Characterization of hepatitis C virus NS3 modifications in the context of replication

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Post-translational modifications (PTMs) of viral proteins regulate various stages of infection. With only 10 proteins, hepatitis C virus (HCV) can orchestrate its complete viral life cycle. HCV non-structural protein 3 (NS3) has many functions. It has protease and helicase activities, interacts with several host-cell proteins and plays a role in translation, replication and virus-particle formation. Organization of all these functions is necessary and could be regulated by PTMs. We therefore searched for modifications of the NS3 protein in the subgenomic HCV replicon. When performing a tag-capture approach coupled with two-dimensional gel electrophoresis analyses, we observed that isolated His6–NS3 yielded multiple spots. Individual protein spots were digested in gel and analysed by mass spectrometry. Differences observed between the individual peptide mass fingerprints suggested the presence of modified peptides and allowed us to identify N-terminal acetylation and an adaptive mutation of NS3 (Q1067R). Further analysis of other NS3 variants revealed phosphorylation of NS3.

The serine protease domain of NS3 is in the N-terminal one-third of the protein. Together with NS4a, it forms a stable complex that cleaves the non-structural proteins downstream of NS3 (Bartenschlager et al., 1995; Failla et al., 1994; Tanji et al., 1995). Besides these cleavages, the NS3/4A protease is known to cleave MAVS/IPS-1/VISA/Cardif and TRIF, which affect signalling of the RIG-I and Toll-like receptor 3 pathways, respectively, thereby abrogating the interferon response (Meurs & Breiman, 2007).

In addition to its protease activity, the C terminus of NS3 can unwind RNA through ATPase and helicase activities (Kim et al., 1995; Suzich et al., 1993). The RNA-unwinding activity seems to be modulated by other viral proteins, e.g. NS5B (Jennings et al., 2008; Zhang et al., 2005) and NS4A (Gallinari et al., 1999).

The helicase domain of NS3 can be methylated post-translationally at Arg1493 (polyprotein) by protein arginine methyltransferase 1 (PMRT1) (Rho et al., 2001).

Non-methylated NS3 helicase can unwind double-stranded (ds) DNA; however, when the helicase domain of NS3 is methylated, unwinding of dsDNA is inhibited (Duong et al., 2005). This shows that methylation of the NS3 helicase domain can change its activity. Besides the interaction of NS3 with PMRT1, several other host proteins have been reported to interact with NS3, including PKA, PKC, TBK1 and p53; these are all proteins involved in cell signalling.
(Borowski et al., 1996, 1999; Ishido & Hotta, 1998; Otsuka et al., 2005).

Recently, a role in virus-particle production has been suggested for NS3, by the identification of adaptation and compensatory mutations in NS3 using the infectious virus system (Kaul et al., 2009; Ma et al., 2008).

Taken together, the NS3 protein has various interaction partners and many functions that need to be regulated. PTMs could direct these processes. A first step towards understanding regulation is the identification of NS3 modifications. Besides methylation, which seems to regulate helicase activity and has been identified by using overexpression of NS3, no other modifications have been reported. We therefore set up an assay to search for other NS3 modifications in the context of functional replication. In order to purify NS3, we introduced a 6×His (His6) tag at the N terminus of NS3 in the replicon RNA genome (genotype 1b containing NS3–NS5B; see Supplementary Methods, available in JGV Online), providing an unlimited source of HCV proteins actively replicating HCV RNA. Addition of the His6 tag did not influence replicon colony-formation efficiency; furthermore, RT-PCR sequencing of the replicon cell line confirmed that the six histidines remained unchanged at passage 30, at which stage our experiments were conducted (see Supplementary Fig. S1, available in JGV Online). In addition, lysates of the His6–NS3 replicon cell line showed that the extra tag resulted in a slightly higher molecular mass than that of the control cell line (Fig. 1a, lanes 1 and 2). Based on Western blotting using an NS3 antibody (NCL-HCV-NS3; Novagen), similar amounts of NS3 are expressed by both cell lines (Fig. 1a).

Identification of NS3 modifications in the replicon is complicated, given that only low amounts of protein are available and a combination of processes can influence the modification states of NS3. An advantage of a His6 tag is that it allows affinity purification under denaturing conditions, using Co2+ beads. Lanes 3 and 4 represent (His6–)NS3 bound to the beads from control or His6–NS3 cell lines, respectively. Lanes 5–8 contain increasing volumes of total cell lysate from His6–NS3 cells, with 100 % corresponding to the total amount of His6–NS3 used for pull-down. (b, c) Isolated His6–NS3 was separated by 2D-PAGE, followed by either silver staining (b) or Western blotting using NS3 antibody (c). Each His6–NS3 spot is labelled with a number. Basic and acidic indicate where the negative and positive poles are positioned.

![Fig. 1. Affinity purification and 2D-PAGE analysis of NS3 isoforms from the Huh7 replicon cell line. (a) Lysates of Huh7 replicon cells containing non-tagged NS3 (Control, lane 1) or His6-tagged NS3 (His6–NS3, lane 2), standardized to protein concentration using Bradford reagent (Bio-Rad), were separated by SDS-PAGE, followed by Western blot analysis with anti-NS3. These lysates were subjected to affinity purification under denaturing conditions, using Co2+ beads. Lanes 3 and 4 represent (His6–)NS3 bound to the beads from control or His6–NS3 cell lines, respectively. Lanes 5–8 contain increasing volumes of total cell lysate from His6–NS3 cells, with 100 % corresponding to the total amount of His6–NS3 used for pull-down. (b, c) Isolated His6–NS3 was separated by 2D-PAGE, followed by either silver staining (b) or Western blotting using NS3 antibody (c). Each His6–NS3 spot is labelled with a number. Basic and acidic indicate where the negative and positive poles are positioned.](image)

Protein isoforms may arise from PTMs, which introduce variation in the molecular mass and/or charge of proteins. To examine possible NS3 variants, purified His6–NS3 was separated on the basis of isoelectric point (pl) and molecular mass in two-dimensional PAGE (2D-PAGE). Isoelectric focusing was performed on an Ettan-IPGphor 3 with 13 cm Immobiline DryStrips pH 3–10, as described by the manufacturer (GE Healthcare), which was followed by separation on SDS-PAGE 9 % gels. His6–NS3 was visualized by means of silver staining (Fig. 1b) or Western blotting using NS3-specific antibodies (Fig. 1c). With both techniques, at least 11 NS3 protein spots were resolved, with a pl ranging from approximately 7 to 9 and with an apparent molecular mass of 70 kDa. Lower-molecular-mass products were considered to be NS3 breakdown products. The split of NS3 into multiple pl variants indicates several NS3 protein modifications in the context of replication.

To unravel possible PTMs, tryptic digestion followed by mass spectrometric (MS) analysis was performed on spots 3–8 (see Supplementary Methods). In total, peptides assigned to HCV NS3 add up to 60 % coverage (Fig. 2a).
Although the sequence coverage was much lower in the digests of spots 7 and 8, NS3-specific peptides were also observed in these spectra (Fig. 2b), confirming the results of Western blotting (Fig. 1). As we observed similar intensities and comparable sequence coverages for spots 3–6, these spectra were used for side-by-side comparison.

In the matrix-assisted laser desorption/ionization–time of flight (MALDI-ToF) MS spectrum of spot 3, a peptide at m/z 2314.2 was observed that was absent in the other spectra (Fig. 2c). This mass could not be matched to an in silico tryptic peptide of NS3. MALDI-ToF-ToF analysis revealed that this ion corresponds to the peptide SFLATCVNGVCWTVHGAGSK, corresponding to aa 51–71 in NS3 (Fig. 2a). (e) Identification of a peptide in the LC-MS run from the digest of protein spot 3, confirming a Gln→Arg substitution at position 50 within NS3.

**Fig. 2.** MS analysis of NS3 isoforms and characterization of an NS3 mutation. His6-tagged NS3 was affinity-purified and separated by using 2D-PAGE. Six individual NS3 spots (3–8; Fig. 1b) were digested with trypsin and analysed by MALDI-ToF MS. (a) Primary sequence of His6-tagged NS3, with the sequence coverage obtained from spot 4 with MALDI-ToF MS shown in bold. (b) Comparison of MALDI-ToF spectra showing two of the most abundant NS3 tryptic peptides in all six protein spots. (c) Comparison of MALDI-ToF spectra from NS3 isoforms in spots 3–6. The unique presence of a peptide at m/z 2314.2 in the tryptic digest of spot 3 and absence of the tryptic peptide at m/z 2343.3 in spot 6 are shown. (d) MALDI-TOF-ToF MS analysis of the peptide at m/z 2314.2, showing that it matches the peptide SFLATCVNGVCWTVHGAGSK, corresponding to aa 51–71 in NS3 (Fig. 2a). (e) Identification of a peptide in the LC-MS run from the digest of protein spot 3, confirming a Gln→Arg substitution at position 50 within NS3.

http://vir.sgmjournals.org 1015
During further analysis of the spots by MALDI-ToF analysis, a unique peptide at position 50 (NQVEGVQVQVSTATR) (Fig. 2e). Altogether, this revealed a Gln—Arg mutation in NS3 at position 50, corresponding to position 1067 of the polyprotein (HCV type 1b; GenBank accession no. AJ238799). A Q1067R mutation would add a significant charge difference to the NS3 protein (pI 7.5 vs 7.8). This Q1067R mutation, located close to the active site of the NS3 protease (Yao et al., 1999), was described previously for an HCV genotype 1a replicon to compensate for a negative effect located in the proximal NS3 protease region and was found to enhance the replication capacity of the viral RNA (Yi & Lemon, 2004). Surprisingly, we now observe this compensating mutation at the protein level in the Con1 HCV genotype 1b, which is normally not downmodulated and does not accumulate mutations at this position.

Protein phosphorylation is an important regulator of diverse intracellular processes. HCV NS5a is a phospho-protein involved in replication and virus-particle formation. In both processes, the phosphorylation status of NS5a has been shown to be critical (Appel et al., 2005; Evans et al., 2004; Masaki et al., 2008; Tellinghuisen et al., 2008). The multifunctional protein NS3 might be regulated in a similar way. We therefore investigated this possibility for NS3. As phosphorylation leads to a net charge change of proteins and thus the migration behaviour during 2D-PAGE, comparison of 2D-gel spot patterns before and after treatment of the sample with a phosphatase can be utilized to assess the phosphorylation state of a protein (Yamagata et al., 2002). Shrimp alkaline phosphatase was added to one sample of purified His6–NS3. Subsequently, the control and treated samples were both incubated at 37 °C (1 h) and analysed by 2D-PAGE and Western blotting using NS3 antiserum (see Supplementary Methods). In the untreated sample, we detected several spots in a pattern similar to that shown before (Fig. 3a). Upon treatment with phosphatase, the two most acidic spots disappeared and one extra spot appeared at the alkaline (basic) side of the gel (compare Fig. 3a and b). This shift of spots towards the basic pole after removing a negatively charged phosphate group clearly indicates phosphorylation of His6–NS3. Distinct spots resembling spots 9, 10 and 11 in Fig. 1(c) were not detected in these experiments, which might be due to the long phosphatase procedure.

To corroborate these results, we stained the 2D gels directly with ProQ Diamond (Invitrogen), a dye that selectively stains phosphorylated proteins in polyacrylamide gels (Fig. 3c). The phosphospecific stain mainly coloured the acidic spots, which are the low-abundance isoforms of NS3. Conversely, highly abundant NS3 isoforms were hardly stained.

Taken together, the phosphospecific staining and the dephosphorylation assay strongly suggest phosphorylation of NS3. Multiple phosphorylation sites, i.e. 17 serine-, 13 threonine- and 4 tyrosine-phosphorylation sites, are suggested within NS3 by using the phosphorylation prediction program NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/).

To try to identify the phosphorylated amino acid in NS3, we enriched phosphorylated peptides by using TiO2 beads prior to analysis by MS. However, using this approach, no phosphorylated peptides could be identified. As illustrated in Fig. 3, phosphorylated NS3 protein is low in abundance. Additionally, lower signal intensities are often observed for phosphopeptides, possibly due to low ionization efficiency (Thingholm et al., 2009). Moreover, the phosphorylation site might be located within a trypic peptide that is not well-suited to our standard MS analysis (Fig. 2a). Usually, tryptic digestions are measured in the mass range of 500–3500 Da. Altogether, this might explain the difficulty to identify the site of phosphorylation.
PL heterogeneity of a protein can be generated by PTMs or chemical reactions such as deamination (Sarioglu et al., 2000), or it could be due to conformational isomers of a protein (Lutter et al., 2001). All result in multiple isomers observed in 2D-PAGE. In contrast, a PTM that does not affect the pl of proteins is methylation (McBride & Silver, 2001). Methylation was described to take place on aa 1493 and/or 1490 of NS3 (Fig. 2a, aa 473 and 475) (Rho et al., 2001). In our experiment, we were not able to detect specific peaks in this region, as digestion with trypsin in the area of NS3 methylation generates very small peptides (5 aa).

Taken together, we identified modifications, such as N-terminal acetylation, a Q1067R point mutation and phosphorylation, of His6–NS3 in the context of self-replicating HCV RNA. Resolving the precise sites of phosphorylation and determining their function will be the next challenging step. Generation of replicons with mutations in the predicted phosphorylation sites, in total around 34 sites, followed by 2D-PAGE analysis could result in identification of specific phosphorylation sites, which might help our understanding of the orchestration of the HCV life cycle.

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References


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