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TOPIC HIGHLIGHT

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Post-translational modifications of hepatitis C viral proteins and their biological significance

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Abstract

Replication of hepatitis C virus (HCV) depends on the interaction of viral proteins with various host cellular proteins and signalling pathways. Similar to cellular proteins, post-translational modifications (PTMs) of HCV proteins are essential for proper protein function and regulation, thus, directly affecting viral life cycle and the generation of infectious virus particles. Cleavage of the HCV polyprotein by cellular and viral proteases into more than 10 proteins represents an early protein modification step after translation of the HCV positive-stranded RNA genome. The key modifications include the regulated intramembranous proteolytic cleavage of core protein, disulfide bond formation of core, glycosylation of HCV envelope proteins E1 and E2, methylation of nonstructural protein 3 (NS3), biotinylation of NS4A, ubiquitination of NS5B and phosphorylation of core and NS5B. Other modifications like ubiquitination of core and palmitoylation of core and NS4B proteins have been reported as well. For some modifications such as phosphorylation of NS3 and NS5A and acetylation of NS3, we have limited understanding of their effects on HCV replication and pathogenesis while the impact of other modifications is far from clear. In this review, we summarize the available information on PTMs of HCV proteins and discuss their relevance to HCV replication and pathogenesis.

Key words: Hepatitis C virus; Hepatitis C virus proteins; Post-translational modifications of proteins; Hepatitis C virus replication; Hepatitis C virus pathogenesis

Core tip: Post-translational modifications (PTMs) are an important step in protein maturation and associated with protein function, activity and/or protein life span. PTMs of viral proteins are often essential for regulation of processes involved in viral infections and can be crucial for infectious virion production. Moreover, identification of PTM sites in viral proteins is particularly useful for the development of antiviral drugs. This overview on PTMs of hepatitis C virus (HCV) proteins discusses how PTMs affect HCV replication and virus-induced pathogenesis.

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INTRODUCTION

Hepatitis C virus (HCV), a member of the genus *Hepacivirus* within the family *Flaviviridae*, is able to establish chronic infection in humans, which eventually leads to liver cirrhosis, hepatocellular carcinoma (HCC) and liver

failure[1,2]. Approximately 3% of the world population are infected with HCV. However, no effective vaccine has been developed and the current antiviral treatments have some limitations[3,4]. In order to develop efficient antiviral therapies, a complete understanding of viral pathogenesis and virus-host interactions is fundamental. Like other positive-stranded RNA viruses, HCV hijacks the host cell's translation machinery for producing viral proteins[5]. Thereby, post-translational modifications (PTMs) of virus encoded proteins occur as a rather natural step during the cell's general protein synthesis process, but concurrently encompass impact on viral replication and infectivity. In this review, we will start with a discussion on the proteolytic cleavage of HCV polyprotein, and give an overview on PTMs of HCV proteins and discuss their influence on viral replication and pathogenesis.

PROTEOLYTIC CLEAVAGE OF HCV POLYPROTEIN

HCV genome consists of a 5'-untranslated region (UTR), a large open reading frame (ORF) encoding a polyprotein precursor of about 3000 amino acids and a 3'-UTR[6]. Proteolytic processing of the HCV polyprotein giving rise to single viral proteins represents an initial step in viral protein modification. There are nine defined proteolytic cleavage sites within the HCV polyprotein precursor, resulting in the generation of at least ten non-overlapping proteins, including structural proteins core, E1 and E2, and nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. Additional viral protein products might be produced by alternative ORFs discovered within the HCV genome[7-10].

Proteolytic processing of the polyprotein precursor occurs co- and post-translationally involving cellular as well as viral proteases[11]. The structural proteins are cleaved off the polyprotein precursor by host cellular signal peptidase (SP) located in the endoplasmic reticulum (ER) of the host cell, while the nonstructural proteins are released from the polyprotein precursor by viral proteases NS2-3 and NS3-4A[9]. The core protein is found to be additionally cleaved inside the ER membrane by host cellular signal peptide peptidase (SPP), thus yielding the mature core variant[12]. This step leads to the release of core from the ER and its trafficking to lipid droplets (LDs) which are believed to serve as a platform for HCV particle assembly[13]. E2, p7 and NS2 are first generated as an E2-p7-NS2 precursor protein. Remarkably, the E2-p7-NS2 precursor is proteolytically processed at the p7-NS2 junction efficiently whereas the E2-p7 junction gets cleaved less frequently, hence resulting in the presence of E2 and p7 proteins as well as the non-cleaved E2-p7 variant in infected cells[14]. The NS2-3 autoprotease cleaves at the NS2-NS3 junction and the NS3-4A protease cleaves at the sites between NS3 and NS4A, NS4A and NS4B, NS4B and NS5A, and NS5A and NS5B. Proteolytic processing by the NS3-4A complex follows a certain order that the cleavage first happens *in cis* at the NS3-NS4A junction, then rapidly *in trans* at NS5A-NS5B followed by proteolysis at NS4A-NS4B, and finally at NS4B-NS5A. NS3 to NS5B mainly function in HCV genome replication[8,15]. Proteolytic processing releases structural and nonstructural HCV viral proteins that take part in different stages of HCV life cycle.

POST-TRANSLATIONAL MODIFICATIONS OF HCV PROTEINS

Core protein

HCV core protein is the most conserved viral protein among different HCV genotypes. It constitutes the viral nucleocapsid that encapsidates the viral RNA genome, and is essential for virus particle assembly[16,17]. HCV core also possesses several regulatory functions, such as cellular transcription, virus-induced transformation, signal transduction, steatosis and HCC. Moreover, core is significantly involved in virus-mediated pathogenesis. It is able to modulate apoptosis and cell growth, but also upregulates reactive oxygen species (ROS) production and has a possible immunoregulatory role[16,18].

The complete core protein is composed of three domains: an N-terminal hydrophilic domain that is essential for RNA binding and homo-oligomerization, a C-terminal hydrophobic domain that associates with LDs and is involved in proper folding, and a hydrophobic signal sequence tail that can target E1 to the ER membrane[16,19-21]. Unlike other HCV proteins, core protein liberation from HCV polyprotein precursor needs sequential proteolytic processing. Following cleavage from HCV polyprotein at the core-E1 junction by host cellular SP, the immature core protein is additionally cut by SPP within its hydrophobic C-terminus to release mature N-terminal amino acids 173-179 core protein and dissociate core from the ER membrane[16,22]. The exact C-terminus of mature core has not been identified yet in mammalian cells, even though it was reported to be Phe^{177} or Leu^{179} in insect cells[23]. This further processing by SPP relies on previously correct cleavage by SP and the sequential processing controls viral protein production rate[24]. Only the mature form of core can attach to LDs and interact with NS5A that transports HCV genome RNA to core[25,26]. Therefore, core maturation by SPP cleavage plays a critical role in virus assembly and regulation of HCV life cycle.

It has been demonstrated that disulfide bonds in nucleocapsid proteins of viruses with icosahedral structure play a role in virus assembly and capsid structure stabilization[27]. Since HCV virion is packaged into a similar spherical structure, its nucleocapsid might resemble the same organization[28]. Mutation analysis discovered that mature core formed a dimeric membrane protein which was linked by disulfide bond at Cys^{128}. This disulfide bond formation stabilizes capsid structure and strengthens the interaction between core and membranes, and is critical for virus assembly and virion production. However, the disulfide bond in core has no effect on HCV RNA rep-

lication, the association with LDs or other functions of core[27]. Because of the low mutation rate of Cys^{128}, drugs targeting Cys^{128} disulfide bond formation may be considered as a candidate to inhibit HCV virion formation.

Phosphorylation is a common type of PTMs, which is also observed in HCV core protein. The phosphorylation of core by protein kinases A (PKA) at Ser^{53} and Ser^{116} and by protein kinases C (PKC) at Ser^{53} and Ser^{99} was reported both *in vitro* and in Huh-7 and HepG2 cells. However, Ser^{99} and Ser^{116} are the major and predominant sites with low phosphorylation efficiency[29,30]. The phosphorylation at these two sites is critical for inhibition of HBV gene expression and replication in Huh-7 cells, but the detailed *trans*-suppression mechanism of HCV core remains unclear. The same study showed that only truncated core is phosphorylated by PKC suggesting structural conformation might be a prerequisite for phosphorylation[30]. The phosphorylation at Ser^{116} by PKA is shown to be responsible for the repressive activity of core on cyclin-dependent kinase inhibitor (CKI) p21 promoter[31]. Reduced p21 may interfere with p53 driven repair mechanism in cell cycle, which may facilitate tumorigenesis. Another role of phosphorylation of core might be involved in modulating nuclear localization of core, although controversial results have been reported[29,30]. Nuclear core is involved in regulating host gene transcription[32].

Moreover, HCV core also undergoes several other types of PTMs. For example, the ubiquitination of core protein by E3 ubiquitin ligase E6AP preferentially at N-terminal lysine residues induces the degradation of core in the cytoplasm by the ubiquitin-proteasome pathway, which could control HCV virion production and have an antiviral effect. The interaction region between core and E6AP is located between amino acids 58 to 71 of the core protein, which are highly conserved in all HCV genotypes[33-35]. Palmitoylation of core at Cys^{172} plays a vital role in targeting the core to smooth ER and ER-associated LDs, but does not affect SPP proteolytic cleavage-induced maturation and LD accumulation. Importantly, it also affects HCV assembly and production[36].

Envelope glycoproteins E1 and E2

HCV envelope glycoproteins E1 and E2 play an important role in virus entry and immune evasion[37]. In infected cells, E1 and E2 are either found as noncovalent heterodimers, which are mainly localized to the ER, or as disulfide-linked aggregates, which were originally thought to represent misfolded protein complexes[38-41]. Heterodimers and oligomers of E1 and E2 are also found in infectious virus particles, whose structure is stabilized by disulfide bonds[38,42,43].

Both E1 and E2 proteins consist of a large N-terminal ectodomain and a C-terminal hydrophobic transmembrane anchor. PTMs of HCV envelope proteins include the attachment of glycans and the formation of disulfide bridges[44,45]. Glycans attached to HCV envelope proteins were shown to modulate virus entry by modifying their receptor binding affinity or fusion activities. They are also involved in protein folding and play a key role in immune evasion by masking potential antigenic sites from binding of neutralizing antibodies[44,46,47]. Because glycosylation sites within HCV glycoproteins are rather highly conserved, glycosylation mutants are considered as immunogens to induce a potent antibody response against HCV[48].

There are four to five N-glycosylation sites in E1 and up to 11 N-glycosylation sites in E2[44,47,49,50]. N-linked glycosylation occurs at asparagine residues and the consensus sequence is Asn-X-Ser/Thr[44,51]. Mass spectrometric analysis of E2 revealed that this protein is mainly modified by high-mannose type oligosaccharides and more complex glycan types are observed for just two glycosylation sites within E2[52]. E1 is believed to be modified only by high-mannose type oligosaccharides since a restricted localization of E1/E2 heterodimers to the ER is confirmed by immunofluorescence[49]. However, more complex type glycosylations generally occur in the cis-Golgi compartment, where indeed a small population of E2 protein has been detected by immunofluorescence[53]. On the other hand, the attachment of complex glycans can happen during the release of viral particles via the exocytotic pathway, which involves the Golgi apparatus. Interestingly, due to the differences in the assembly process, more mature glycoproteins containing complex type glycans could have been observed with HCV pseudoparticles (HCVpp) compared to cell culture-derived HCV particles (HCVcc)[38]. HCVpp is found to assemble in post-Golgi compartments[54], while HCVcc assembly takes place in ER-derived compartments[55]. HCVpp glycoproteins might also be more accessible to Golgi glycosyltransferases than HCVcc glycoproteins, which are components of high-order virion complexes[38]. Differences in the glycosylation pattern of HCVpp and HCVcc might be relevant for studying HCV immune evasion strategies.

Furthermore, the carbohydrate composition of envelope glycoproteins vary to some extent depending on the cell line the virus infected[56]. Changes in the glycosylation pattern of HCV glycoproteins have a major impact on virus particle assembly, entry and immunogenicity[44,50], thus affecting virus pathogenesis and virulence. Mutations of glycosylation sites N1 and N4 in HCV glycoprotein E1 (E1N1, E1N4) as well as N8 and N10 in HCV glycoprotein E2 (E2N8, E2N10) strongly interfere with the incorporation of both envelope proteins into HCVpp, suggesting the importance of these sites for protein folding and E1/E2 heterodimerization[42,44]. Additionally, mutation of glycosylation site E2N2 or E2N4 leads to the decreased infectivity of HCVpp, confirming a role of both glycans in virus entry[44]. Moreover, glycans at positions E2N1, E2N6 and E2N11 are shown to decrease the binding affinity of E2 to the cluster of differentiation 81 (CD81) receptor and to reduce the sensitivity of pseudotyped HCV particles to antibody neutralization, hence contributing to humoral immune evasion[47]. These findings are supported and extended by studies with HCVcc glycosylation mutants[42]. Apparently, glycosylation sites

E2N1, E2N2, E2N4 and E2N6 seem to surround the CD81 receptor binding site within E2, therefore "protecting" this site from recognition by neutralizing antibodies. Helle *et al*[42] provided structural evidence for glycans attached to HCV envelope proteins to modulate the humoral immune response.

Besides N-glycosylation, little information is available on O-glycosylation of HCV E1 or E2. Supposedly, there is one potential O-glycosylation site within E1 and four potential O-glycosylation sites within E2[48]. So far, 3 O-linked glycosylation sites in E2 have been shown to be important for HCV entry, with two of them apparently decreasing E2 binding affinity to CD81 receptor[48].

Virion-associated HCV glycoproteins are assembled into large oligomeric protein complexes which are stabilized by disulfide bonds[38,42]. These complexes are able to bind conformation-sensitive neutralizing antibodies and recombinant CD81[38], and therefore can be considered functionally significant rather than the result of a misfolding event.

Proper folding of glycoprotein E1 is dependent on E2 coexpression and *vice versa*[57,58]. E1 and E2 consist of eight and 18 highly conserved cysteine residues, respectively[43]. Structural information is mainly available for HCV E2 protein, where nine intramolecular disulfide bonds have been identified[59]. Because of the difficulties in expressing E1 in the absence of E2, disulfide arrangement of cysteine residues in E1 has not been determined[43]. Beside their apparent impact on virus particle structure and infectivity, it is conceivable that disulfide-linked glycoprotein oligomers may play an active role in HCV budding by assisting protein-protein interactions[38]. Furthermore, it is possible that the presence of disulfide bridges in HCV envelope proteins could be responsible for the lack of sensitivity of HCVcc to low-pH treatment[60]. This suggests their direct influence on virus internalization by affecting the presentation of HCV fusion peptide[38]. Additionally, the impact of disulfide rearrangement and the oxidation state of cysteine residues in E1 and E2 glycoproteins on HCV entry and membrane fusion was confirmed by Fraser *et al*[43]. Here the presence of free thiol groups has been shown to be essential for HCV infectivity.

Altogether, PTMs of HCV glycoproteins by glycosylation and disulfide bond formation have a strong impact on several steps in viral life cycle, more specifically entry, fusion of viral membrane with the host cell's endosomal membrane and budding.

p7

HCV p7 represents a small integral membrane protein which is able to oligomerize and form proton channels within the HCV particle envelope. The precise role of p7 in HCV life cycle has not been determined, even though it has been shown to be essential for infection, but not for viral replication[61,62]. Due to incomplete or delayed proteolytic processing, the generation of a p7 species linked to the E2 glycoprotein has been observed. The role of E2-p7 precursor during HCV infection is not known so far. However, it is speculated that E2-p7 might be involved in regulating the production of native p7 and formation of ion channel complexes[63]. The optimal cleavage at the E2-p7 junction is shown to be important for virus production probably due to the increased NS2-associated virus assembly complex formation in close proximity of LDs. It also enhances NS2 interaction with NS3 and E2, but does not affect HCV genome replication[64].

Structural analysis revealed that HCV p7 protein consists of two membrane-spanning α-helices connected by a short cytoplasmic loop[65]. PTMs of p7 have not been demonstrated.

NS2

HCV NS2 is a transmembrane protein. Together with the N-terminal domain of NS3, NS2 forms the NS2-3 autoprotease. The NS2-3 cysteine autoprotease is a zinc-dependent metalloprotease that cleaves the HCV polyprotein at the NS2-NS3 junction. After its self-cleavage from NS3, NS2 is quickly degraded[66,67]. Like p7, NS2 is known to be essential for virus assembly. Even though NS2 is part of the HCV replication complex, which is composed of NS2, NS3, NS4A, NS4B, NS5A and NS5B, NS2 is not essential for virus replication[62,68,69]. The interaction of NS2 with E1, E2, NS3 and NS5A results in co-localization of these viral proteins to dot-like structures near LDs, which are the sites for virus particle assembly[64,70]. Moreover, proper cleavage at the NS2-NS3 junction is important for an active HCV replication complex formation, but is not required for NS3 protease activity[71,72]. Other functions linked to NS2 include the inhibition of apoptosis and modulation of host cellular gene transcription[73-76].

The highly hydrophobic N-terminus of NS2 consists of three trans-membrane segments which form the protein membrane binding domain[77]. No attachment of fatty acids or prenyl groups by modifications typically involved in membrane targeting, like farnesylation, myristoylation, palmitoylation or prenylation[78], has been associated with membrane anchoring of NS2 so far. Though NS2 is located to the ER membrane, the protein is not glycosylated[79]. The protease activity of NS2 is located within its C-terminal domain, which is able to homodimerize and thus creates two composite active sites[80]. Regarding the role of NS2 in HCV particle formation, the overall structural integrity rather than the protease activity of NS2 itself appears to be crucial[81,82].

The C-terminal globular domain of NS2 facing towards the cytoplasm of the infected cell was shown to be modified by phosphorylation. Phosphorylation of NS2 is presumably mediated by host cellular casein kinase 2 (CK2)[79]. NS2 is a short-lived protein that is rapidly degraded by the proteasome. Proteasome-mediated degradation of NS2 is regulated in an ubiquitin-independent manner by phosphorylation within its C-terminal domain. Ser^{168} as part of a CK2 consensus recognition site (Ser/Thr-X-X-Glu) is shown to be vital for NS2 degra-

dation. It is highly conserved between all HCV genotypes and single point mutation of Ser^{168} confers resistance to NS2 degradation[79]. Therefore, phosphorylation of NS2 is strongly connected to its abundance within the host cell and can have a strong impact on HCV pathogenesis, more particularly on assembly and virion production.

NS3-4A complex

HCV NS3-4A is a noncovalent complex composed of the serine protease NS3 and its cofactor NS4A[83]. The NS3-4A mediated cleavage releases NS3, NS4A, NS4B, NS5A and NS5B from the HCV polyprotein in a specific order. The NS3-4A protease complex also has three identified cellular targets so far, including mitochondrial antiviral signaling protein (MAVS), T-cell protein tyrosine phosphatase (TC-PTP) and toll/IL-1 receptor homology domain-containing adaptor inducing IFN-β (TRIF), which may be involved in the development of persistent infection and HCC[10,15]. Therefore, the NS3-4A protease is a prime target for antiviral drug design. For example, the two recently approved direct-acting antivirals (DAAs), telaprevir and boceprevir, are oral NS3-4A protease inhibitors[4,84].

NS3 protein consists of an N-terminal serine protease domain with its catalytic triad composed of His^{57}, Asp^{81} and Ser^{139}, and a C-terminal RNA helicase/NTPase domain. The NS3 helicase/NTPase couples NTP hydrolysis to unwind extensive RNA secondary structure and is important for RNA replication and virus assembly[85,86].

The two domains of NS3 can function independently from each other, and the reason for their physical linkage remains unclear[83,85]. The intracellular NS3 protease shows structure homology with extracellular serine proteases, but does not possess disulfide bonds to stabilize its structure as extracellular serine proteases[87]. A Zn^{2+} ion together with its binding site formed by Cys^{97}, Cys^{99}, Cys^{145} and His^{149} stabilizes NS3 protease, activates NS3 hydrolysis and facilitates NS2 processing at the NS2-NS3 junction. Binding of NS4A further stabilizes NS3 by restructuring the N-terminus of NS3 protease through interaction with the central hydrophobic portion of NS4A, increases catalytic efficiency by influencing the spatial configuration of the catalytic triad and directs the cellular membrane localization because of the high hydrophobicity of the N-terminal transmembrane α-helix of NS4A. In addition, the C-terminal acidic portion of NS4A plays a role in regulating HCV genome replication and virus assembly by interacting with other viral proteins in the replication complex[15,87-89]. NS4A also regulates HCV replication by modulating NS5A hyperphosphorylation[90].

Liefhebber *et al*[91] has shown that NS3 might get phosphorylated in subgenomic HCV replicon cells through phosphospecific staining and dephosphorylation assay. However, phosphorylation efficiency is low and the phosphorylation sites are hard to identify. In addition, N-terminal acetylation of NS3 was identified by this research group through mass spectrometric analysis. The role of NS3 phosphorylation and acetylation in HCV life cycle needs to be further investigated.

It has been reported that protein arginine methyltransferases (PRMTs) can irreversibly and post-translationally methylate arginine residues in the arginine-glycine (RG)-rich region of many RNA-binding proteins[92,93]. Since NS3 protein can bind to RNA through its RNA helicase domain and contains seven RG motifs including two RG motifs in the helicase domain, it is a potential methylation target for PRMTs. Full-length NS3 and NS3 helicase domain are shown to be methylated at Arg^{1493} in the ^{1486}Gln-Arg-Arg-Gly-Arg-Thr-Gly-Arg-Gly^{1494} motif by PRMT1, but no methylation is found in NS3 protease domain[94]. Mutation analysis has demonstrated that Arg^{1490} and Arg^{1493} are determinants for the helicase activity[95]. Methylation of NS3 at Arg^{1493} inactivates the helicase by inhibiting unwinding of double-stranded DNA[96]. The reason that arginine methylation is involved in protein-nucleic acid interaction is that methyl modification may affect the binding affinity, protein stability, transcription and signal transduction[92]. Negative regulation of PRMT1 by protein phosphatase 2A (PP2A) increases NS3 helicase activity and enhances HCV RNA replication, therefore PP2A is considered a potential target for HCV drug development[96].

The cofactor activity of NS4A is mediated by its central region and especially the hydrophobic Ile^{25} and Ile^{29} residues, since an I25A/I29A double mutant cannot form a complex with NS3[97]. To reactivate NS4A cofactor activity, the double mutant requires biotinylation at the N-terminus by biotin-aminohexanoic acid (ahx). However, the N-terminal biotin fusion alone without ahx or C-terminal biotin-ahx fusion cannot restore NS4A cofactor activity. On the other hand, N-biotinylation of wild-type NS4A by biotin-ahx can dramatically promote cofactor activity. Based on these data and the crystal structure, it is predicted that N-biotinylation by biotin-ahx resembles a hydrophobic environment that enhances the stabilization of NS3-4A complex and C-biotinylation may sterically interfere with the substrate binding pocket[98].

NS4B

HCV NS4B is relatively poorly understood compared to other HCV proteins. The liberation of NS4B happens at last during HCV polyprotein precursor processing in a strictly defined position[99]. It is a highly hydrophobic integral membrane protein that induces the formation of the membranous web around ER membrane where HCV genome replication takes place and functions by anchoring the HCV replication complex through an unknown mechanism[100]. It has been reported that NS4B can interact with other viral proteins such as NS5A, binds viral RNA and has NTPase activity. It is involved in RNA replication, virus assembly and release[101,102]. The multifunctional NS4B is also shown to activate ER stress pathways, contributes to steatosis by altering lipid metabolism and escape from innate immune system by inhibiting interferon[99]. Moreover, its anti-apoptosis function might be associated with HCC development[103].

There are two amphipathic helices (AH1 and AH2) located in the N-terminal region of NS4B with their hydrophobic sides facing the cytoplasmic side of ER membrane. AH2 is a membrane interacting domain that is essential for membrane trafficking, HCV genome replication and protein oligomerization. NS4B oligomerization is critical for replication complex formation[104-106]. The highly hydrophobic central core region of NS4B contains four transmembrane domains and the highly conserved C-terminus is a membrane binding domain that consists of two α-helical elements and plays a role in NS4B self-interaction, thus being important for replication complex formation[107,108].

There are three common lipid modifications of protein located in lipid raft, including palmitoylation, N-terminal myristoylation and palmitoylation, and glycosylphosphatidylinositol modification[109]. So far, only palmitoylation is detected in NS4B at Cys^{257} and Cys^{261} in the C-terminus and these two sites are relatively conserved among HCV genotypes. Site-directed mutagenesis confirmed that Cys^{261} palmitoylation is more crucial for protein-protein interaction and replication complex formation. Palmitoylation can enhance the polymerization activity of NS4B through its N-terminus[110].

NS5A

HCV NS5A is a phosphorylated zinc-metalloprotein without any enzymatic activity, but required for RNA replication and virion morphogenesis[111]. However, the precise mechanism of how NS5A functions is not clear. It is demonstrated that NS5A can bind to HCV RNA, other HCV proteins such as NS5B and cellular proteins such as human vesicle-associated membrane-associated protein of 33 kDa (hVAP-33), thus contributing to replication complex formation[8]. Several other functions of NS5A include interferon resistance, transcriptional activation and signaling pathway regulation[112,113].

NS5A is composed of three domains. Domain I contains a zinc-binding motif and is the determinant for HCV RNA replication. It is a nucleic acid-binding domain that binds to the 3' G/C rich sequence in HCV RNA. It also functions in LD association. Domain Ⅱ may play a role in evading innate immune response as well as RNA replication. Domain Ⅲ participates in virus assembly and core protein interaction[83,86,114]. In addition, there is an amphipathic α-helix in the N-terminal region responsible for ER membrane anchoring[111].

NS5A is a phosphoprotein that exists in two forms, a basally phosphorylated form (56 kDa) and a hyperphosphorylated form (58 kDa), which is conserved among HCV genotypes[115]. The basally phosphorylated sites are mainly serine residues and the minority are threonine residues located in the central and C-terminal region. Major hyperphosphorylated sites are identified in a serine-rich region in the central portion of NS5A[112,115]. The basally phosphorylated form may be affected by NS2 and NS4A, whereas hyperphosphorylation of NS5A requires NS3, NS4A and NS4B. Cellular protein kinases in the CMGC kinase family are involved in NS5A phosphorylation, including cyclin-dependent kinase (CDK), mitogen-activated protein kinase (MAPK), glycogen synthase kinase 3 (GSK3) and casein kinase Ⅱ (CKⅡ)[90,112,116-118]. Since the subcellular distributions of both NS5A forms are similar, the degree of phosphorylation does not affect NS5A localization to the ER membrane[119]. However, the degradation of NS5A is enhanced by increased degree of phosphorylation[115]. Mutation analysis revealed that reduced NS5A hyperphosphorylation promotes HCV RNA replication, whereas reduced basal phosphorylation does not have an effect on HCV RNA replication in a replicon system. This suggested that the ratio of these two NS5A phosphorylation forms may be important for viral RNA replication[120,121]. NS5A is also involved in virion production through its interaction with core protein, which requires basal phosphorylation of NS5A[122].

NS5B

HCV NS5B is a conserved RNA-dependent RNA polymerase (RdRp) that initiates complementary negative-strand RNA synthesis and then synthesizes positive-strand RNA using the newly synthetic negative-strand RNA as template. Due to the lack of proofreading of RdRp, HCV replication is error-prone[83,86]. NS5B can interact with other viral proteins such as NS3, NS4A and NS5A, and cellular proteins like hVAP-33, which facilitates the formation of the viral RNA replication complex[123,124]. Furthermore, it can form a complex with the retinoblastoma tumor suppressor protein (pRb) and promote pRb degradation in an ubiquitin dependent manner, therefore contributing to HCC development[125].

Like other polymerases, the crystal structure of NS5B reveals that it resembles the configuration of a right hand. The finger, thumb and palm domains compose a unique shape. The active site located in the palm domain has a highly conserved GDD motif. There are four allosteric sites within the thumb and palm domains which serve as targets for antiviral development[126-128]. Besides, NS5B is a tail-anchored protein with its C-terminal hydrophobic tail associated to the ER membrane[86].

The function of many cellular enzymes for DNA and RNA metabolism and viral RdRps is often regulated by phosphorylation[129]. Hwang *et al*[130] demonstrated that NS5B is a phosphoprotein in insect cells. Kim *et al*[129] discovered that the protein kinase C-related kinase 2 (PRK2) is the specific enzyme for NS5B phosphorylation within the N-terminal finger domain. Knock-down and over-expression of PRK2 demonstrated PRK2 up-regulates HCV RNA replication in HCV subgenomic replicon cells, suggesting that NS5B phosphorylation can enhance HCV replication.

Gao *et al*[131] identified an interaction between ubiquitin-like protein hPLIC1 (human homolog 1 of protein linking integrin-associated protein and cytoskeleton) and NS5B. Since hPLIC1 interacts with both proteasome and

E3 ubiquitin protein ligases E6AP and βTrCP, the ubiquitination modification of NS5B through hPLIC1 binding promotes ubiquitin-dependent proteasome degradation, resulting in decreased level of NS5B. NS5B mainly functions in RNA replication, so decreased NS5B leads to HCV genome RNA reduction[131,132]. Although the ubiquitination sites within NS5B and the detailed mechanism of hPLIC1-induced NS5B degradation are still not clear, up-regulating NS5B ubiquitination may represent a target for anti-viral development.

SUMMARY AND PERSPECTIVES

PTMs of HCV viral proteins include phosphorylation, glycosylation, disulfide bridging, methylation, palmitoylation, acetylation, and ubiquitination. These protein modifications ensure proper protein functions by regulating protein activity, subcellular localization, protein-nucleic acid interaction, and protein-protein interactions. Among the already identified PTMs of HCV proteins, some are essential for HCV virion production such as sequential proteolytic cleavage of core protein, whereas others have regulatory roles in virus replication such as phosphorylation of NS5A and ubiquitination of NS5B. PTM sites and PTM pathways are potential pharmacological targets for antiviral drug development. However, much work remains to be done to unveil the precise PTM sites and the underlying mechanisms.

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