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Recombinant HCV NS3 and NS5B enzymes exhibit multiple posttranslational modifications for potential regulation

Sergio Hernández[1,2] · Ariel Díaz[1] · Alejandra Loyola[1] · Rodrigo A. Villanueva[1]

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Abstract

Posttranslational modification (PTM) of proteins is critical to modulate protein function and to improve the functional diversity of polypeptides. In this report, we have analyzed the PTM of both hepatitis C virus NS3 and NS5B enzyme proteins, upon their individual expression in insect cells under the baculovirus expression system. Using mass spectrometry, we present evidence that these recombinant proteins exhibit diverse covalent modifications on certain amino acid side chains, such as phosphorylation, ubiquitination, and acetylation. Although the functional implications of these PTM must be further addressed, these data may prove useful toward the understanding of the complex regulation of these key viral enzymes and to uncover novel potential targets for antiviral design.

Keywords Hepatitis C virus · HCV · NS3 · NS5B · Posttranslational modification · Protein regulation

The hepatitis C virus (HCV) contains a genome of single-stranded, positive-polarity RNA. The expression of its RNA in the endoplasmic reticulum produces a precursor, which is proteolytically cleaved into mature viral proteins, comprising structural, and nonstructural (NS) polypeptides [1]. Whereas structural proteins will package into particles for viral progeny, NS proteins will direct the assembly of a multi-subunit RNA replication complex on intracellular membranes [2–4]. Amongst the viral NS proteins, two critical enzymatic activities for the viral cycle, NS3, and NS5B have been targets of intense research efforts to design new anti-viral compounds.

HCV NS3 is a multifunctional protein composed of two domains: a chymotrypsin-like serine-protease activity, residing in its N-terminal third, and an NTPase/helicase activity, located in its two C-terminal thirds (Fig. 1, left) [5]. Both enzymatic activities are modulated by interdomain interactions and by the NS3-cofactor, NS4A, whose N-terminal hydrophobic helix provides a membrane anchor for the NS3–NS4A complex [6, 7]. The NS3–NS4A complex catalyzes cleavages on the HCV polyprotein precursor to generate viral NS proteins. The helicase activity of NS3 is essential for HCV RNA replication in replicons and productive infection in chimpanzees [8–10].

HCV NS5B is the viral RdRP. Analysis of the NS5B amino acid sequence has indicated the existence of five conserved motifs [11, 12]. The crystal structure of NS5B uncovers a catalytic domain, continued by a C-terminal region connecting to the transmembrane domain through the active-site groove [13, 14]. The catalytic domain folds into the classical right-hand structure, with "*fingers*", "*palm*", and "*thumb*" subdomains (Fig. 1, right) [15]. After the expression of NS5B, this protein is detected in association with

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✉ Rodrigo A. Villanueva
rvillanueva@cienciavida.org

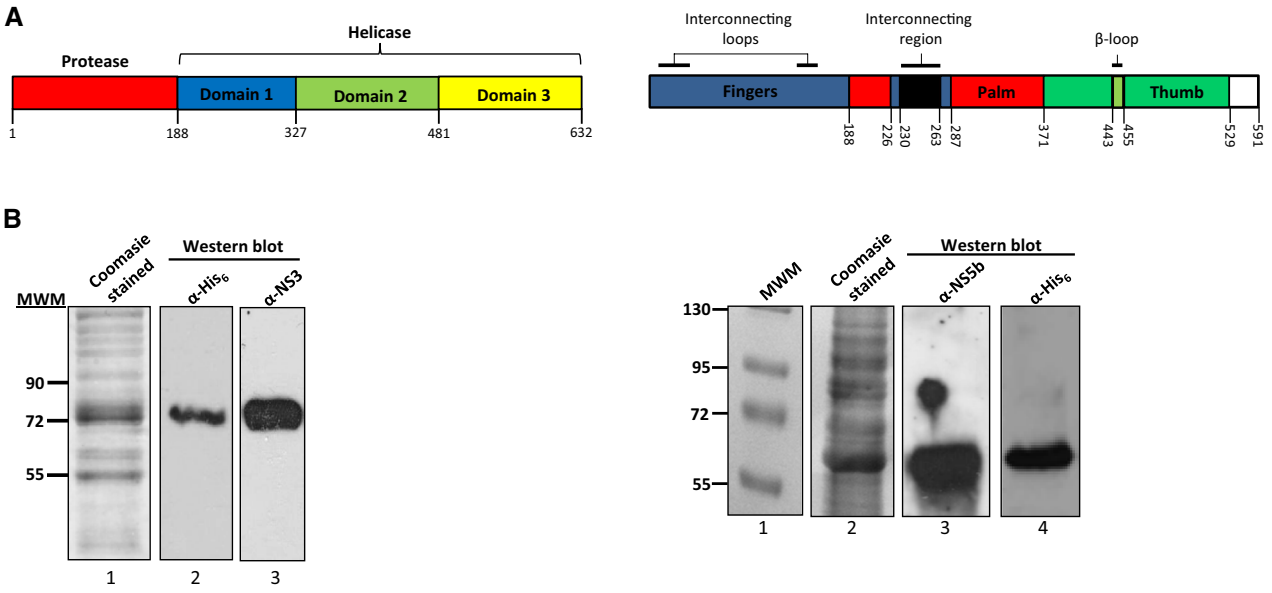
Sergio Hernández
Sergio.Hernandez@afmb.univ-mrs.fr

Ariel Díaz
ariel.e.diaz@emory.edu

Alejandra Loyola
aloyola@cienciavida.org

[1] Fundación Ciencia &, Vida, Avda. Zañartu 1482, 7780272 Ñuñoa, Santiago, Chile

[2] Present Address: Architecture et Fonction des Macromolécules Biologiques, CNRS UMR7257, Department of Medicinal Chemistry, Aix Marseille Universite, Marseille, France



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SLEPCKCGAVDLYLVTRNADVIPARRRGDKRGALLSPRPISTLKGSSGGPVLCPRGH
VVGLFRAAVCSRGVAKSIDFIPVETLDVVTRSPTFSDNSTPPAVPQTYQVGYLHAPT
GSGKSTKVPVAYAAQGYKVLVLNPSVAATLGFGAYLSKAHGINPNIRTGVRTVMTGE
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AVDFSLDPTFTITTQTVPQDAVSRSQRRGRTGRGRQGTYRYVSTGERASGMFDSVVL
CECYDAGAAWYDLTPAETTVRLRAYFNTPGLPVCQDHLEFWEAVFTGLTHIDAHFLS
QTKQAGENFAYLVAYQATVCARAKAPPPSWDAMWKCLARLKPTLAGPTPLLYRLGPI
TNEVTLTHPGTKYIATCMQADLEVMT

MSGSHHHHHHGSSGENLYFQSLSMSYSWTGALITPCSPEEEKLPINPLSNSLLRYHN
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PPHSARSKYGFGAKEVRSLSGRAVNHIKSVWKDLLEDPQTPIPTTIMAKNEVFCVDP
AKGGKKPARLIVYPDLGVRVCEKMALYDITQKLPQAVMGASYGFQYSPAQRVEYLLK
AWAEKKDPMGFSYDTRCFDSTVTERDIRTEESIYQACSLPEEARTAIHSLTERLYVG
GPMFNSKGQTCGYRRCRASGVLTTSMGNTITCYVKALAACKAAGIVAPTMLVCGDDL
VVISESQGTEEDERNLRAFTEAMTRYSAPPGDPPRPEYDLELITSCSSNVSVALGPR
GRRRYYLTRDPTTPLARAAWETVRHSPINSWLGNIIQYAPTIWVRMVLMTHFFSILM
VQDTLDQNLNFEMYGSVYSVNPLDLPAIIERLHGLDAFSMHTYSHHELTRVASALRK
LGAPPLRVWKSRARAVRASLISRGGKAAVCGRYLFNWAVKTKLKLTPLPEARLLDLS
SWFTVGAGGGDIFHSVSRARPRSLLFGLLLLFVGVGLFLLPAR

Fig. 1 Recombinant HCV NS3 and NS5B proteins. **a** Left: domain organization of NS3 protease and NTPase/helicase. The one-third N-terminal domain, exhibiting protease activity, and the two-thirds C-terminal domains, displaying NTPase/helicase activity are shown. As indicated, the NTPase/helicase domain is further functionally dissected into three domains, named Domains 1–3 [5–7]. Right: domain organization of NS5B RdRP. The HCV RNA polymerase is organized into *fingers, palm*, and *thumb* domains, as shown. Interconnecting loops, interconnecting region, and the β-loop are indicated on top of the diagram [12–14]. **b** Left: recombinant HCV NS3 protein. A fraction of partially purified protein was stained with Coomassie stain (lane 1). Western blots where anti-His_6, in lane 2, and anti-NS3 specific antibodies, in lane 3, respectively, were used to identify the recombinant HCV NS3 protein. Right: recombinant HCV NS5B protein. A fraction of partially purified protein was stained with Coomassie stain (lane 2). Western blots, where anti-NS5B (lane 3), and anti-His_6-specific antibodies (lane 4), were used to identify the recombinant HCV NS5B protein. **c** Left: amino acid sequence of the recombinant HCV NS3 protein (JFH1 virus, HCV genotype 2a, AB237837.1), and protein coverage with trypsin digestion. The first 22 residues are contributed by the vector, introducing a His_6-tag utilized for purification, followed by the full amino acid sequence of NS3. Sequence coverage is 74%, which is indicated by the underlined amino acids. Right: amino acid sequence of the recombinant HCV NS5B protein (JFH1 virus, HCV genotype 2a, AB237837.1), and protein coverage with trypsin digestion. The first 22 residues are contributed by the vector, introducing a His_6-tag utilized for purification, followed by the full amino acid sequence of NS5B. Sequence coverage is 81%, which is indicated by the underlined residues

ER membranes, being directed to membranes by an anchor peptide located at its C-terminal end [15, 16].

Posttranslational modification (PTM) can regulate a viral protein's subcellular localization, stability, biochemical and/or enzymatic activity, protein/nucleic acid interactions, and interactions with other cellular and viral partners, thereby improving protein functional diversity. Amongst the HCV proteins, several mature polypeptides have been shown to be posttranslationally modified. The trans-suppression activity of the Core protein has been shown to be modulated by phosphorylation [17], and the transmembrane NS2 protease protein is also phosphorylated, which is involved in its turnover [18, 19]. The NS3 protease and helicase viral protein have been shown to be N-terminal acetylated, methylated, and phosphorylated, and all these modifications are thought to somehow regulate their functions [20, 21]. The transmembrane NS4B *membranous web* protein has been shown to be palmitoylated; this lipid modification facilitates the self-association of the protein [22]. In cells where HCV RNA replication is ongoing, two phosphorylated forms of NS5A

have been identified, the ratio of which correlates with the level of HCV RNA replication [23, 24]. Finally, it is known that the viral RNA polymerase NS5B is a phosphoprotein [25], and the protein involved in the phosphorylation of several residues has been identified as the cellular protein kinase C-related kinase 2 (PRK2) [26, 27]. However, other phosphorylation sites have been also functionally identified and characterized [28]. Since HCV NS3 and NS5B are multifunctional enzyme proteins, and both likely subjected to regulation mechanisms, we investigated the profile of PTM of these proteins upon their individual expression in eukaryotic Sf9 insect cells infected with their recombinant baculoviruses.

HCV recombinant NS3 and NS5B proteins were partially purified from infected Sf9 cell extracts using Ni–NTA beads, and imidazole elution procedures. Partially purified proteins were probed with specific antibodies, as shown for both NS3 and NS5B in Fig. 1b (left and right, respectively). Coomasie stained protein bands were then excised from gels. Isolated protein bands were digested with trypsin and subjected to high-resolution tandem mass spectrometry analyses (MS/MS) to identify PTM utilizing an AB Sciex 5600 Triple Tof mass spectrometer in the Targeted Metabolomics and Proteomics Laboratory (TMPL) at the University of Alabama at Birmingham.

Posttranslational modifications found in the HCV NS3 are shown in Table 1. In its protease domain, sites of deamidation (N110) and phosphorylation (S128) were identified. For Ser128 phosphorylation spectra, the trypsin digested peptide was identified with a precursor charged at 716.8998 m/z. On the other hand, in the helicase domain 2, a site of ubiquitination (C374) was found, whereas in domain 3, sites of ubiquitination and allysine (K589) and deamidation (N607) were confirmed (Supplementary Material, Figure S1).

Posttranslational modifications identified in the HCV NS5B protein are listed based on its domain organization. Table 2 top shows PTMs found in the *fingers* domain, where sites of phosphorylation (S27, and S29), dimethylation (N28), ubiquitination (C89, and K151), and nitration

Table 1 PTMs identified in recombinant HCV NS3 protein

Amino acid[a]	Modification[b]	Modified peptide[c]
N110	Deamidation	N(Deamidated)ADVIPAR
S128	Phosphorylation	GALLS(Phospho)PRPISTLK
C374	Ubiquitination	C(Ubiquitin)DELAAALR
K589	Ubiquitination	LK(Ubiquitin)PTLAGPTPLLYR
K589	Allysine	LK(Allysine)PTLAGPTPLLYR
N607	Deamidation	LGPITN(Deamidated)EVTLTH PGTK

[a]Amino acid residue number of HCV NS3, according to the indicated in Fig. 1

[b]PTM identified by LC–MS/MS

[c]Trypsin-digested peptide where modified residue is shown underlined

Table 2 PTMs identified in recombinant HCV NS5b protein

Amino acid[a]	Modification[b]	Modified peptide[c]
Fingers		
S27	Phosphorylation	LPINPLS(Phospho)NSLLR
N28	Dimethylation	LPINPLSN(Dimethyl)SLLR
S29	Phosphorylation	LPINPLSNS(Phospho)LLR
C89	Ubiquitination	LLTLEEAC(GlyGly)QLTPPHSAR
K151	Ubiquitination	NEVFC(Propionamide)VDPAK(GlyGly)GGK
Y162	Nitration	LIVY(Nitro)PDLGVR
Palm		
S190	Acetylation	LPQAVM(Oxidized)GAS(Acetyl)YGFQYSPAQR
S190	Decanoylation	PQAVM(oxidized)GAS(Decanoyl)YGFQYSPAQR
K206	Ubiquitination	VEYLLK(GlyGly)AWAEK
C243	Ubiquitination	TEESIYQAC(GlyGly)SLPEEAR
Thumb		
T390	Phosphorylation	DPTT(Phospho)PLAR
S473	Decanoylation	HGLDAFS(Decanoyl)M(oxidized)HTYSHHELTR
Y524	Nitration	Y(Nitro)LFNWAVK
K531	Ubiquitination	YLFNWAVK(GlyGly)TK
K535	Ubiquitination	LK(GlyGly)LTPLPEAR

[a]Amino acid residue number of HCV NS5B, according to the indicated in Fig. 1

[b]PTM identified by LC–MS/MS

[c]Trypsin-digested peptide where modified residue is shown underlined

(Y162) were identified (Supplementary Material, Figure S2). Table 2 middle lists PTMs found in the *palm* domain, where sites of acetylation (S190), decanoylation (S190), and ubiquitination (K206 and C243) were found (Supplementary Material, Figure S3). Finally, Table 2 bottom shows the PTMs found in the *thumb* domain, where sites of phosphorylation (T390), decanoylation (S473), nitration (Y524), and ubiquitination (K531, and K535) were found (Supplementary Material, Figure S4). For the identification of Ser27, Ser29, and Thr390, the indicated peptides were identified in the spectra with precursors charged at 708.8860 m/z, 708.8821 m/z, and 475.7215 m/z, respectively.

Reversible phosphorylation is the best studied PTM that regulates the biological role of proteins [29]. HCV NS3 has been preliminarily reported as N-terminal acetylated, methylated, and phosphorylated [20, 21]. Herein, we have identified that HCV NS3 S128 is phosphorylated. This residue is located within the protease domain, and since the protease and helicase domains are regulated by interdomain interactions, and NS4A, it is possible that S128 phosphorylation may also be involved in the regulation of both enzymatic activities [30]. Additionally, phosphorylated S128 might have a role in functionally regulating interactions with NS2 protease [31]. For HCV NS5B, it has been proposed that the cellular kinase PRK2 can modulate the polymerase by phosphorylation on serine residues at the N-terminus, in the *fingers* subdomain, in vivo [27, 28]. Consistently, we have found that HCV NS5B S27 and S29 residues within the *fingers* subdomain are also phosphorylated. However, we have also discovered that T390, located at the *thumb* subdomain, is phosphorylated as well, thereby expanding the complexity of the regulation of the HCV RdRP by phosphorylation.

Ubiquitin is a small regulatory polypeptide, and its conjugation onto proteins often directs the labeled protein to degradation in the proteasome [32]. However, there are several non-proteolytic functions associated with the mono-ubiquitination of proteins. It has been shown that this is a reversible, non-proteolytic protein mark that can be involved in processes as diverse as the modulation of histones, DNA repair, protein trafficking, endocytosis, and virus exit [33–37]. Herein, we have shown that both recombinant HCV NS3 and NS5B proteins become ubiquitinated. For both HCV proteins, it is possible that several of these sites might have a role as sites for degradation targeting. However, it is also possible that some of them can perform regulatory functions on their enzymatic activities or on modulating protein–protein interactions. Interestingly, HCV NS5B K151, which we have found here to be ubiquitinated, has been identified as critical for genome replication and infectious virus production [38].

Similar to histones, cytoplasmic proteins have been also reported to be acetylated, and acetylation seems to play a role in cell biology, not only for transcriptional regulation [39]. Furthermore, cross talk between acetylation, and other PTM, including phosphorylation, ubiquitination, and methylation, can modify the function of the acetylated protein [39–41]. For recombinant HCV NS5B, we found that residue S190 is acetylated, however, whether this particular acetylation can functionally interplay with other modifications remains to be determined.

Deamidation is a PTM resulting in the conversion of an asparagine residue to a mixture of isoaspartate and aspartate. Deamidation of glutamine residues can also occur, but does so at a much lower rate [42]. In the current analysis, we found that recombinant HCV NS3 is deamidated at two different asparagine sites, and it has been postulated that deamidation may provide a signal for protein degradation, thereby regulating intracellular levels and protein turnover. The role of protein deamidation during HCV replication remains to be determined.

Protein fatty acid acylation is the PTM of proteins via the attachment of functional groups through acyl linkages. One important kind is fatty acylation, where two modifications are well known. In myristoylation, a myristoyl group (derived from myristic acid, $CH_3(CH_2)_{12}COOH$) is covalently attached to an N-terminal residue of a nascent protein, commonly on glycine residues [43]. Palmitoylation is the covalent attachment of palmitoyl group (derived from palmitic acid, $CH_3(CH_2)_{14}COOH$) to cysteine, and less frequently to serine and threonine residues of proteins [43, 44]. In both cases, these PTMs display significant roles in targeting proteins to membranes. In the current analyses, we have found that recombinant HCV NS5B exhibited two serine residues (S190 and S473) that were modified by decanoylation, probably derived from decanoic or capric acid ($CH_3(CH_2)_8COOH$). A similar kind of acylation has been previously well documented [45–48]. Since HCV NS5B is known to be targeted to membranes through its C-terminal hydrophobic region, the role and relevance of this new modification for the association of the protein with membranes needs to be experimentally addressed. However, this finding might be noteworthy, since it has been shown that HCV replication is regulated by fatty acids [49].

Both, limitations and advantages of protein PTM found in baculovirus expression systems have been recently reviewed [50–52]. Regarding to this, a whole field of research to produce human vaccines in baculovirus expression system is currently in full development, which validates and guarantees the utilization of such an expression system.

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Author's contributions RAV contributed to the study conception and design. SH and AD performed the experiments. RAV wrote the manuscript. SH, AD, RAV, and AL checked and revised it. RAV and AL contributed with funding. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals that required ethical approval.

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