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Review

NS5A interacting proteins and progress in anti-hepatitis C virus research

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ABSTRACT

The non-structural protein 5A (NS5A), which is unique among hepatitis C virus (HCV) non-structural proteins, is a proline-rich hydrophilic phosphoprotein that is essential for viral RNA replication and play a role in disrupting host intracellular signaling pathways. Although no intrinsic enzymatic activity has been ascribed to NS5A, this protein likely exerts its functions through interaction with viral and host cellular factors, as numerous NS5A-interacting cellular proteins have been identified. Recent studies using an HCV-infected cell culture system to model HCV RNA replication have suggested that NS5A inhibition is a promising therapeutic strategy for the treatment of HCV. Most selected inhibitors against HCV replication derived from genotype 1b were targeted to NS5A. This review summarizes the progress made towards understanding the role of NS5A in viral replication and HCV-mediated hepatocarcinoma for the development of possible inhibitors for HCV therapy.

KEYWORDS: hepatitis C, non-structural protein 5A, viral replication, inhibitor, host factors

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INTRODUCTION

Chronic infection with hepatitis C virus (HCV) carries with it a substantial risk of severe liver conditions such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The interactions between cellular proteins and HCV gene products may provide clues to novel approaches for interfering with viral propagation and pathogenesis. In particular, the HCV non-structural protein 5A (NS5A) is a phosphorylated 56- to 58-kD protein that promotes viral pathogenesis and has been reported to interact with Grb2, p53, Cdk1, Pitx1, TRAF-2, karyopherin b3, SNARE-like protein, hVAP-33 (the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein), and amphiphysin II [1-10]. The interferon sensitivity-determining region (ISDR) of NS5A may be associated with the sensitivity of viral isolates to IFN treatment [11], however, this observation is controversial. The complex multi-mechanistic role of NS5A in promoting viral persistence, pathogenesis and, indirectly, virus-related hepatocarcinogenesis, suggests that this protein is important for HCV pathobiology. The search for anti-HCV replication inhibitors has identified several agents that target NS5A, suggesting that NS5A is important for HCV replication [12-14]. The structure of NS5A, including its phosphorylation and amino acid substitutions, affects HCV replication, which has led to the development of several NS5A inhibitors. Although numerous interacting proteins with NS5A have been reported, the effect of NS5A interaction with other HCV NS proteins and host

cell factors on NS5A function and hepato-carcinogenesis remain unclear. To better understand these interactions, it may be necessary to evaluate NS5A inhibitors on cellular transformation by HCV.

Hepatitis C virus

HCV is an enveloped, positive-stranded RNA virus that is a member of the Flaviviridae family, which includes the genera Flavivirus, Pestivirus, and Hepacivirus. The single-stranded 9.6 kbp HCV RNA genome consists of a single open reading frame that encodes a polyprotein of ~3010 amino acids (aa). The flanking 5' and 3' untranslated regions (UTRs) of the HCV RNA genome contain cis-acting signals that are important for the initiation of viral RNA translation and replication [15].

Upon translation, the HCV polyprotein is processed proteolytically by both cellular and viral proteases into at least 10 individual proteins, including four structural (core, E1, E2, and p7) and six non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig.1). NS3 forms a complex with NS4A, which acts as a proteinase to cleave other NS proteins, and also displays helicase activity and NS5B is thought to function as a viral RNA polymerase, while NS5A is a pleiotropic viral regulatory protein that is important for viral RNA replication and modulating host cell physiology. NS5A is localized primarily in the ER membrane and is a major component of the HCV replicase, a multi-component replication complex that comprises NS4 and NS5B, in addition to several other host cell factors [16, 17]. As described in this review, NS5A has recently been shown to interact with numerous host regulatory proteins and factors to modulate the host cell cycle, apoptosis, and stress-responsive pathways, including cell transformation [18]. The study of the cellular interacting partners with NS5A will lead to the identification of functionally relevant aspects, which provides

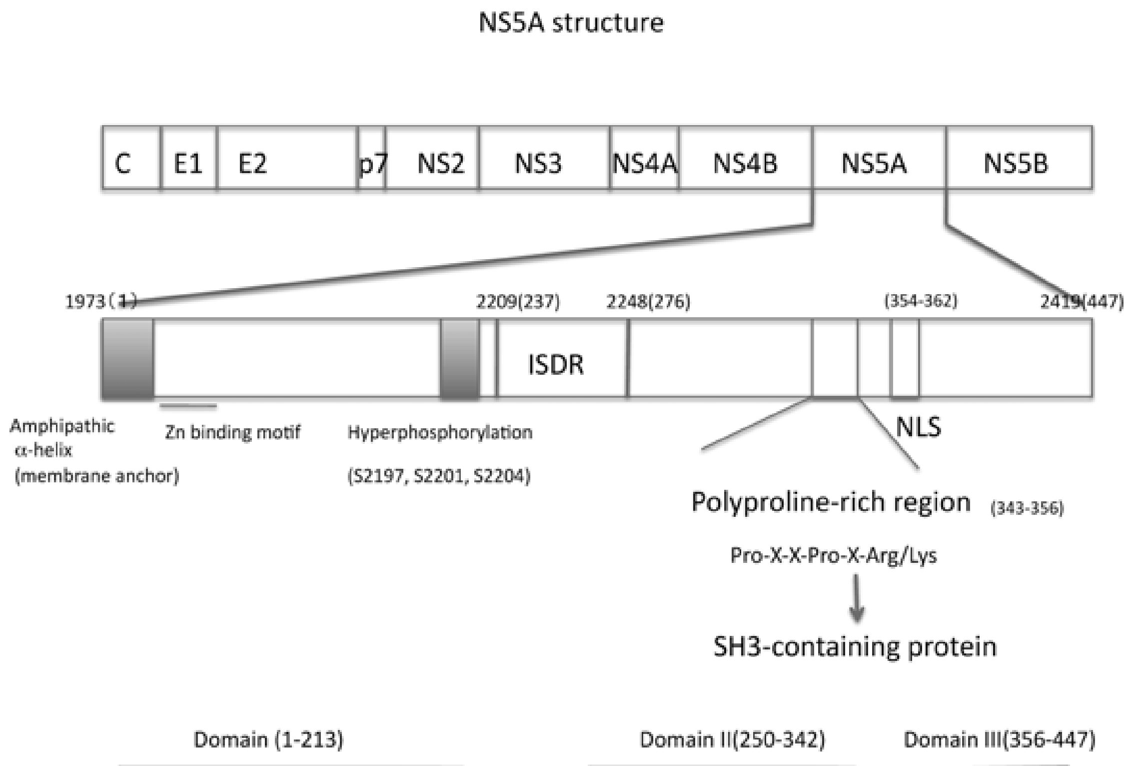

Fig. 1. A schematic representation of the HCV virus genome and nonstructural NS5A protein. HCV genome processes the structural and non-structural proteins. The amino acid positions of NS5A protein are shown based on HCV genotype 1b.

a deeper understanding of HCV biology and the development of novel antiviral strategies.

Here, in this review, we describe the interactions between NS5A and human host factors, and discuss possible NS5A inhibitors with therapeutic potential.

Structural features of NS5A

NS5A is a proline-rich hydrophilic phosphoprotein of 447 aa that forms dimers [19] and displays a variety of functions, including participation in polyprotein cleavage and interaction with host proteins (Fig. 1). A 30-aa sequence within the N-terminus of NS5A is highly conserved and is predicted to form an amphipathic alpha helix. This conserved region is necessary for the association of NS5A with endoplasmic reticulum (ER) membranes, and the amphipathic characteristics of the helix are retained in all HCV genotypes [20]. NS5A also has an unconventional, cysteine-rich zinc-binding motif within the N-terminal domain that is vital for HCV replication. NS5A contains four serine residues that are phosphorylated [21, 22], which are found in a region termed a hyperphosphorylation cluster and represent basal phosphorylation. The ISDR, which may be associated with the sensitivity of viral isolates to IFN treatment, in combination with 24 residues within the C-terminus binds and inactivates protein kinase R (PKR) [23]. In addition, NS5A has polyproline cluster and nuclear localization signal (NLS). The polyproline cluster within NS5A contains two proline-rich motifs in close proximity. Two classes of these motifs have been defined, which bind to the SH3 domain within several cellular signaling proteins, including Grb2 (1), amphiphysin II (10), and Src-family tyrosine kinases [24].

NS5A consists of three domains I, II and III. Domains I and II of NS5A interacts with the polypiridine region of 3'UTR RNA [25, 26] and this binding is reported to be important for RNA replication.

NS5A phosphorylation

Two phosphorylated forms of NS5A, termed p56 and p58, have been distinguished on the basis of their electrophoretic mobilities. A well established ratio between NS5A p56 and p58 is reported to be required for productive replication/infection of HCV [27]. NS5A p56 is constitutively phosphorylated at residues located in the center and C-terminus of the protein, while p58 represents a hyperphosphorylated form.

NS5A stably associates with casein kinase II (CK2) and cAMP-dependent protein kinase (PKA), with both kinases phosphorylating NS5A *in vitro*. In addition, NS5A is phosphorylated by Akt, p70S6K, casein kinase I (CKI), and the mitogen-activated protein kinases (MAPK) kinases MEK1, MKK6, and MKK7-1 [28]. Quintavalle *et al.* demonstrated that the isoform of protein kinase CKI is a target for NS5A hyperphosphorylation [29, 30]. NS5A hyperphosphorylation in cells is inhibited by CKI inhibitors and silencing of CKI-α inhibitors and inhibition of NS5A-p58 was rescued by CKI-α overexpression [30]. NS5A p58 is hyperphosphorylated within a serine-rich region in the center of the protein; however, the lack of putative phosphorylation site consensus sequences has made it difficult to definitively identify the phosphorylated residues. Despite this challenge, Katze *et al.* demonstrated that Ser 222 (corresponding to Ser 2194) is a conserved phosphorylation site [31], while the centrally located serine residues Ser 225, Ser 229, and Ser 232 (corresponding to Ser 2197, Ser 2201, and Ser 2204, respectively of the HCV polyprotein) are reported to be NS5A hyperphosphorylation sites [22, 23].

Although differential NS5A phosphorylation may modulate HCV RNA replication, it is not known how NS5A phosphorylation affects the binding to other NS proteins such as NS4A or NS5B, and host factors.

NS5A-dependent modulation of HCV RNA replication

Phosphorylated NS5A binds human vesicle-associated membrane protein-associated protein A (hVAP-A) [8, 32], which in turn may facilitate or stabilize NS5A interaction with NS5B to regulate the polymerase activity of this NS protein. Hyperphosphorylation prevents NS5A from constitutively binding to hVAP-A, resulting in disruption of the replicase complex and decreased viral RNA replication. Thus, hVAP-A is a critical regulator of HCV RNA replication,

and the expression of truncated hVAP-A dominant-negative mutants or siRNA against hVAP-A decreases HCV RNA and protein levels in HCV replicon cells [8]. Therefore, it is necessary to examine whether hVAP-A modulates CKI-mediated NS5A hyperphosphorylation. hVAP is one of typical interacted proteins with NS5A, which is well examined to participate in both RNA replication and phosphorylation of NS5A.

Role of NS5A in the IFN response

The role of NS5A in the interferon (IFN) response has been the subject of previous reviews, and will therefore only be briefly described here [23, 33]. Molecular epidemiological studies of HCV-infected patients identified a 40-aa stretch of NS5A that is conserved in IFN-resistant HCV isolates of genotype 1b. Patients with HCV variants harboring mutations within this region are more sensitive to therapy, suggesting that NS5A confers IFN resistance. Thus, this region within NS5A is termed the IFN sensitivity-determining region (ISDR). Significantly, mutations within the ISDR accumulate in HCV-infected patients during IFN therapy [34] and in chimpanzees that develop chronic infection following HCV inoculation [35]. Although patients with number of mutations within the ISDR achieved a sustained virologic response (SVR) to IFN-α therapy [36], the involvement of the ISDR of NS5A in HCV antiviral responses remains controversial. Gale *et al.* reported that NS5A binds and subsequently inactivates PKR, an IFN-induced gene product that is activated upon binding to dsRNA produced by viral genome replication [37]. The interaction between NS5A and PKR is mediated through the ISDR and an additional 26 amino acids distal to the ISDR, which is termed the PKR binding domain (PKRBD). The introduction of multiple mutations within the PKRBD region abrogated the ability of NS5A to bind PKR [38, 39].

To determine the influence of ISDR sequence variation on HCV replication, HCV isolates containing various ISDR mutations were examined both *in vitro* and *in vivo*. The SVR rate to IFN therapy varied significantly in patients infected with different HCV genotypes. However, in cell culture systems, IFN-α-induced ISRE-luciferase activity was inhibited by both genotypes 1b and 2a NS5A protein expression [40]. Furthermore, the inhibitory effect of NS5A on IFN-α signaling was enhanced by IL-8 induced by NS5A from both genotypes in the cultured cells [40]. No significant differences of IFN responsiveness in HCV patients between genotypes 1b and 2a were detected in the *in vitro* culture system. The differences between the patients and cell culture system in a genotypic IFN resistant analysis suggest the role of other host factors, which is implicated in IFN responsiveness of HCV patients.

NS5A interacts with SH3 domains

HCV NS5A has a conserved proline-rich (PxxP) region that forms a class II Src homology 3 (SH3) binding motif. Therefore, it is not surprising that NS5A interacts with several SH3 domain-containing proteins. The first such identified interacting protein was Bin1/amphiphysin II (box-interacting protein-1 or bridging integrator protein-1), a pro-apoptotic tumor suppressor. The function of Bin1 was initially identified based on its ability to interact with c-myc and inhibit malignant transformation by this oncogene [41, 42]. Amphiphysin II has several isoforms, and muscle type amphiphysin II is a ubiquitously expressed isoform that localizes to the cytoplasm and nucleus, and is known to activate a caspase-independent apoptotic process [43, 44]. Although widely expressed in normal cells, amphiphysin II has been reported to exist in a functional form in 50% of carcinoma cell lines and primary breast carcinomas examined.

Bin1/amphiphysin II contains an N-terminal Bin/Amphiphysin/Rvs (BAR) domain, a C-terminal SH3 domain, and a c-Myc-binding domain preceded by a nuclear localization signal (NLS) in the central region. As expected, deletion of the Bin1 SH3 domain alone abolishes the protein's interaction with NS5A [9, 45]. To further evaluate the role of the SH3-binding motif of NS5A in this interaction, three conserved proline residues within this region were mutated. The resulting NS5A mutant was unable to interact with Bin1, which indicates that the proline-rich region is the primary mediator of the NS5A interaction [10].

Previously, we also demonstrated that an SH3 domain is required for NS5A interaction with

Bin1 in human cell cultures (9). Another SH3-containing protein c-Src is involved in an NS5A interaction. Macdonald *et al.* showed that NS5A interacts with the SH3 domains of members of the Src family of tyrosine kinases, Hck, Lck, Lyn and Fyn, but not Src itself (24). NS5A binds c-Src in cell culture but the NS5A interaction with c-Src may be weaker than its interaction with other Src family members. Macdonald *et al.* also demonstrated that NS5A interacted with native Src-family kinases *in vivo* and differentially modulated their kinase activity: NS5A inhibited Hck, Lck and Lyn but activated Fyn, which suggested a role for the interactions between NS5A and Src-family kinases in viral pathogenesis.

In vitro kinase assays demonstrated that Bin-1-NSA interaction inhibits the phosphorylation of NS5A [9]. In addition, Neddermann *et al.* demonstrated that hyperphosphorylation of NS5A inhibits HCV RNA replication [27]. Furthermore, Evance *et al.* reported that hyperphosphorylation of NS5A disrupts its interaction with hVAP-A and negatively regulates viral RNA replication [32]. Thus, interaction with Bin1 may also be necessary to limit the hyperphosphorylation of NS5A and promote HCV RNA replication in HCV-infected cells. To gain insight into NS5A regulation, it is necessary to further examine the function of Bin1 and other NS5A-interacting proteins, such as hVAP-A. These proteins may be regulated through conformational changes upon interaction with NS5A. Bin1, which is thought to regulate NS5A phosphorylation, may regulate NS5A-hVAP-A interaction through its SH3 region. Therefore, the reduction of NS5A phosphorylation through interaction with Bin1 may support the NS5A-hVAP-A interaction and promote HCV replication. However, Zech *et al.* demonstrated that the disruption of NS5A-Bin1 interaction does not inhibit subgenomic HCV replication and found that NS5A-Bin1 complex formation appears to be dispensable for HCV RNA replication [10]. Nanda *et al.* reported that NS5A-Bin1 interaction is implicated in productive HCV infection and may contribute to the pathogenesis of hepatocellular carcinoma [45]. Given that Bin1 is an oncoprotein that enhances cellular transformation, NS5A may induce hepatocarcinogenesis through its interaction with Bin1.

Hughes *et al.* studied interaction of two poplyproline motifs in NS5A with HCV replication [46]. N-terminal polyproline motif, which is conserved in genotype 1 isolates, is only required for genotype 1b RNA replication. Mutation of proline 346 within N-terminal motif abrogated genotype 1b replicon replication, but the corresponding proline 342 mutation was not required for the genotype 2a replication [46]. In addition, mutational analysis of C-terminal polyproline motif, which is conserved throughout all HCV isolates and important region for binding amphiphysin II/Bin1, showed that the interactions between NS5A and SH3 adapter proteins are involved in pathogenesis or persistence of the virus in host rather than viral replication [46].

NS5A inhibitors for HCV therapy

HCV viral RNA replication and the screening of antiviral compounds have been examined by cell-based high-throughput HCV replicon screening, which identified several anti-HCV agents [12-14]. Among these agents, BMS-858 and BMS-824 were identified as potent HCV replication inhibitors. To determine the HCV target of these inhibitors, an *in vitro* replicon system was used to identify HCV inhibitor-resistant variants. During the isolation of the inhibitor-resistant replicons, three mutated clones were found to be resistant and these mutations were present in the NS5A N-terminal region [12]. Notably, the N terminus of NS5A was found to be important for drug sensitivity: this is a region that is believed to be involved in dimer formation (particularly the first 76 amino acids), RNA replication, RNA binding, and membrane association of the replication complex. In addition, chimeric analyses have revealed that the 76 N-terminal residues of genotype 1b, but not genotype 1a, are sensitive to BMS inhibitors [12]. These inhibitors also block the formation of p58, the NS5A hyperphosphorylation form, which suggests that a correlation exists between the blockage of p58 formation and the inhibition of replicon replication. CKI-α isoform inhibitors reduce NS5A hyperphosphorylation and HCV RNA replication [29, 30]. However, because it was previously concluded that reduced NS5A hyperphosphorylation activates HCV RNA replication, the hypothesis that the well established

ratio between NS5A-p56 and NS5A-p58 is required for productive replication/infection was formulated [27].

Upon screening of a panel of kinase inhibitors, three compounds that inhibited NS5A phosphorylation *in vivo* and the formation of NS5A p58 in cell culture were identified [27]. These kinase inhibitors, which were isolated from compounds with a general structure similar to that of the ATP-competitive 2,4,5-trisubstituted imidazole inhibitors, inhibited NS5A-p58 formation both *in vitro* and in cell culture systems. Using these kinase inhibitors, they demonstrated that high levels of NS5A-p58 cause inhibition of HCV replication in cell culture, but this inhibition can be relieved by kinase inhibitors. Taken together, low amounts of NS5Ap58 is necessary for viral replication, but the kinase inhibitors could completely inhibit NS5A hyperphosphorylation to reduce HCV RNA replication.

Gao *et al*. investigated the activity of BMS-790052, a potent NS5A inhibitor and showed its potential activity towards all genotypes tested (1a, 1b, 2a, 3a, 4a and 5a) [14]. Clinical validation of BMS-790052 offered potential as part of therapeutic regimen based on combination of HCV inhibitors. BMS-790052 exhibited an additive to synergistic effect when combined with IFN-α, NS3 protease or NS5B polymerase inhibitors respectively.

Another group of potential NS5A inhibitors that are potential candidates for HCV therapy are the cyclophilin inhibitors, such as cyclosporine A, which block HCV replication both *in vitro* and *in vivo*. Cyclosporine A inhibits the direct interaction between cyclophilins and hepatitis C NS5A [47, 48]. Cyclosporine A binds to the hydrophobic pocket of cyclophilin A, which inhibits the isomerase activity of cyclophilin A and effectively prevents it from binding to NS5A. A single point mutation in NS5A, D320E, was found to confer resistance to cyclosporine A [49], but still allowed interaction with cyclophilin A that was similar to wild-type NS5A [48]. HCV resistance to cyclosporine A does not correlate with the resistance of the interaction of NS5A with cyclophilin inhibitors. The NS5AD320E mutant will lead to the new avenues for HCV therapeutic methods.

In a recent study, the type I IFN-inducible, ubiquitin-like protein ISG15 modified the NS5A protein, which may contribute to an anti-HCV effect. The inhibitory effect of ISG15 and ISGylation on NS5A was efficiently blocked by the lysine to arginine substitution at residue 379 within the C-terminal region of NS5A [50]. K379 is conserved in most HCV genotypes and exists in the polyproline cluster of NS5A. ISGylation directly controls NS5A protein stability: thus ISG15 represents a therapeutic tool for combined therapy with IFN against HCV infections.

It is tempting to speculate that NS5A could be an optimal target for developing therapeutic agents against HCV, based on the interaction with host factors. However, the correlation between the chemical inhibitors and most of the identified NS5A-interacting host factors is not very convincing. Even if HCV RNA is completely eliminated in infected patients, signals for promoting liver cancer can remain in host cells. It is necessary to develop the anti-liver-cancer therapeutic agents with NS5A as a potential target.

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