bovine viral diarrhea virus), and *Pegivirus* (e.g., human pegivirus, also known as GB virus C) (Kapoor et al. 2011 and references therein) (see also chapter "The Origin of Hepatitis C Virus" by Simmonds, this volume). HCV contains a 9.6 kb positive-strand RNA genome composed of a 5′ noncoding region (NCR), which includes an internal ribosome entry site (IRES), an open reading frame (ORF) that encodes the structural and nonstructural proteins, and a 3′ NCR (Fig. 1). The structural proteins, which form the viral particle, include the core protein and the envelope glycoproteins E1 and E2. The nonstructural proteins include the p7 viroporin, the NS2 protease, the NS3-4A complex harboring protease and NTPase/RNA helicase activities, the NS4B and NS5A proteins, and the NS5B RNA-dependent RNA polymerase (RdRp).

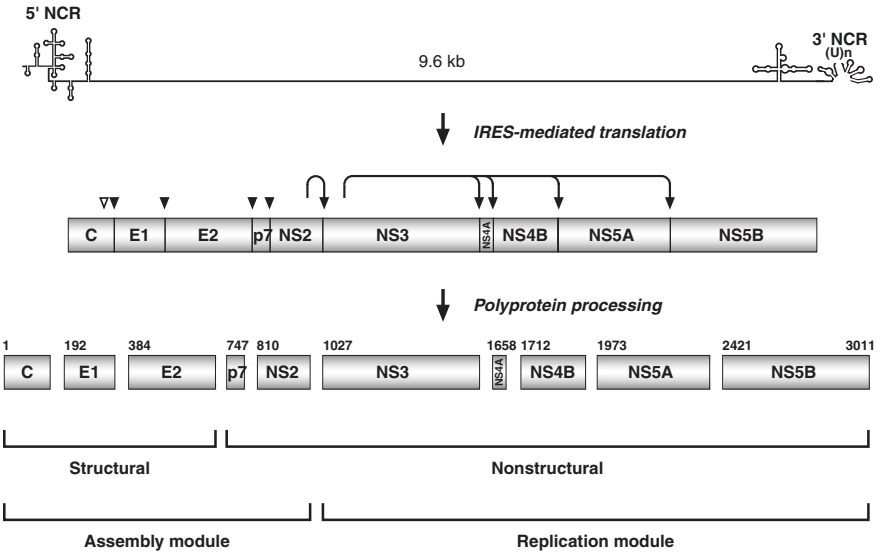
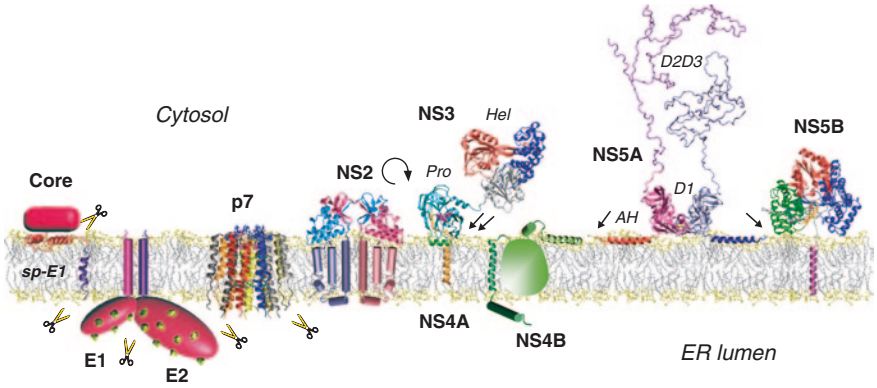


Fig. 1 Genetic organization and polyprotein processing of HCV. The 9.6 kb positive-strand RNA genome is schematically depicted at the *top*. Simplified RNA secondary structures in the 5′ and 3′ noncoding regions (*NCRs*) as well as the core and NS5B coding regions are shown. Internal ribosome entry site (*IRES*)-mediated translation yields a polyprotein precursor that is processed into the mature structural and nonstructural proteins. Amino acid numbers are shown above each protein (HCV H strain; genotype 1a; GenBank accession number AF009606). *Solid arrowheads* denote cleavages by the endoplasmic reticulum signal peptidase. The *open arrowhead* indicates further C-terminal processing of the core protein by signal peptide peptidase. *Arrows* indicate cleavages by the HCV NS2 and NS3-4A proteases. Note that polyprotein processing, illustrated here as a separate step for simplicity, occurs co- and posttranslationally. Also note that all components of the replication module (replicase) are also involved in assembly



IRES-mediated translation of the HCV ORF (see also chapter "Hepatitis C Virus RNA Translation" by Niepmann, this volume) yields a polyprotein precursor that is co- and posttranslationally processed by cellular and viral proteases into the mature structural and nonstructural proteins (Fig. 1). The structural proteins and the p7 polypeptide are processed by the endoplasmic reticulum (ER) signal peptidase and signal peptide peptidase (SPP) while the nonstructural proteins are processed by the viral NS2 and NS3-4A proteases. The nonstructural proteins are cleaved in a preferential order (Pietschmann et al. 2001 and references therein). The first cleavages by the viral proteases occur cotranslationally at the NS2/NS3 and NS3/NS4A sites and liberate NS3 from the remainder of the polyprotein. Subsequent NS3-4A protease-mediated processing events occur *in trans*, with rapid processing at the NS5A/NS5B site to produce an NS4A-5A intermediate, followed by cleavage between NS4A and NS4B, to produce a relatively stable NS4B-5A precursor, and subsequently between NS4B and NS5A. These kinetics appear to be important, and some precursors may have time-dependent functions (Herod et al. 2012 and references therein).

From a functional point of view, HCV proteins can be divided into an assembly module (core-NS2) and a replication module (NS3-NS5B, making up the replicase) (Fig. 1). However, it has become apparent that each of the replicase proteins is also involved in HCV assembly, although the precise mechanisms are elusive (Appel et al. 2008; Ma et al. 2008; Tellinghuisen et al. 2008; Jones et al. 2009; Gouklani et al. 2012; reviewed in Murray et al. 2008). Current evidence indicates that NS5A might function as a "molecular switch" between RNA replication and assembly, possibly by tethering the viral RNA to membranes and/or by providing a physical link between replication complexes and viral assembly sites on lipid droplets (LD) or the ER-LD interface and/or by engaging in interactions with host factors involved in replication and assembly (see below). However, the mechanisms governing HCV RNA translation and replication versus the assembly and release of newly formed viral particles are still poorly understood (see also chapter "Virion Assembly and Release" by Lindenbach, this volume).

◀ **Fig. 2** Structure and membrane association of HCV proteins. Scissors indicate cleavages by the endoplasmic reticulum (*ER*) signal peptidase, except on the cytosolic side where it indicates the processing of core by signal peptide peptidase (*SPP*). The *cyclic arrow* denotes cleavage by the NS2 protease. *Black arrows* indicate processing by the NS3-4A protease. Known protein structures are shown as ribbon diagrams. The structures and the membrane bilayer are shown at the same scale. Proteins or protein segments of unresolved structure are represented as colored *spheres* or *cylinders* with their approximate sizes. From *left* to *right* are shown: (1) Core protein (*red*) includes the N-terminal natively unfolded domain (D1) and two amphipathic α-helices connected by a hydrophobic loop (D2 domain; Boulant et al. 2006) as well as the core-E1 signal peptide (PDB entry 2KQI) (Oehler et al. 2012) cleaved by SPP. (2) E1-E2 glycoprotein heterodimer associated by the C-terminal transmembrane domains. *Green spots* denote glycosylation of the envelope proteins. (3) Oligomeric model of p7 based on the structure of the monomer solved by nuclear magnetic resonance (Montserret et al. 2010) and molecular dynamics simulations in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) phospholipid bilayer (Chandler et al. 2012). (4) NS2 catalytic domain (dimer subunits in *blue* and *magenta*; PDB entry 2HD0; Lorenz et al. 2006) connected to their N-terminal membrane domains constituted of three putative transmembrane segments (PDB entries 2JY0, 2KWT, and 2KWZ; Jirasko et al. 2008, 2010). The active site residues His 143, Glu 163, and Cys 184 are represented as spheres. (5) NS3 serine protease domain (*cyan*) associated with the central protease activation and the N-terminal transmembrane domains of NS4A (*yellow*). The catalytic triad of the NS3 serine protease (His 57, Asp 81, and Ser 139) is represented as *spheres* (*magenta*). NS3 helicase domains 1, 2, and 3 are colored in *silver*, *red* and *blue*, respectively. This representation of NS3 (derived from PDB entry 1CU1; Yao et al. 1999) indicates that the helicase domain can no longer interact with the NS3 protease domain when the latter is associated with the membrane through its amphipathic α-helix 11–21 (*green*) and the transmembrane domain of NS4A (BMRB entry 15580; Brass et al. 2008). (6) NS4B with the N-terminal part, including two amphipathic α-helices of which the second has the potential to traverse the membrane bilayer (PDB entry 2KDR; Gouttenoire et al. 2009a), the central part harboring multiple predicted transmembrane segments, and the C-terminal cytosolic part, including a predicted highly conserved α-helix and an amphipathic α-helix interacting in-plane with the membrane (PDB entry 2JXF; Gouttenoire et al. 2009b). (7) NS5A domain 1 dimer (D1; PDB entry 1ZH1 (Tellinghuisen et al. 2005); subunits colored in *magenta* and ice *blue*) as well as intrinsically unfolded domains 2 and 3 (D2D3; Liang et al. 2007; Hanoulle et al. 2009; Verdegem et al. 2011). The N-terminal amphipathic α-helix in-plane membrane anchor (PDB entry 1R7E; Penin et al. 2004; helices colored in *red* and *blue*) are shown in relative position to the phospholipid membrane (adapted from Tellinghuisen et al. 2005). (8) NS5B RNA-dependent RNA polymerase (RdRp) catalytic domain (PDB entry 1GX6; Bressanelli et al. 2002) associated with the membrane via its C-terminal transmembrane segment (F.P. et al. unpublished data). The fingers, palm, and thumb subdomains of the catalytic domain are colored *blue*, *red*, and *green*, respectively. The catalytic site of the RdRp lies within the *center* of the cytosolic domain and the RNA template-binding cleft is located vertically on the *right* along the thumb subdomain β-flap (*orange*) and the C-terminal part of the linker segment (*silver*), connecting the cytosolic domain to the transmembrane segment (*magenta*). The membrane is represented as a simulated model of a POPC bilayer (obtained from Tieleman, http://moose.bio.ucalgary.ca/). Polar heads and hydrophobic tails of phospholipids (stick structure) are colored *light yellow* and *light gray*, respectively. The positions of the NS5A in-plane membrane helices at the membrane interface as well as that of the transmembrane domain of NS5B were deduced from molecular dynamics simulations in POPC bilayer (F.P., D.M. et al. unpublished data). The positioning of the NS3-4A membrane segments and of the amphipathic α-helices in core and NS4B are tentative. The figure was generated from the structure coordinates deposited in the PDB using Visual Molecular Dynamics (*VMD*) (http://www.ks.uiuc.edu/Research/vmd/) and rendered with POV-Ray (http://www.povray.org/)

An important aspect of the HCV proteins is their multifunctionality, as exemplified by the multiple functions of the NS3-4A complex in the replication and pathogenesis of HCV (see below) or the involvement of replicase components in viral assembly. The diverse functions of HCV proteins may be governed by different structural conformations and/or interactions with viral and/or cellular partners (de Chassey et al. 2008). In addition, it is likely that some or most nonstructural proteins exert "structural" functions beyond their enzymatic activities, forming a specific microenvironment through protein cluster, array, or lattice formation on intracellular membranes. Indeed, as all positive-strand RNA viruses investigated thus far, HCV replicates its genome in a membrane-associated replication complex composed of viral proteins, replicating RNA, rearranged intracellular membranes, and additional host factors (Moradpour et al. 2007; Miller and Krijnse-Locker 2008; den Boon et al. 2010). In fact, each viral protein is anchored to intracellular membranes via specific determinants that are essential to protein function in the cell (Fig. 2).

In the following, we will summarize the current knowledge of the structure and function of the HCV proteins (Table 1) and highlight recent advances in the field.

2 Structural Proteins

2.1 Core

The first structural protein encoded by the HCV ORF is the core protein, which forms the viral nucleocapsid. An internal signal sequence located between core and E1 targets the nascent polypeptide to the translocon of the ER membrane, followed by translocation of the E1 ectodomain into the ER lumen (Santolini et al. 1994). Of note, the signal sequence at the C terminus of core is the only true signal sequence in the HCV polyprotein, while other transmembrane segments may act as stop transfer sequences or reinitiation of translocation signals. Cleavage of the core-E1 signal sequence by signal peptidase yields an immature 191-amino-acid (aa) core protein. Further C-terminal processing by the intramembrane cleaving protease SPP yields the mature 21 kDa core protein of ~177 aa (McLauchlan et al. 2002; Okamoto et al. 2008; Oehler et al. 2012 and references therein). Mutagenesis and *trans*-complementation studies suggest that at least 177 residues of core protein are needed for infectious particle production (Kopp et al. 2010). However, the precise C terminus of mature core has not yet been identified unequivocally. The recently determined nuclear magnetic resonance (NMR) structure of a synthetic core-E1 signal peptide provides the structural basis for further studies on the mechanism of SPP cleavage and its role in HCV infection (Oehler et al. 2012).

Mature core is a dimeric membrane protein stabilized through disulfide bond formation at Cys 128 (Boulant et al. 2005; Kushima et al. 2010). However, the signals and processes that mediate RNA packaging and the assembly of core into nucleocapsids are largely unknown.

Table 1 Structure and function of hepatitis C virus proteins

Protein	aa	MW (kDa)	Structure	Function
C	191	21	N-terminal basic, RNA-binding domain 1 (aa 1-117); hydrophobic, lipid droplet-binding domain 2 (aa 118 to ~177, harboring 2 amphipathic α-helices connected by a hydrophobic loop); C-terminal signal sequence. Maturation via signal peptide peptidase-mediated removal of the C-terminal signal sequence. Dimeric protein (stabilized by disulfide bond formation through Cys 128)	Nucleocapsid formation
E1	192	35	Highly glycosylated (up to 6 glycosylation sites), 4 potential disulfide bonds, C-terminal transmembrane domain	Envelope glycoprotein. Heterodimer formation with E2
E2	363	70	Highly glycosylated (up to 11 glycosylation sites), 9 potential disulfide bonds, C-terminal transmembrane domain. Hypervariable region 1 (aa 1–28). Binds directly to CD81 and scavenger receptor BI	Envelope glycoprotein. Heterodimer formation with E1
p7	67	7	Two transmembrane α-helices connected by a cytoplasmic loop. Forms oligomers (hexamer and heptamer)	Viroporin
NS2	217	23	N-terminal membrane domain with 3 predicted transmembrane passages. C-terminal catalytic domain (aa 94–217) forms a dimeric cysteine protease with two composite active sites	Membrane-associated dimeric cysteine protease
NS3	631	70	N-terminal serine protease domain (aa 1–180) with a chymotrypsin-like fold with two β-barrel subdomains. Includes a structural zinc binding site. NTPase/RNA helicase domain (aa 181–631) with 3 subdomains. Membrane association through helix α_0 and N-terminal transmembrane segment of NS4A	Serine protease and NTPase/RNA helicase activities. Forms a non-covalent complex with NS4A
NS4A	54	8	N-terminal transmembrane α-helix (aa 1–21); central part (aa 21–32) forms a β-sheet as part of the N-terminal β-barrel of NS3; C-terminal acidic portion (aa 40–54) interacts with other replicase components, including NS3 NTPase/RNA helicase domain	Cofactor for NS3
NS4B	261	27	Integral membrane protein comprising an N-terminal portion (aa 1 to ~69, including amphipathic α-helices AH1 and AH2, extending from aa 3–35 and aa 42–66, respectively), a central part harboring four predicted transmembrane passages (aa ~70 to ~190), and a C-terminal portion (aa ~191–261, including amiphipathic α-helices H1 and H2, extending from aa 201–213 and 229–253, respectively)	Induction of the membranous web. NTPase activity? RNA binding?

(continued)

Table 1 (continued)

Protein	aa	MW (kDa)	Structure	Function
NS5A	447[1]	56–58	N-terminal amphipathic α-helix as membrane anchor (aa 1-31); domain 1 (aa 36–213) includes a zinc binding site and forms either a 'claw-like' dimer with a basic groove or a side-by-side dimer; both natively unfolded domains 2 (aa 250–352) and 3 (aa 356–447) exhibit intrinsic α-helical propensity	Serine phosphoprotein; RNA binding; phosphorylation-dependent modulation of RNA replication (domains 1 and 2) and viral assembly (domain 3)
NS5B	591	68	Fingers, palm, and thumb subdomains. Interactions between fingers and thumb subdomains result in encircled active site. Membrane association mediated by C-terminal transmembrane α-helix (tail-anchored protein)	RNA-dependent RNA polymerase

[1]NS5A from genotype 2 isolates contains a 20-aa insertion in domain 3, close to the C terminus

HCV core is composed of two domains (Boulant et al. 2005) (Fig. 3). The N-terminal hydrophilic domain 1 (D1, aa 1–117) contains a high proportion of basic aa residues and has been implicated both in RNA binding and homooligomerization, thereby promoting nucleocapsid assembly. As a general feature of nucleocapsid proteins, core D1 exhibits RNA chaperone activity likely required for the structural remodeling and packaging of the RNA genome in the viral particle (Cristofari et al. 2004). D1 is also involved in interactions with numerous cellular factors (de Chassey et al. 2008) and may thereby contribute to alterations of host cell functions upon HCV infection. Core D1 behaves as an intrinsically unstructured protein when isolated (Boulant et al. 2005), but it includes several potential α-helices, notably a helix-loop-helix motif involving aa 15–41, constituting an immunodominant antigenic site (Jolivet et al. 1997) and harboring residues critical for core function (Angus et al. 2012). Moreover, D1 folds upon interaction with the C-terminal hydrophobic domain 2 (D2, aa 118 to ~177) (Boulant et al. 2005), which mediates association with LDs (Boulant et al. 2006). D2 consists of a central hydrophobic loop connecting two amphipathic α-helices (comprising aa 119–136 and 148–165) interacting in-plane with the LD phospholipid interface (Boulant et al. 2006).

Recent fluorescent tagging and live cell imaging studies have provided elegant insights into the trafficking of functional core to putative assembly sites and the interaction with viral nonstructural proteins (Counihan et al. 2011; Coller et al. 2012). The association of core with LDs (Boulant et al. 2007; Miyanari et al. 2007; Shavinskaya et al. 2007) and interaction with NS5A (Masaki et al. 2008) play central roles in nucleocapsid assembly (see also chapter "Virion Assembly and Release" by Lindenbach, this volume). NS5A is indeed thought to deliver the HCV genome RNA to core protein (see below), and as its membrane anchor is able to associate with the phospholipid monolayer of LDs, RNA transfer likely occurs on LDs or the ER-LD interface. Because of the particular features of the core membrane-binding domain, the resulting RNA-containing nucleocapsid is expected to be surrounded by a lipid monolayer, allowing its full immersion into the hydrophobic core of LDs and, ultimately, of lipo-viro-particles (reviewed in Bartenschlager et al. 2011).

Additional translation products from an alternative reading fame overlapping the core coding region (alternative reading frame protein, F protein, core +1; reviewed in Branch et al. 2005) as well as a family of minicores ranging in size from 8 to 14 kDa have been detected (Eng et al. 2009), but the role of these proteins, if any, in the life cycle and pathogenesis of HCV remains to be elucidated.

2.2 Envelope Glycoproteins

The envelope glycoproteins E1 and E2 play pivotal roles at different steps of the HCV life cycle, including the assembly of the infectious particle, virus entry, and fusion with the endosomal membrane (see also chapter by "Hepatitis C Virus Entry" Zeisel et al., this volume). To fulfill these functions, E1 and E2 likely adopt

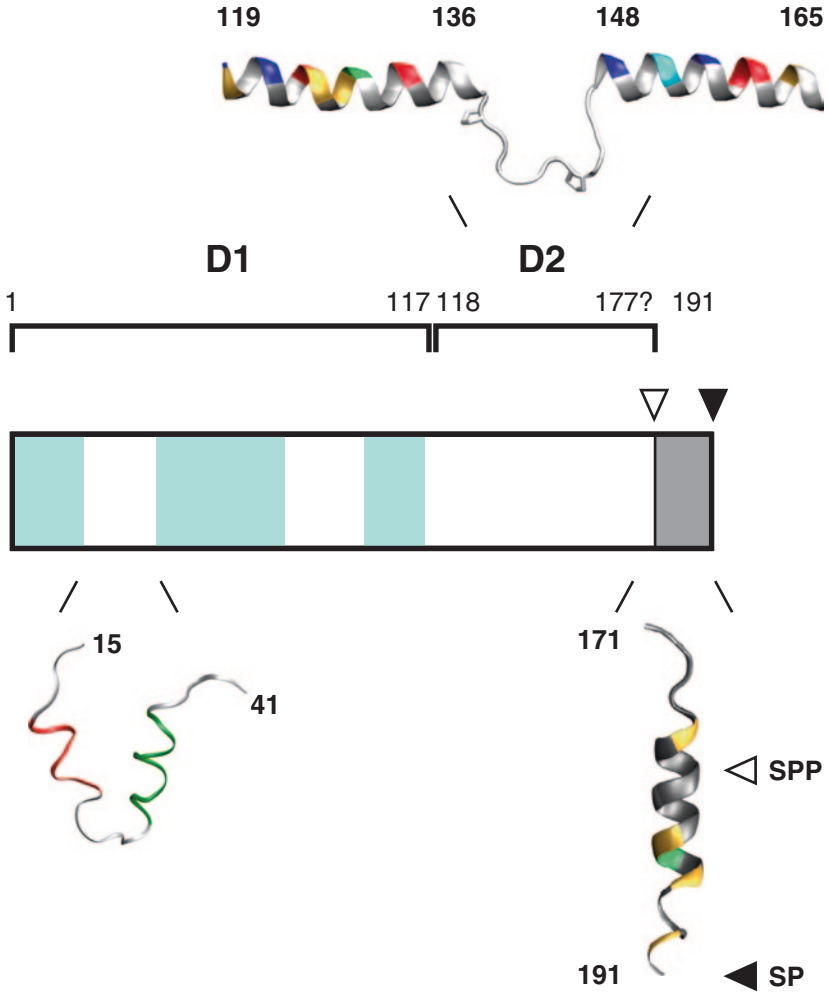


Fig. 3 Schematic representation of the HCV core protein. Core protein is composed of two domains (Boulant et al. 2005). The N-terminal hydrophilic domain 1 (D1, aa 1–117) contains three hydrophilic, highly basic segments (*blue boxes*) separated by two more hydrophobic segments. D1 is an intrinsically unstructured protein when isolated but includes several potential α-helices, notably a helix-loop-helix motif involving aa 15–41 (ribbon representation (PDB entry 1CWX; Jolivet et al. 1997; Angus et al. 2012). The α-helices 1 (aa 19–24) and 2 (aa 30–37) are *red* and *green*, respectively. The C-terminal hydrophobic domain 2 (D2, aa 118 to ~177) consists of a central hydrophobic loop connecting two amphipathic α-helices (comprising aa 119–136 and 148–165; ribbon representation; Boulant et al. 2006). At the C terminus of core is the core-E1 signal peptide (PDB entry 2KQI; Oehler et al. 2012), which is cleaved by signal peptidase (SP; after aa 191; *solid arrowhead*) and signal peptide peptidase (SPP; after aa ~177; *open arrowhead*) to yield mature core protein. Hydrophobic residues are *gray*, neutral residues (Gly and Ala) are *light gray*, and hydrophilic residues are colored accordingly to their physico-chemical properties: Ser, Thr, Asn, and Gln in *yellow*; Asp and Glu in *red*; Arg and Lys in *blue*; His in *cyan*; and Cys in *green*

markedly different conformations that should be tightly controlled to occur at the appropriate moment during the viral life cycle.

E1 and E2 are type I transmembrane proteins with an N-terminal ectodomain (~160 and ~360 aa for E1 and E2, respectively) and a short C-terminal transmembrane domain (TMD) of ~30 aa. During their synthesis, the E1 and E2 ectodomains are translocated into the ER lumen and their TMDs are inserted into the ER membrane. Both TMDs are composed of two stretches of hydrophobic aa separated by a short polar segment containing fully conserved charged residues. The second hydrophobic stretches function as reinitiation of translocation signals for the downstream protein. Before signal sequence cleavage, both the E1 and E2 TMDs form a hairpin structure. After signal peptidase cleavage, the signal sequence is reoriented toward the cytosol, resulting in a single transmembrane passage (Cocquerel et al. 2002).

The TMDs contribute importantly to the functions of E1 and E2, including membrane anchoring, ER retention, and E1–E2 noncovalent heterodimer formation, which is believed to represent the building block for the viral envelope (reviewed in Voisset and Dubuisson 2004). However, it was shown recently that virion-associated E1 and E2 form large covalent complexes stabilized by disulfide bonds (Vieyres et al. 2010). The presence of disulfide bonds between the envelope glycoproteins suggests that lateral protein–protein interactions might play an active role in the budding of HCV. The ectodomains of E1 and E2 indeed contain numerous highly conserved cysteine residues that may form 4 and 9 disulfide bonds, respectively. In addition, E1 and E2 contain up to 6 and 11 glycosylation sites, respectively. Thus, E1 and E2 maturation and folding is a complex and interdependent process that involves the ER chaperone machinery and disulfide bond formation as well as glycosylation.

Secondary and tertiary structures of the glycoproteins are supposed to be similar among members of the *Flaviviridae* family, suggesting that the HCV envelope glycoproteins belong to the class II fusion proteins (reviewed in Kielian and Rey 2006). Based on disulfide mapping studies and modeling on the known flavivirus glycoprotein E structure, a model of the structural organization of the E2 ectodomain was recently proposed (Krey et al. 2010). This model comprises three domains: Domain 1 (D1) is predicted to contain eight β-strands, is extended at the N terminus by hypervariable region 1 (HVR1), and harbors the majority of known determinants for CD81 interaction. Domain 2 (D2) includes HVR2, and its most conserved part is suggested to act as a fusion loop (aa 119–137). D1 is connected to domain 3 (D3) by a linker region called the intergenotypic variable region (igVR). D3, which includes part of the CD81-binding domain, is connected to the TMD by a flexible stem region. Remarkably, both D2 and D3 appear to be largely intrinsically unstructured, contrasting with the classical view of well-structured viral envelope proteins reported so far. Similar to other intrinsically unstructured proteins, which are expected to adopt different conformations depending on their interaction with specific biological partners, this feature is likely essential for the interaction with E1 as well as the intramolecular interactions required for receptor engagement and membrane fusion. Determination of

the three-dimensional structures of the E1 and E2 ectodomains in association with their biological partners would be key in elucidating the receptor binding and fusion processes mediated by these proteins.

Recent work identified a segment (aa 322–332) in the stem region of E2 (aa 279-331) which is essential for virus entry and harbors a central amphipathic α-helix (see also chapter "Hepatitis C Virus Entry" Zeisel et al., this volume). Due to its location in the stem region, segment 322–332 is likely involved in the reorganization of the glycoprotein complexes taking place during the fusion process (Albecka et al. 2011).

The genes encoding E1 and E2 are particularly variable, and several HVRs have been identified in E2, differing by up to 80 % among HCV genotypes. HVR1 comprises the first 27 aa of E2 and is an immunodominant region that elicits type-specific neutralizing antibodies. Interestingly, despite high variability at the sequence level, HVR1 is a globally basic region with positively charged residues located at specific sequence positions, and the structural properties of this intrinsically unstructured region were found to be quite conserved (Penin et al. 2001). HVR2 (aa 91–97) shows up to 100 % sequence diversity and forms a loop-like structure via flanking cysteine residues within domain 2. The igVR (aa 187–197) varies widely between genotypes but contains a single conserved N-linked sugar moiety and, similar to HVR2, likely forms a disulfide-constrained loop. In contrast to HVR1, HVR2 and igVR are not known targets of the humoral immune response but are essential for structural integrity and function of the HCV glycoprotein heterodimer and may modulate E2 receptor binding (McCaffrey et al. 2011).

3 Nonstructural Proteins

3.1 p7

p7 is a 63-aa integral membrane polypeptide comprising two transmembrane α-helices connected by a positively charged cytosolic loop, while the N and C termini are oriented toward the ER lumen (Carrère-Kremer et al. 2002). It forms hexamers or heptamers with cation channel activity and facilitates virus production, indicating that it belongs to the viroporin family (Clarke et al. 2006; Luik et al. 2009; Chandler et al. 2012 and references therein).

p7 is not required for RNA replication in vitro but is essential for the assembly and release of infectious HCV in vitro as well as productive infection in vivo (reviewed in Steinmann and Pietschmann 2010) (see also chapter "Virion Assembly and Release" by Lindenbach, this volume). However, its precise function is unknown and the role of p7 ion channel activity in viral assembly and release has yet to be elucidated. One possibility is that p7 acts by suppressing acidification of cellular organelles, thereby protecting nascent virions from premature acid-induced conformational changes (Wozniak et al. 2010). Furthermore, there is a growing body of evidence suggesting that p7 is critical for other functions in virus assembly and egress unrelated to its

channel activity (reviewed in Steinmann and Pietschmann 2010). As discussed above and below, p7 likely acts in concert with core, E1 and E2, as well as NS2, especially the first transmembrane segment of NS2, to exert its function(s) (Jirasko et al. 2010; Boson et al. 2011; Ma et al. 2011; Popescu et al. 2011; Stapleford and Lindenbach 2011).

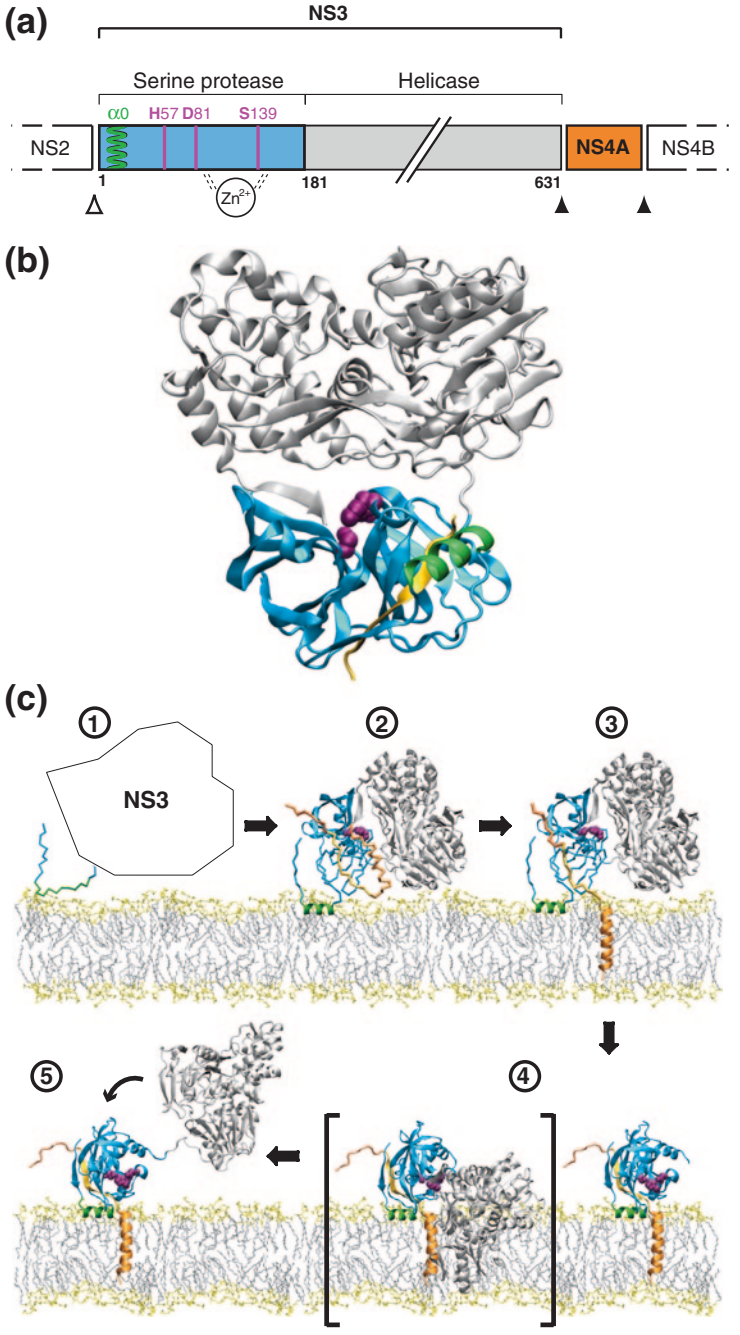
Combined NMR experiments and molecular dynamics (MD) simulations allowed the identification of the secondary structure elements of p7 and the construction of a three-dimensional model of the monomer in a phospholipid bilayer (Montserret et al. 2010). This model revealed an N-terminal α-helix (aa 2–16), a turn involving fully conserved Gly 18, transmembrane α-helix 1 (aa 19–33), a cytosolic loop harboring two fully conserved basic residues at positions 33 and 35, and transmembrane α-helix 2 (aa 40–56), which is slightly bent due to Pro 49, and an unstructured seven-residue C-terminal segment.

The three-dimensional structure of a hexameric p7 channel in a 1,2-diheptanol-sn-glycero-3-phosphocholine (DHPC) short-tail (C7) phospholipid bilayer was solved at 16 Å resolution by single-particle electron microscopy, revealing a highly tilted, flower-shaped protein architecture with six protruding petals oriented toward the ER lumen (Luik et al. 2009). In the modelled structure, the first transmembrane α-helix lines the pore and the N and C termini occupy the "petal tips". More recent modeling of p7 oligomers in a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) long-tail (C16:C18) phospholipid bilayer, reflecting the thickness of the ER membrane, revealed a more cylindrical, upright complex (Chandler et al. 2012). Interestingly, comparative evaluation by MD simulations in short- and long-tail phospholipid bilayers revealed the adaptability of the p7 oligomeric structure to membrane thickness. These simulations suggest that p7 forms structurally plastic, minimalist ion channels, compatible with the coexistence of hexameric and heptameric states.

3.2 NS2

Cleavage of the polyprotein precursor at the NS2/NS3 junction is accomplished by a cysteine protease encoded by NS2 and whose function is strongly enhanced by the N-terminal one-third of NS3 (Schregel et al. 2009 and references therein). The NS2 protease itself is dispensable for RNA replication, but cleavage at the NS2/NS3 junction is essential to liberate fully functional NS3 protein and, hence, promote viral RNA replication. The catalytic activity resides in the C-terminal half of NS2 (aa 94–217, $NS2^{pro}$, with His 143, Glu 163, and Cys 184 representing the catalytic triad) while the N-terminal part represents a membrane domain with three putative transmembrane segments (Jirasko et al. 2008, 2010 as well as references therein).

The crystal structure of $NS2^{pro}$ revealed a dimer with two composite active sites (Lorenz et al. 2006) (Fig. 2). Each active site is composed of residues from the two monomers, i.e., His 143 and Glu 163 are contributed by one monomer and Cys 184 by the other. The membrane domain is believed to comprise 3 transmembrane segments (aa 4–23, 27–49, and 72–94) and a small α-helix (aa 61–70)


(a)
NS3
Serine protease
Helicase
α0
H57 D81
S139
NS2
NS4A
NS4B
1
181
631
Zn2+
(b)
(c)
NS3
1
2
3
4
5

◀ **Fig. 4** Structure and membrane association of the HCV NS3-4A complex. **a** Schematic representation of the NS3-4A region of the HCV polyprotein. The NS3 serine protease and NTPase/RNA helicase domains are depicted in *cyan* and *grey*, respectively. Serine protease active site residues His 57, Asp 81, and Ser 139 are highlighted in *purple* and N-terminal amphipathic helix α_0 in *green*. A Zn^{2+} atom coordinated by three cysteine residues and one histidine stabilizes the serine protease structure. NS4A is shown in *orange*. The *white arrowhead* denotes cleavage by the NS2 protease while the *black arrowheads* illustrate *cis* (NS3/NS4A site) and *trans* (NS4A/NS4B site) cleavages by the NS3-4A protease. **b** Structure of the NS3-4A complex, as resolved for a single chain construct comprising NS3 and the central part of NS4A (Yao et al. 1999). The same colors as in panel A are used to highlight the different elements. **c** Dynamic model for the membrane association and structural organization of NS3-4A. (1) Translation of NS3 occurs at the membrane. (2) Amphipathic helix $\alpha_0$folds upon interaction with the membrane interface, followed by folding of the serine protease and helicase domains. (3) Processing at the NS3/NS4A site and posttranslational insertion of the N-terminal segment of NS4A into the membrane. (4) Complete folding of the protease domain and membrane association through helix α_0 and the N-terminal α-helix of NS4A lock the serine protease in a strictly defined position onto the membrane. (5) At the same time, the helicase domain has to move away from the serine protease domain through a rotation of the linker segment connecting the two domains. Adapted from (Brass et al. 2008), where a video illustrating the different steps can be found, and (Morikawa et al. 2011)

whose atomic structures have been solved by NMR, allowing to propose a topology model of full-length, membrane-associated NS2 (Jirasko et al. 2010).

NS2 plays a central organizing role in HCV infectious virus assembly that is independent of its protease activity, but may involve a complex network of interactions with structural and other nonstructural viral proteins (E1 [mediated by E2], E2, p7, NS3, NS5A) (Jirasko et al. 2010; Boson et al. 2011; Stapleford and Lindenbach 2011; Ma et al. 2011; Popescu et al. 2011 and references therein).

3.3 NS3-4A Complex

NS3-4A is a noncovalent complex made of NS3 and the cofactor NS4A. NS3 is a 70 kDa multifunctional protein, with a serine protease located in the N-terminal one-third (aa 1–180) and an NTPase/RNA helicase in the C-terminal two-thirds (aa 181–631). Both enzyme activities have been well characterized, and high-resolution structures have been solved (Yao et al. 1999) (reviewed in De Francesco and Steinkühler 2000; Raney et al. 2010; Morikawa et al. 2011) (Fig. 4).

The NS3-4A protease adopts a chymotrypsin-like fold with two β-barrel subdomains. The structure is stabilized by a Zn^{2+} ion that is coordinated by Cys 97, Cys 99, Cys 145, and His 149. This Zn^{2+} binding site also plays an important role in facilitating processing of the NS2/NS3 site by the NS2 protease (Schregel et al. 2009). The catalytic triad is formed by His 57, Asp 81, and Ser 139 (Fig. 4). The 54-aa NS4A polypeptide functions as a cofactor for the NS3 serine protease. Its central portion (aa 21–32) comprises a β-strand that is incorporated into the N-terminal β-barrel of NS3. The N-terminal hydrophobic portion of NS4A

(aa 1–21) forms a transmembrane α-helix required for the integral membrane association of the NS3-4A complex (Brass et al. 2008) while the C-terminal acidic portion (aa 40–54) comprises a highly negatively charged α-helix that has been shown to interact with other replicase components and to contribute to HCV RNA replication and virus particle assembly (Lindenbach et al. 2007; Phan et al. 2011).

Determinants of NS3-4A protease substrate specificity include an acidic aa at the P6 position, a P1 cysteine (*trans*-cleavage sites) or threonine (*cis*-cleavage site between NS3 and NS4A), and an aa with a small side chain, i.e., alanine or serine, at the P1′ position (consensus cleavage sequence D/E-X-X–X-X-C/T | S/A–X-X–X). However, the recent identification of cellular substrates of the NS3-4A protease has revealed a much more complex scenario. On the one hand, a vast number of cellular proteins display the consensus cleavage sequence and yet only very few are cleaved by NS3-4A. On the other hand, the cellular substrates identified so far have less canonical cleavage sites (reviewed in Morikawa et al. 2011). Therefore, substrate specificity appears to be conferred by additional mechanisms such as the positioning of the NS3-4A protease active site with respect to the membrane (see below).

The NS3 NTPase/RNA helicase is a member of the superfamily 2 DExH/D-box helicases (reviewed in Raney et al. 2010). It couples ATP hydrolysis to the unwinding of double-stranded RNA or of single-stranded RNA regions with extensive secondary structure. The NS3 helicase is essential for HCV RNA replication and also plays a role in viral particle assembly (reviewed in Murray et al. 2008). However, its precise function(s) in the viral life cycle remain(s) elusive. The NS3 helicase unwinds RNA in an "inchworm" or "ratchet-like" fashion (Gu and Rice 2010 as well as references therein). It is unknown why the serine protease and NTPase/RNA helicase domains are physically linked, but evidence for cross-talk between these two essential enzymatic activities is emerging (Beran et al. 2009 and references therein).

Membrane association and structural organization of the NS3-4A complex are ensured in a sequential manner by two determinants: an in-plane amphipathic α-helix at the N terminus of NS3, designated helix α_0 (aa 12–23), and the N-terminal 21 aa of NS4A, which form a transmembrane α-helix (Brass et al. 2008). As shown in Fig. 4, sequential membrane association by these two determinants plays an active role in the processing and structural organization of NS3-4A and positions the serine protease active site in a strictly defined topology with respect to the membrane (Fig. 4). Moreover, one should postulate a second conformation of the NS3-4A complex, with the protease and helicase domains interacting by contacts different from the ones identified in the *cis*-cleavage structure. Indeed, recent studies performed in the NS2B-3 protease of flaviruses and the NS3-4A protease of HCV are in support of a conformational flexibility of the NS3 protease and helicase domains mediated through the interdomain linker segment (Assenberg et al. 2009; Luo et al. 2010; Ding et al. 2011).

Interestingly, NS3-4A is located not only on membranes of the ER and in replication complexes but also, to a minor extent, on mitochondrial or mitochondria-associated membranes, which are thought to be specialized ER sites in close proximity of mitochondria (Wölk et al. 2000; Horner et al. 2011). These

observations may explain how the NS3-4A protease can cleave and thereby inactivate a mitochondrial host protein, the RIG-I adaptor MAVS (also known as Cardif, IPS-1, and VISA) (Meylan et al. 2005) (see also chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume). The NS3-4A protease also cleaves another crucial adaptor protein in innate immune sensing, the TLR3 adaptor TRIF (Li et al. 2005), as well as T cell protein tyrosine phosphatase (Brenndörfer et al. 2009), a modulator of the epidermal growth factor receptor. Hence, the NS3-4A protease plays essential roles not only in the replication but also in the persistence and pathogenesis of HCV (reviewed in Morikawa et al. 2011).

Two first-generation NS3-4A protease inhibitors, telaprevir and boceprevir, have been approved recently for the treatment of chronic hepatitis C (see also chapter "Treatment of Chronic Hepatitis C: Current and Future" by Pawlotsky, this volume). These directly acting antivirals have to be combined with pegylated interferon-α and ribavirin in order to avoid the rapid selection of antiviral-resistant HCV strains (Halfon and Locarnini 2011). Triple therapy comprising pegylated interferon-α, ribavirin, and telaprevir or boceprevir increases sustained virological response rates to ~70 % and allows shortening treatment duration in ~½ of treatment-naïve patients with HCV genotype 1 infection (Jacobson et al. 2011; Poordad et al. 2011).

3.4 NS4B

NS4B is a relatively poorly characterized, hydrophobic 27 kDa protein of 261 aa (reviewed in Gouttenoire et al. 2010a). It is an integral membrane protein comprising an N-terminal portion (aa 1 to ~69), a central part harboring four predicted transmembrane passages (aa ~70 to ~190), and a C-terminal portion (aa ~191 to 261). The N-terminal portion comprises two amphipathic α-helices, AH1 and AH2, extending from aa 3–35 and 42–66, respectively. AH2 has the potential to traverse the membrane bilayer, likely upon oligomerization (Lundin et al. 2003; Gouttenoire et al. 2009a). Site-directed mutagenesis showed that, similar to other HCV nonstructural protein membrane segments (Moradpour et al. 2004a; Penin et al. 2004; Brass et al. 2008), this segment plays an important role in the assembly of a functional replication complex (Gouttenoire et al. 2009a). Interestingly, transmembrane orientation of the N terminus was found to be reduced by coexpression of the other nonstructural proteins, particularly of NS5A, suggesting that the membrane topology of the N-terminal part of NS4B may be dynamic and modulated by protein–protein interactions within the HCV replication complex (Lundin et al. 2006; Gouttenoire et al. 2009a). The C-terminal part comprises a highly conserved amphipathic α-helix (H1, aa 201–213) and a second, membrane-associated 'twisted' amphipathic α-helix (H2, aa 229–253) (Gouttenoire et al. 2009b) as well as two reported palmitoylation sites at the C terminus (Yu et al. 2006). Therefore, membrane association of HCV NS4B is mediated not only by transmembrane domains in its central part but also by determinants for membrane association in the N- and C-terminal portions (AH2, H2, possibly palmitoylation).

NS4B induces the formation of the membranous web, a specific membrane alteration consisting of locally confined membranous vesicles that serves as a scaffold for the HCV replication complex (Egger et al. 2002; Gosert et al. 2003). NS4B interacts with other viral nonstructural proteins and has been reported to bind viral RNA (Einav et al. 2008). In addition, NS4B was reported to harbor NTPase activity (Einav et al. 2004; Thompson et al. 2009) and has been shown to have a role in viral assembly (Jones et al. 2009). Much work remains to be done to further dissect these multiple functions which may be governed by distinct membrane topologies and/or interactions with other viral as well as cellular proteins. Clearly, obtaining a three-dimensional structure of full-length NS4B will be key to gaining further insight into the functions of NS4B.

Similar to the other HCV nonstructural proteins, NS4B has been reported to form oligomers (Yu et al. 2006; Gouttenoire et al. 2010b). Indeed, cross-linking studies provided evidence for formation of at least trimers and suggested that C-terminal palmitoylation plays an important role in this process (Yu et al. 2006). More recently, fluorescence resonance energy transfer and confirmatory coimmunoprecipitation analyses provided evidence for an oligomerization of NS4B in the membrane environment of intact cells (Gouttenoire et al. 2010b). Several conserved determinants were found to be involved in NS4B oligomerization through homotypic and heterotypic interactions. N-terminal amphipathic α-helix AH2 and C-terminal conserved elements were identified as major determinants for NS4B oligomerization (Gouttenoire et al. 2010b; Paul et al. 2011). Mutations that affected the oligomerization of NS4B disrupted membranous web formation and HCV RNA replication, implying that oligomerization of NS4B is required for the creation of a functional replication complex, likely through the induction of membrane curvature and vesicle formation. Indeed, the inducers of replication complexes of other positive-strand RNA viruses have been shown to exert their function as multimeric complexes (reviewed in den Boon et al. 2010).

Taken together, evidence from biochemical, structural and genetic studies as well as electron microscopy indicates that NS4B is a master organizer of HCV replication complex formation. It is no surprise, therefore, that NS4B is also actively being pursued as antiviral target (Rai and Deval 2011).

3.5 NS5A

NS5A is a 447-aa membrane-associated phosphoprotein that plays an important role in modulating HCV RNA replication and particle formation (see also chapter "Virion Assembly and Release" by Lindenbach, this volume). Comparative sequence analyses and limited proteolysis of recombinant NS5A have defined, in addition to its N-terminal membrane anchor, three domains separated by two low complexity sequences (LCS) (Tellinghuisen et al. 2004) (Fig. 5). Domains 1 (D1, aa 36–213; genotype 1b Con1 strain) and 2 (D2, aa 250–342) are primarily involved in RNA replication whereas domain 3 (D3, aa 356–447) is essential for viral assembly (Appel et al. 2008; Tellinghuisen et al. 2008; Kim et al. 2011). In addition, D1 is

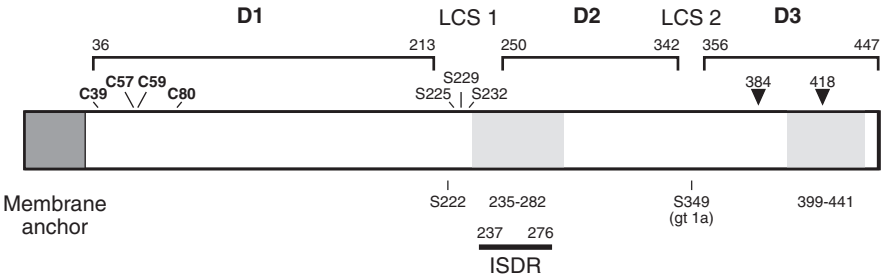


Fig. 5 Schematic representation of HCV NS5A. NS5A is drawn to scale as a *box*. Amino acid positions relate to the HCV Con1 sequence (genotype 1b; GenBank accession number AJ238799; add 1972 amino acids to obtain positions relative to the HCV polyprotein). The domain organization proposed by Tellinghuisen et al. (2004) is shown. Domains 1–3 (D1–D3) are connected by low complexity sequences (*LCS*) 1 and 2. Cysteine residues 39, 57, 59, and 80 coordinate one zinc atom per NS5A protein. The N-terminal amphipathic α-helix, mediating membrane association of NS5A, is highlighted by a *gray box*. Phosphoacceptor sites mapped for genotype 1b (Ser 222; Katze et al. 2000) and genotype 1a HCV isolates (Ser 349; Reed and Rice 1999) are highlighted. Serine residues 225, 229, and 232 that affect hyperphosphorylation of NS5A are also indicated. Two examples each of deletions (Blight et al. 2000; Appel et al. 2005) and *green* fluorescent protein insertions (Moradpour et al. 2004b) found to be tolerated with respect to HCV RNA replication are indicated by *light gray boxes* and *arrowheads*, respectively. ISDR, interferon sensitivity determining region

involved in LD binding (Miyanari et al. 2007) whereas D3 is involved in interaction with the core protein (Masaki et al. 2008). Interestingly, deletions in D2 and D3 are tolerated with respect to RNA replication (Blight et al. 2000; Appel et al. 2005), and viable replicons and viruses harboring GFP insertions in D3 have been established (Moradpour et al. 2004b; Schaller et al. 2007) (Fig. 5).

HCV NS5A can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms. Basal phosphorylation occurs in central and C-terminal parts of NS5A while serine residues 225, 229, and 232 (i.e., Ser 2197, Ser 2201, and Ser 2204 of the genotype 1a strain H polyprotein) in LCS 1 are important for NS5A hyperphosphorylation (Fig. 5). Cell culture-adaptive changes often cluster in the central part of NS5A and target these residues. This observation and the results of studies with kinase inhibitors have led to the assumption that the phosphorylation state of NS5A modulates HCV RNA replication, possibly via regulating interactions with replication—versus assembly-specific host factors (Evans et al. 2004; Neddermann et al. 2004; Appel et al. 2005). The α isoform of casein kinase I (CKIα) (Quintavalle et al. 2007) and casein kinase II (CKII) (Tellinghuisen et al. 2008) can phosphorylate NS5A on serine residues in LCS 1 and on Ser 457 (genotype 2 JFH-1 strain) in D3, respectively. CKII-mediated phosphorylation of Ser 457 (NS5A from the genotype 2 JFH-1 strain is 20 aa longer than that from genotype 1 strains) was found to be essential for assembly of infectious virus particles, at least in the case of the JFH-1 isolate (Tellinghuisen et al. 2008). However, it is likely that additional cellular kinases are involved in generating the different phosphoforms of NS5A (Huang et al. 2007).

NS5A is a monotopic protein anchored to the membrane by an N-terminal amphipathic α-helix embedded in-plane into the cytosolic leaflet of the membrane (Penin et al. 2004 and references therein). This specific feature allows NS5A to associate with a phospholipid monolayer and thus to interact with core on LDs or the LD-ER interface. The crystal structure of D1 immediately following the N-terminal amphipathic α-helix revealed a 'claw-like' dimer with a groove that faces away from the membrane and could accommodate either single- or double-stranded RNA (Tellinghuisen et al. 2005). Each molecule of the dimer coordinates one zinc atom through the four fully conserved cysteine residues, Cys 39, Cys 57, Cys 59, and Cys 80 (Fig. 5). As predicted from the three-dimensional structure, D1 (as well as LCS 1 and part of D2) have been shown to bind RNA as a dimer (Hwang et al. 2010 and references therein). According to one hypothesis, multiple NS5A dimers may form a 'basic railway' on intracellular membranes that would allow tethering as well as sliding of the viral RNA on intracellular membranes and coordination of its different fates during HCV replication (Moradpour et al. 2005). It has been shown that only a small proportion of the HCV nonstructural proteins expressed in cells at a given time are actively engaged in RNA replication (Miyanari et al. 2003; Quinkert et al. 2005). One can easily conceive, therefore, that these proteins may have additional structural functions as arrays or lattices on membranes. The formation of such higher-order structures may explain the extraordinary potency of small molecule inhibitors targeting D1 of NS5A (Gao et al. 2010). Interestingly, a second X-ray structure of NS5A D1 revealed a dimer with a different conformation (Love et al. 2009). It is tempting to speculate that a switch between these alternative conformations may modulate the different roles of NS5A in viral RNA replication and particle assembly.

NS5A D2 (Liang et al. 2007; Hanoulle et al. 2009) and D3 (Verdegem et al. 2011) are natively unfolded monomeric conformers in fast interconversion, with an intrinsic α-helical propensity, suggesting that nascent secondary structures constitute molecular recognition elements and, as such, promote the interaction and stabilization of the conformations with specific viral or host proteins. For example, D2 and D3 were found to interact with and serve as a substrate for cyclophilin A (Hanoulle et al. 2009; Verdegem et al. 2011) (see below).

HCV NS5A has attracted considerable interest because of its potential role in modulating the response to IFN-α therapy (see also chapter "Innate Immune Responses to Hepatitis C Virus" by Schoggins and Rice, this volume). Studies performed in Japan first described a correlation between mutations within a discrete region of NS5A, termed interferon sensitivity determining region (ISDR), and a favorable response to IFN-α therapy (Enomoto et al. 1996). However, this remains a controversial issue. Numerous additional potential functions and interactions have been attributed to NS5A (Macdonald and Harris 2004; de Chassey et al. 2008 and references therein). The remarkably high number of viral and cellular interactants for NS5A is likely due to the natively unfolded nature of D2 and D3. NS5A thus appears to be a hub for protein interactions with high specificities and low affinities. However, only few of these postulated properties of NS5A have been validated in the context of the entire viral life cycle in vitro or of natural HCV infection in vivo.

Interestingly, siRNA screens performed by several independent groups identified phosphatidylinositol 4-kinase IIIα (PI4KIIIα) as an essential host factor involved in HCV RNA replication. Knockdown of PI4KIIIα interferes with membranous web formation and inhibits HCV RNA replication. NS5A interacts with and stimulates PI4KIIIα activity and phosphatidylinositol 4-phosphate (PI4P) production that appears to be essential for HCV replication complex formation (Berger et al. 2011; Lim and Hwang 2011; Reiss et al. 2011 and references therein).

Starting with the observation that cyclosporin A (CsA) inhibits HCV RNA replication in vitro, cyclophilin B was intially identified as target of CsA action (Watashi et al. 2005). More recently, cyclophilin A (CypA) was found to play an essential role in HCV RNA replication and assembly, through interactions with NS5A and eventually also in assembly in an NS2-dependent manner (Ciesek et al. 2009; Kaul et al. 2009; Liu et al. 2009 and references therein). NS5A D2 and D3 were found to interact directly with the active site of CypA, and NMR studies revealed that proline residues in D2 and D3 form a substrate for the peptidyl-prolyl *cis/trans* isomerase activity of CypA (Hanoulle et al. 2009; Verdegem et al. 2011). Moreover, mutations conferring resistance to cyclophilin inhibitors often map to NS5A D2 (Coelmont et al. 2010 and references therein). The exact role of CypA in HCV RNA replication has yet to be defined, although various mechanisms have been proposed, including alterations of NS5A folding that is required to activate the viral replicase (Kaul et al. 2009) and the recruitment of NS5B into the replicase (Liu et al. 2009). Based on the above findings, non-immunosuppressive CsA analogues are currently being developed as antivirals against hepatitis C (Flisiak et al. 2009).

3.6 NS5B

HCV replication proceeds via synthesis of a complementary negative-strand RNA using the genome as a template and the subsequent synthesis of genomic positive-strand RNA from this negative-strand RNA template (see also chapter "Hepatitis C Virus RNA Replication" by Lohmann, this volume). The key enzyme responsible for both of these steps is the NS5B RdRp. This 68 kDa viral enzyme composed of 591 aa has been extensively characterized (Behrens et al. 1996; Lohmann et al. 1997; Lesburg et al. 1999; Simister et al. 2009 and references therein), and NS5B has emerged as a major target for antiviral intervention. The NS5B catalytic domain, formed by the N-terminal 530 aa, contains motifs that are shared by all RdRps, including the hallmark GDD sequence within motif C, and the classical fingers, palm, and thumb subdomain organization of a right hand. A 40-aa linker between the catalytic domain and the C-terminal membrane anchor (aa 570-591, see below) occludes the active site. In vitro, the NS5B catalytic domain is capable of performing copy-back, *de novo* and primer-dependent RNA synthesis, requiring only divalent metals (magnesium or manganese) as cofactors. The catalytic aspartic acids (Asp 220 and Asp 318), located in the palm subdomain, chelate two catalytic metal ions that are responsible

for the polymerization reaction. The single-strand RNA template or primer-template duplex binds in a groove between the fingers and thumb domains that leads directly to the active site. NTPs access this site via a specific tunnel beginning at the backside periphery and extending into the active site located in the palm domain.

A special feature of most HCV RdRp three-dimensional structure models is that extensive interactions between the fingers and thumb subdomains result in a completely encircled active site, with the linker occluding the active site via interaction with the so-called β-flap element of the thumb domain. This likely corresponds to a closed conformation of the enzyme that is adopted for the initiation step of RNA synthesis by a *de novo* mechanism. It was recently reported that the linker is directly involved in the formation of the first dinucleotide primer (Harrus et al. 2010). Transition from this first dinucleotide primer state to the elongation phase of RNA replication requires the removal of the linker and the β-flap element. This is supported by the recently reported co-crystal structure of β-flap-mutated NS5B with primer-template RNA (Mosley et al. 2012). Current structural models do not include an obvious exit path for double-stranded RNA. It is unknown whether the newly synthesized strand emerges base-paired to the template (as double-stranded RNA) or if it is forced to unwind after a few nucleotides in order to leave the active site region.

NS5B is a so-called tail-anchored protein. Posttranslational membrane association is mediated by the C-terminal 21 aa residues, resulting in integral membrane association and cytosolic orientation of the catalytic domain (Schmidt-Mende et al. 2001). The NS5B membrane anchor is dispensable for polymerase activity in vitro but indispensable for RNA replication in cells (Moradpour et al. 2004a). Molecular modeling of membrane-associated NS5B suggests that the RNA-binding groove is stacked onto the membrane interface, thereby preventing access to the RNA template. This inactive form of the RdRp may be activated by a conformational change of the 40-aa linker which connects the catalytic domain and the C-terminal transmembrane segment of NS5B. Such a conformational change would liberate the RNA-binding groove and move the NS5B catalytic domain away from the membrane, while the connecting segment might be involved in binding of viral or cellular factors required for replication complex formation.

4 Conclusions and Perspectives

Much has been learned over the past years about the structure and function of HCV proteins, and most of them are actively being pursued as antiviral targets. However, much work remains to be done with respect to high-resolution structures of full-length membrane proteins, especially core, the envelope glycoproteins, NS2, and NS4B. The study of membrane protein structures still represents a formidable challenge with respect to sample preparation and structural methods. It is likely that novel techniques, such as solid-state NMR, will allow to advance on these important unresolved issues. With respect to the nonstructural proteins, a higher order

structure of the viral replicase will represent an ultimate goal. Such a structure may also reveal insights into the determinants of multifunctionality of the nonstructural proteins, e.g., NS3-4A and NS5A, as well as the different interactions with host factors engaged at a given moment of the HCV life cycle. Ultimately, these efforts should not only yield important new insights into the pathogenesis of hepatitis C, but they should translate into innovative therapeutic and preventive strategies for one of the most common causes of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide.

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