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Non-structural protein NS4B: HCV replication web inducer

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ABSTRACT

Hepatitis C virus (HCV) genome consists of a positive strand RNA encoding a polyprotein, having 3 structural and 6 non-structural components including non structural protein 4B (NS4B). NS4B is a 27 kDa protein, of 261 amino acids, released after polyprotein cleavage by NS3 serine protease and localized on endoplasmic reticulum (ER). NS4B has 2 alpha helices each in its N and C terminal domains and 4 transmembrane domains in its central region. N-terminal domain resides in the ER-lumen while C-terminal domain resides in the cytoplasm. Around its middle it has a nucleotide binding motif (NBM) which plays a role in ATP and GTP hydrolysis. It is involved in hyperphosphorylation of the NS5A protein and is also thought to be involved in production of various cytokines by the activation of NF- κ B pathway. NS4B plays a major role in HCV replication by inducing membranous web and facilitating other HCV proteins necessary for replication. Here we discuss various functional aspects of this protein and their potential for targeted antiviral approaches.

1. Introduction

HCV is a positive strand RNA virus belonging to family flaviviridae, encoding a polyprotein of ~3000 amino acids. It causes chronic HCV infection which can ultimately result in liver cirrhosis, hepatic failure or hepatocellular carcinoma[1]. The open reading frame (ORF) encodes 10 proteins, including 3 structural proteins (C or core, E1 and E2), a small protein, p7, whose function has not yet been definitively defined and 6 nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B). Nonstructural protein 4B of HCV is a 27 kDa hydrophobic protein produced as a result of cleavage by NS3 protease[2] having a half-life of approximately 11 h[3]. NS4B functions remained unknown for many years, however with recent studies NS4B is now known to modulate NS5A hyperphosphorylation[4,5] transactivate interleukin 8 promoter[6], suppress HCV translation[7] and modulate the ER stress response through interaction with activating transcription factor 6 β [8,9].

2. Cleavage of HCV NS4B from the viral polyprotein

The genome of HCV translates as a single polypeptide which is later cleaved into individual proteins by action of the host enzymes and the HCV NS3 protease[10,11]. The NS2-3 auto protease cleaves at the NS2/3 junction, and ultimately

NS3 serine protease is responsible for the cleavage of other non-structural proteins[12]. NS3 protease in the presence of HCV NS4A cofactor cleaves at the 3/4A, 4A/4B and 4B/5A junctions[13–15]. As a result, this polyprotein processing leads to the production of individual proteins as depicted in Figure 1.

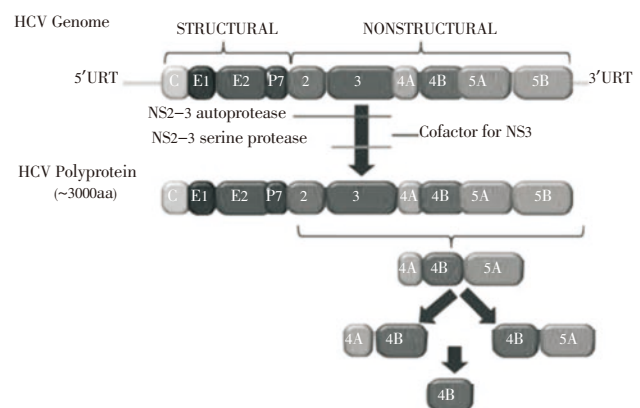


Figure 1. Schematic representation of cleavage of HCV NS4B from polyprotein of HCV by NS3 protease. NS3 serine protease with its cofactor 4A cleaves at the junction of NS3/4A and NS4A/4B. They further cleave to produce either 4A/B or 4B/5A. At last the HCV NS4B is released.

3. Sub cellular localization

Initially indirect immunofluorescence and green

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fluorescent protein (GFP) fusion experiments were helpful in determining that NS4B is cytoplasmically-localized in the perinuclear region where it adopts a chicken wire-like, speckled pattern, typical of a membrane-associated protein[16,17].

Later, GFP tagging and immuno-staining followed by phase separation by Triton X-114 experiments confirmed the localization of HCV NS4B on endoplasmic reticulum (ER), when expressed in recombinant form[2], moreover the co-expression of other HCV proteins like NS3 or NS5B does not influence the subcellular localization of HCV NS4B[18]. Hugel *et al* also showed that NS4B cotranslationally integrates into ER membranes rather than post translationally by adding microsomal membranes to the reaction either during or after completion of *in vitro* transcription and translation experiments. NS4B, therefore, is tightly associated with the ER membrane and behaves as an integral membrane protein. Protease sensitivity of NS4B indicates that majority of the protein is localized towards the cytoplasmic face of the ER membrane[18].

A recent study using fluorescence recovery after photobleaching (FRAP) experiments examined the localization of NS4B in live cells using a chimeric NS4B-GFP fusion protein where NS4B appeared to be distributed in a thread-like pattern, consistent with ER localization[19]. This localization was concentrated at small foci similar to those described by others and termed as membrane-associated foci (MAFs)[20,21].

4. Structural features

The HCV NS4B comprises of 261 amino acids, of which amino acids 1 to ~69 form the N-terminal part, amino acids ~70 to ~190 comprise the central part having four transmembrane domains and amino acids ~191 to 261 constitute the C-terminal[22]. Presence of transmembrane domains in the central region of NS4B was previously confirmed by hydrophobicity plots and glycosylation studies[2]. Lundin *et al* also proposed the presence of a fifth transmembrane domain (residues 60–80) in the N-terminal portion whose orientation is influenced by the NS5A protein[2]. The first 29 amino acids form an amphipathic helix while a basic leucine zipper is present between residues 20 and 55 which could be involved in protein-protein interactions[23,24].

Computational studies predict that the C-terminal region of NS4B is present in the cytoplasm. This region (residues 135 to 261) is composed of two helical domains, is highly conserved among HCV isolates and likely to be engaged in protein-protein interactions required for NS4B functions[25]. Amongst the known functions, C terminal domain (CTD) has been shown to interact with NS3 protein[26], play a role in NS4B oligomerization[27], interact with viral RNA[28], and also play a role in hyperphosphorylation of NS5A protein[4]. Two cysteine residues (cysteines 257 and 261) at the CTD are now known to be involved in lipid modifications (palmitoylation). Site-specific mutagenesis showed that modification at Cys261 is important for protein-protein interaction in the formation of the HCV RNA replication complex. These lipid modifications on the C terminus also facilitate the polymerization process. Lipid modification and polymerization activity could be two properties associated with NS4B mediated induction of the specialized membrane structures, involved in viral RNA replication[27].

The glycosylation pattern of HCV NS4B mutants indicate the presence of its N-terminal region in the ER lumen,

which contradicts the fact that N-terminus is cleaved by NS3 protease whose localization has been proved to be cytoplasmic. Therefore NS4B is said to have dual topology which may involve the presence of a fifth transmembrane domain that helps orient the N-terminal region either towards the luminal or cytoplasmic side[2]. N-terminal domain alpha helices are also known to be involved in NS4B host membranes association[23,29].

Towards the middle, NS4B has a nucleotide binding motif (NBM) that resembles NBM of several viral proteins and GTP binding members of G protein superfamily. Studies using glycosylation markers suggest that this domain is present within the cytoplasm as opposed to the ER lumen; however the secondary structure of NBM within NS4B is not known[22]. Within this NBM, highly conserved elements called A-motif (GxxxxGK) and B-motif (DxxA) are present which are separated by a variable number of amino acids, depending on the particular protein[30]. Presence of NBP and the fusion of NS4B with GFP was confirmed with experiments using western blotting and autoradiography for direct visualization[31]. This NBM has been implicated in the binding and hydrolysis of GTP[31,32] and ATP[33,34]. Figure 2 depicts the topology of HCV NS4B protein along with examples of the recognized roles of each domain.

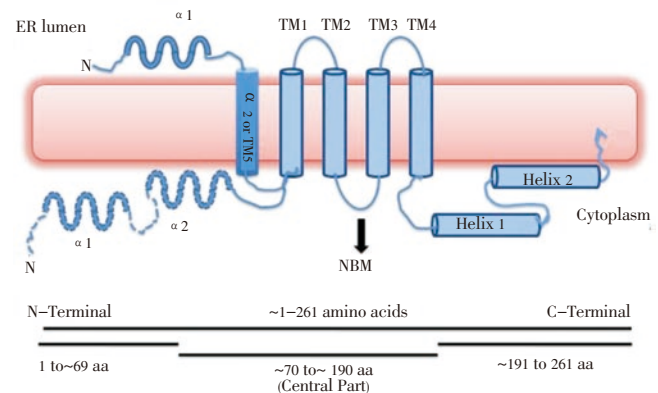


Figure 2. Schematic representation of HCV NS4B topology. It has 2 alpha helices at its N-terminal domain, four transmembrane domains in central region and 2 helices at its C-terminal domain. At its middle it contains a nucleotide binding motif (NBM). The N-terminal alpha helix 2 may reside in the ER membrane to form 5th transmembrane domain. Its N-terminal resides in ER lumen while C-terminal resides in cytoplasm.

5. Immunological response

HCV NS4B was used in the first generation of anti-HCV commercial assays[35]. NS4B significantly activated the NF- κ B-associated signaling pathway and might modulate the production of various cytokines and inflammatory responses in HCV-infected liver[36]. The activation of NF- κ B may result in the production of IL-8 since IL-8 promoter contains the binding site for NF- κ B and Polyak *et al*[37] reported that the patients infected with HCV have elevated levels of IL-8[6]. Himoudi *et al*[38] showed that two HCV encoded epitopes (NS4B 1769–1777 and NS4B 1807–1816) displayed a predominant and particularly potent capacity to induce the proliferation of interferon (IFN) producing cells. These observations have been corroborated in at least one study performed with patients' cells[39]. Thus HCV NS4B also has a potential to be used as a peptide vaccine.

Another study indicates that HCV strains having NS4B (Q1737H) substitution confer IFN resistance thus suggesting

a possible role of NS4B in causing interferon resistance^[40,41]. Further studies are needed however to confirm this hypothesis.

6. Role in HCC

The HCV NS4B has been reported to be involved in the formation of tumor^[42]. Recently, it has been suggested that NS4B GTPase activity might play a role in NS4B-induced cellular transformation and tumor formation^[28]. Clinical studies in this context remain to be investigated.

7. NS4B as a membrane web inducer and its role in replication complex

Plus stranded RNA viruses are known to induce alterations in cellular membranes and then utilize it to replicate their own genomes. In replicon-bearing cells, HCV non-structural proteins, RNA and RNA dependent RNA polymerase (RdRP) activity associate with ultrastructural vesicular structures termed the ‘membranous web’, which also resembles the membrane alterations seen in hepatocytes from HCV-infected liver^[20,43]. Studies indicate that both HCV NS4A/B and NS4B alone caused the accumulation of clustered, aggregated membranes and localized to these membranes. These swollen vesicles were suggested to be ER-derived membranes^[44].

NS4B protein has been found to interact with other nonstructural (NS) proteins (NS3, NS4A, NS5A and NS5B), all of which are involved in HCV RNA synthesis^[20,43,45–47]. Membranous web structures were also found in cells harboring HCV replicons^[20]. Colocalization of HCV NS4B with other HCV nonstructural proteins suggest that NS4B might provide the scaffold for the formation of the HCV replication complex (RC)^[18]. Mechanism and evidence about physical interactions amongst these replication complex proteins are yet to be fully understood.

NS4B of bovine viral diarrhea virus (BVDV) has also been demonstrated to be chemically cross-linked to both NS3 and NS5A exhibiting their association^[48]. Egger *et al.*^[43] showed that expression of NS4B alone also induces formation of the membranous web. NS3 is known to modulate NS5B’s RNA dependent RNA polymerase (RdRp) activity via its helicase function thus facilitating NS5B activity, while NS4B acts as an antagonist^[49]. NS4B, NS3 and NS5A are mislocalized in cells expressing NS4B V233R and L237E mutant proteins^[50] suggesting that NS4B might be involved in the localization of these proteins as well.

Egger *et al.*^[43] using electron microscope has shown that the entire HCV polyprotein induced a novel membrane structure termed as the “membranous web” derived from the ER which appeared to consist of vesicles within a membranous matrix. Similar structures have also been described by Pfeifer *et al.*^[51] in livers of HCV-infected chimpanzees. NS4B AH2 is positioned in the membrane in such a way that it helps in the formation of membranous web. Any change in the primary sequence can disrupt its hydrophobicity as well as the interaction with membrane^[52]. It has also been hypothesized that NS4B’s AH2 is a key determinant for the formation of MAFs during HCV replication. Studies with NS4B inhibitor anguizole showed that it disrupt the assembly of lipid membrane into these MAFs and results in the cytoplasmically distributed elongated snake-like structures of HCV NS4B^[53].

Studies have shown that NS4A and NS4B proteins inhibited both cap-dependent (host) translation and cap-independent

(virus internal ribosome entry site (IRES) mediated) translation from HCV IRES. It has been suggested that the latter might be a viral self regulation mechanism limiting the amount of viral protein^[7]. In contrast, in other experiments using similar constructs, IRES-mediated translation was found to be specifically up regulated in HCV replicon cells. The discrepancies in results of both experiments may be due to differences in duration and levels of expression of NS4B, or the presence of a critical host cell factor^[54]. Further investigations are needed in this context which will lead to better understanding of these mechanisms.

HCV NS4B has a predicted N-terminal amphipathic helix (AH) similar to the one present in HCV NS5A, possibly involved in its anchorage to the ER membrane, conserved in all HCV isolates, suggesting that it plays a critical role in productive natural infections^[55]. Mutational studies confirm that any change in sequence causes loss of membrane association, resulting in disruption of HCV RNA replication partially or completely^[23]. In the presence of mutated NS4B AH, it retains a reticular staining pattern suggestive of ER localization, but it is unable to be further sub localized into the characteristic speckles. Thus the NS4B AH may be responsible for mediating the association of NS4B and replication complex components with lipid rafts. The AH is also hypothesized to play a role in membranous web formation. Interestingly, a second AH has also been identified within NS4B, which may also play an important role in the viral life cycle but needs further investigation^[23,56].

As described earlier, NS4B contains a NBM, which specifically binds to GTP. Binding of NS4B to GTP and its capacity to hydrolyze nucleotides has been confirmed through mutational studies. These studies indicate that mutations not only disrupt its GTPase activity but also abolished replication^[31]. Moreover NBM is conserved among all HCV isolates of all genotypes. NBM mediates binding of nucleotides not only as single molecules but also as part of a polynucleotide structure such as RNA. By simultaneously binding cellular membranes and RNA, NS4B might contribute to the structural integrity of the replication complex by facilitating anchorage to other membranes^[31]. Altogether these studies suggest that the NBM within NS4B is somehow essential for mediating NS4B’s role in HCV replication *in vitro*.

HCV has also been shown to induce ER stress^[57]. In a recent study which utilized yeast two hybrid assay, cyclic AMP-response-element-binding protein-related protein (CREB-RP), also called ATF6 β was identified to interact with NS4B where ATF6 is a transcription factor activated to alleviate ER stress when protein folding is disrupted^[8]. The N-terminal half of NS4B and a central portion of CREB-RP/ATF6 β containing the basic leucine zipper (bZIP) domain were shown to be involved in this interaction. It was also observed that NS4B strongly interacts with ATF6 β and only weakly interacts with ATF6 α . Interestingly, ATF6 β suppresses transcription of ER stress-inducible genes while ATF6 α enhances it^[58]. Such interactions of HCV NS4B with ATF6 may cause the divergence of ER components to form membranous web.

NS4B has also been reported to bind to the 3’ end of negative strand viral RNA^[28], suggesting that NS4B might tether HCV RNA onto the membranous web and facilitate positive sense RNA synthesis. That’s why the inhibitors against NS4B are hot topic for the study as anti HCV therapy now a days^[59], like (clemizole) that can disrupt the binding of NS4B to negative strand RNA molecule^[60,61]. The binding of NS4B to negative strand is shown to mediate through arginine rich-like motif within NS4B^[62].

The CTD of NS4B is shown to interact with viral RNA[28]. A recent report has also shown that the NS4B CTD is partially responsible for NS4B association with host membranes[55], while N-terminal contains two alpha helices involved in NS4B host membranes association[23,29]. It is also reported that NS4B interacts with Rab[5], an early endosome protein with a putative role in the formation of the HCV replication complex[64].

Recently it has been studied that charged residues in the HCV NS4B N and C terminal domain are involved in many functions like formation of membranous web which lead towards the HCV replication[65]. Qingxia *et al* mapped a conserved transmembrane domain (GXXXG) in NS4B which is required for HCV Replication[66].

Collectively several NS4B structural elements are involved in membrane anchoring and formation of web beside the TM1 and TM2. Sequences upstream of TM1 including two putative amphipathic alpha-helices and a Leucine Rich Repeat-like motif which is conserved in all HCV genotypes are required. The N-terminal 55 peptidic sequence, containing the 1st amphipathic helix, mediates association with cellular membranes including the ER membrane while the C-terminal 70 peptidic sequence is also required for anchorage[67].

NS4B, a non-structural protein of hepatitis C virus, is a poorly characterized protein. Ambiguities are found about its structure and membrane anchorage especially about its N-terminal domain. It seems to play a crucial role in HCV replication, development of HCC and in the initiation of immune responses. It has acquired a prominent position in the viral life cycle as it induces formation of the membranous web. Moreover, the selective inhibition of NS4B-induced membranous web formation could represent a specific antiviral strategy. Highly focused investigations of this viral protein are needed in order to fully characterize HCV NS4B and ultimately use the findings for development of antiviral approaches as well as its use as a potential peptide vaccine candidate.

Conflict of interest statement

We declare that we have no conflict of interest.

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