Minireview

Phosphorylation of hepatitis C virus NS5A nonstructural protein: A new paradigm for phosphorylation-dependent viral RNA replication?

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Abstract

The hepatitis C virus (HCV) nonstructural 5A (NS5A) phosphoprotein has been intensely studied due to its ability to subvert the host interferon-induced antiviral response. However, more recent studies suggest that it may also play an important regulatory role in HCV RNA replication as well as modulate host intracellular signaling pathways. Phosphorylation of NS5A appears to be a highly regulated process and several cellular protein kinases responsible for NS5A phosphorylation have been identified *in vitro*. Studies utilizing the HCV replicon cell culture system have suggested a provocative role for the differential phosphorylation of NS5A in the regulation of viral RNA replication through its association with the viral replication complex, including several host cell factors. Importantly, recent *in vivo* data linking loss of NS5A hyperphosphorylation to non-productive HCV replication in the chimpanzee model have provided high validation for targeting the cellular kinases involved, particularly the kinases responsible for NS5A phosphorylation, for antiviral therapeutic intervention. Understanding the process of NS5A phosphorylation and the definite identification of the culprit cellular protein kinase(s) will shed light on the mechanisms of HCV RNA replication and/or pathogenesis.
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Introduction

HCV is an enveloped, positive-stranded RNA virus and member of the Flaviviridae family, which includes three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. The single-stranded RNA genome of HCV codes for a single open reading frame, resulting in the translation of a single polyprotein of ~3010 amino acids (aa). The flanking 5′ and 3′ untranslated regions (UTRs) of the viral RNA genome contain *cis*-acting signals important for the initiation of viral RNA translation and replication (Reed and Rice, 2000). Upon translation, the HCV polyprotein is processed proteolytically by both cellular and viral proteases into at least 10 individual proteins, including four structural proteins (core, E1, E2 and p7) and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig. 1). An additional HCV protein, named F (frameshift) has been recently shown to be produced as a result of a −2/+1 ribosomal frameshift in the N-terminal core (C)-encoding region, but its role in the HCV lifecycle is not entirely clear. As the penultimate protein processed from the HCV polyprotein precursor, NS5A is a proline-rich hydrophilic phosphoprotein and may exist as a dimeric form (Tellinghuisen et al., 2005) (for a recent review of the NS5A protein, see He et al., 2006) (Fig. 1). Although no intrinsic enzymatic activity has been ascribed to
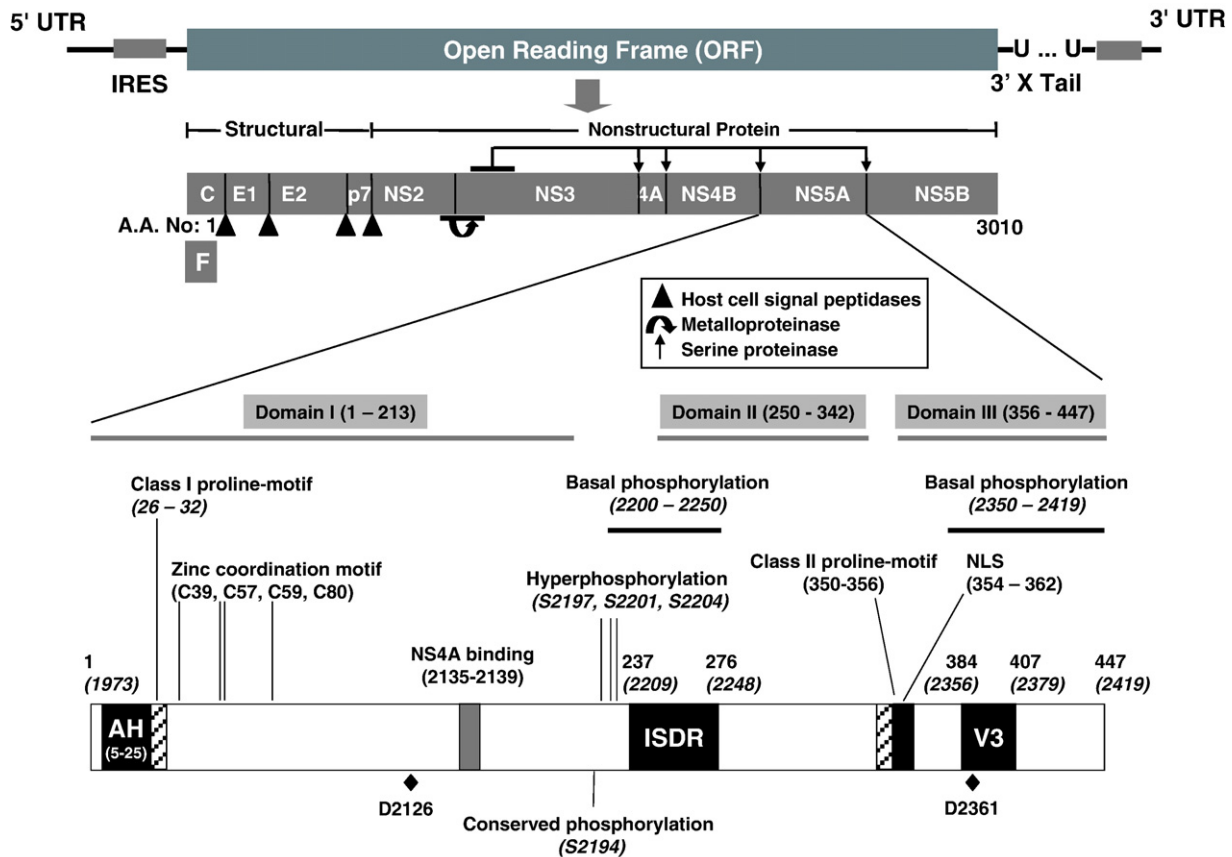
Fig. 1. Genome organization of HCV and functional structure of the NS5A protein. A schematic representation of the HCV genome indicating the position and processing of the structural and nonstructural proteins as well as the 5′- and 3′-UTRs, including the internal ribosomal entry site (IRES) and the poly-U-rich 'X' region, respectively. The cleavage enzymes are described in the above thick line box. The amino acid positions of NS5A protein are based on HCV genotype 1b (HCV-J). The three domain structure of NS5A (Tellinghuisen et al., 2004) is depicted. The N-terminal amphipathic α-helix (AH), NS4A-binding site, the IFN-sensitivity-determining region (ISDR), the class I and class II proline-rich motifs, the variable region V3, and NLS sequences are also shown. In addition, zinc coordination motif, conserved phosphorylation, basal and hyperphosphorylation sites, as well as caspase-mediates cleavage sites (D2126 and D2361) are noted.

NS5A, it likely functions through interaction(s) with other HCV NS proteins and host cell factors. Here, we review our current understanding of NS5A phosphorylation and the emerging evidence supporting a model for NS5A phosphorylation-dependent modulation of HCV RNA replication.

NS5A: a viral Swiss army knife

NS5A first became a focal point of HCV research when it was reported that mutations within a region of *NS5A*, termed the IFN-sensitivity-determining region (ISDR), from HCV geno-type 1b isolates correlated with the treatment efficacy of IFN-α (for a historical account on the role of NS5A in confounding IFN-mediated antiviral responses, see Tan and Katze, 2001). This finding was important because (i) IFN-α, in combination with the nucleoside analogue ribavirin, remains the current standard treatment for HCV infection and (ii) sustained viro-logical response to the therapy differs substantially among HCV genotypes in that patients infected with HCV genotypes 2 and 3 generally respond better to IFN-α than those infected with HCV genotype 1. While the predictive value of the ISDR in patient's response to IFN therapy has been called into question, this nevertheless provided the first hint of a potential functional role

for NS5A. Indeed, subsequent studies have supported the role of NS5A in counteracting the host IFN response; potential mechanisms include up-regulation of IL-8 and inhibition of the IFN-induced dsRNA-dependent protein kinase (PKR) (see discussion below).

More recently, NS5A has been implicated in the modulation of host cell cycle, apoptosis, and stress-responsive pathways. Numerous cellular interacting proteins of NS5A have been identified, including TATA box binding protein (TBP) (Qadri et al., 2002), p53 (Lan et al., 2002; Majumder et al., 2001; Qadri et al., 2002), a novel cellular transcription factor SRCAP (Ghosh et al., 2000), karyopherin β3 protein (Chung et al., 2000), apo-liprotein A1 (Shi et al., 2002), and a human vesicle-associated membrane protein A of 33 kDa (hVAP-A or hVAP-33) (Evans et al., 2004; Tu et al., 1999), to name a few. Recently, several laboratories have demonstrated interactions between the proline-rich motifs (PxxP) in NS5A and the Src homology 3 (SH3) domains of a variety of proteins involved in signal transduction. These include growth factor receptor-binding protein 2 (Grb2) (Tan et al., 1999), the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (He et al., 2002), Src tyrosine kinases (Macdonald et al. 2004), Bin-1 (Nanda et al., 2006), and amphiphysin II (Zech et al., 2003). However, the functional consequences of these

NS5A–host protein–protein interactions during HCV infection have not been elucidated.

NS5A is localized mostly to the endoplasmic reticulum (ER) membrane and believed to be a component of the HCV replication complex comprising NS2 to NS5B, also known as the replicase. The membrane-anchor region of NS5A has been mapped to the amino-terminal 30 aa, which contains an amphipathic α-helix that is highly preserved among HCV isolates (Brass et al., 2002). Interestingly, nuclear localization of NS5A has also been reported (Ide et al., 1996), and caspase-mediated cleavage products of NS5A, which lack the membrane-anchor region, are also detected in the nucleus under certain apoptotic conditions (Goh et al., 2001; Kalamvoki and Mavromara, 2004; Satoh et al., 2000). Coincidentally, the carboxyl-terminal domain of NS5A, including the ISDR, has been shown to exert potent *trans*-activating activity, suggesting that NS5A might function as a viral transcriptional activator (Chung et al., 1997; Kato et al., 1997; Tanimoto et al., 1997). Moreover, the transcriptional activity of NS5A appeared to be affected by mutations in the ISDR. However, a connection between NS5A transcriptional activity and IFN response has not been demonstrated.

The subcellular localization of NS5A in the HCV replication compartments suggests that it may play a role in HCV RNA replication. Furthermore, NS5A was shown to bind and regulate the polymerase activity of HCV RNA-dependent RNA polymerase, NS5B (Shimakami et al., 2004; Shirota et al., 2002). Additionally, NS5A has been shown to bind to the 3′-UTR of HCV plus and minus strand RNAs and may represent a new class of RNA-binding protein (Huang et al., 2005). In the HCV replicon systems, cell culture-adapted mutations clustering in the *NS5A* region were found to confer higher RNA replication efficiency (Blight et al., 2000; Guo et al., 2001; Krieger et al., 2001; Lanford et al., 2003; Lohmann et al., 2003). Interestingly, some of these mutations affected NS5A phosphorylation, suggesting that phosphorylation may play a role in HCV RNA replication. In light of these and aforementioned features of NS5A, the precise roles of the different phospho-forms of NS5A as well as the identification of the responsible host cell protein kinases have been the subject of active investigation.

NS5A phosphorylation: a functional role or red herring?

NS5A primarily exists as two phosphorylated forms, migrating as 56 and 58 kDa proteins on SDS-PAGE gels (Kaneko et al., 1994; Tanji et al., 1995b). The two major phospho-isoforms are also referred to as basally phosphorylated and hyperphosphorylated, respectively. Pulse-chase and mutational analysis indicate that both the basal and hyperphosphorylation of NS5A only occur after completion of the proteolytic cleavages at the N- and C-termini of NS5A, implying that phosphorylation depends on proper cleavage and/or NS5A protein conformation (Kaneko et al., 1994; Koch and Bartenschlager, 1999; Neddermann et al., 1999; Tanji et al., 1995b). In addition, the association of other HCV NS proteins, including NS4A (Asabe et al., 1997) and NS2 (Hijikata et al., 1993), may affect the phosphorylation of NS5A. The NS4A interactive region on NS5A has been mapped to aa 2135 to 2139 (Asabe et al., 1997).

It is likely that a conformational change accompanies NS5A phosphorylation, which may make it vulnerable to further phosphorylation by the same or different cellular protein kinases. These additional phosphorylation events, leading to the formation of p58, apparently requires the presence of NS3, NS4A and NS4B (Koch and Bartenschlager, 1999; Neddermann et al., 1999; Tanji et al., 1995a). While the functional significance is not clear, this type of sequential phosphorylation has been proven significant in the replication of other RNA viruses.

The degree of NS5A phosphorylation does not appear to determine its localization within the cell as there was no significant difference noted in the subcellular location of the two major phospho-isoforms of NS5A (Tanji et al., 1995b). Interestingly, the degradation and half-life of the NS5A protein appear to be affected by phosphorylation; NS5A protein level has an inverse relationship with its hyperphosphorylation (Pietschmann et al., 2001). Finally, the phosphorylation of NS5A is a conserved trait among divergent HCV isolates, and among other members of the Flaviviridae (for NS5) (Reed et al., 1998), suggesting that it may play an important role in the *Flavivirus* life cycle. Taken together, these complex patterns and regulation of NS5A phosphorylation suggest NS5A could have an important role in HCV biology.

As mentioned above, some of the cell culture-adapted mutations in NS5A that render reduced NS5A hyperphosphorylation confer higher RNA replication efficiency in the HCV replicon system. Consistent with this notion, suppression of NS5A hyperphosphorylation through either the use of small-molecule kinase inhibitor (Neddermann et al., 2004) or mutagenesis studies (Appel et al., 2005) also allowed higher levels of RNA replication in non-culture-adapted replicons. However, HCV RNA replication was inhibited when cells carrying adapted replicons were treated with the same kinase inhibitor, thus strongly suggesting that NS5A phosphorylation is an important regulatory mechanism for the viral RNA replication. Surprisingly, mutations within the region affecting basal phosphorylation and a deletion removing aa 2380–2409 of NS5A did not have a significant impact on viral RNA replication in a replicon system (Appel et al., 2005). It was thus proposed that perhaps a critical ratio between the p56 and p58 phospho-forms of NS5A is required for productive HCV RNA replication (Neddermann et al., 2004). In keeping with this notion, cell-culture-adaptive mutations known to decrease the level of hyperphosphorylated NS5A prevented efficient infection of a full-length RNA in the chimpanzee model (Bukh et al., 2002). Importantly, these data linking loss of NS5A hyperphosphorylation to defective HCV replication *in vivo* arguably provide the highest validation, in the absence of human or clinical data, for targeting the cellular kinases responsible for NS5A phosphorylation for HCV therapy. So, what are the relevant sites of NS5A phosphorylation and the responsible kinases?

Identification of NS5A basal and hyperphosphorylation sites

Initial studies focusing on the determination of HCV NS5A phosphorylation sites have been limited by the lack of an

efficient cell culture system for HCV infection and the poor recovery of phosphorylated NS5A from HCV-infected liver cell lysates. Labeling experiments and phosphoamino acid analysis revealed that NS5A in transfected cells is phosphorylated primarily on serine residues and, to a much lesser extent, threonine residues (Kaneko et al., 1994; Reed et al., 1997; Tanji et al., 1995b). NS5A deletion mutants indicated that at least two distinct regions, located around the center and nearby the carboxyl terminus (residues 2200 to 2250 and 2350 to 2419, respectively) of NS5A (Asabe et al., 1997; Kaneko et al., 1994; Reed et al., 1997; Tanji et al., 1995b), are required for basal protein phosphorylation (Fig. 1). Although the phosphate acceptor sites have not directly been mapped, site-directed mutagenesis suggested that three serine residues at positions 2197, 2201 and 2204 (upstream of the ISDR) are important for hyperphosphorylation of NS5A (Koch and Bartenschlager, 1999; Tanji et al., 1995b) (Fig. 1). Two of the serines, S2197 and 2204, when mutated concomitantly in the HCV replicon system led to reduced NS5A hyperphosphorylation and significantly higher viral RNA levels (Blight et al., 2000).

In order to map the phosphorylation sites more directly, a study using recombinantly expressed and purified NS5A combined with two-dimensional (2D) phosphopeptide mapping and sequencing identified a major phosphorylation site in the HCV 1a genotype NS5A (Reed and Rice, 1999). However, the phosphorylation site, Ser-2321, is not conserved across HCV genotypes. Using a different approach that combines 2D phosphopeptide mapping and electrospray ionization mass spectrometry, another group identified Ser-2194 as a predominant phosphorylation site in the NS5A protein derived from HCV 1b genotype (Katze et al., 2000). Both Ser-2194 and the phosphopeptide sequence containing Ser-2194, which spans NS5A residues GSPPSLASSSASQLSAPSLK, are highly conserved across HCV genotypes. However, since both studies used non-hepatocyte-derived cell lines as a source of mammalian protein kinases, it will be critical to determine the relevance of phosphorylation of Ser-2321 or/and Ser-2194 in HCV RNA replication in the natural host cell.

The search for NS5A-associated protein kinases

In studies utilizing chemical inhibitors of protein kinases, the kinase(s) associated with the NS5 protein of other *Flaviviruses*, including dengue virus type-2 (DEN-2), tick-borne encephalitis virus, Yellow Fever virus (YF), and the NS5A protein of the *Pestivirus* bovine viral diarrhea virus (BVDV) all displayed similar inhibition profiles (Reed et al., 1998). These similarities further suggest that these viral proteins are phosphorylated by one or more related cellular serine/threonine kinases. The properties of the NS5A-associated protein kinase (s) are summarized in Table 1.

Highly purified preparations of NS5A protein derived from HCV genotype 1b were phosphorylated efficiently when subjected to an *in vitro* protein kinase assay (Katze et al., 2000). This is consistent with previous reports that NS5A is associated tightly with a cellular protein kinase(s) (Ide et al., 1997). Host cell transcription does not appear to be necessary for NS5A phosphorylation since NS5A phosphorylation in transfected cells can occur in the presence of actinomycin D. Phosphorylation of NS5A *in vitro* reaches the highest level when the pH of the reaction is neutral or slightly alkaline. Interestingly, the NS5A-associated kinase activity has an unusual preference for Mn^{2+} over Mg^{2+}, is inhibited by the addition of Ca^{2+}, and appears somewhat resistant to the general protein kinase inhibitor staurosporine (Reed et al., 1997). The IFN-induced PKR protein kinase would fit these criteria as the putative NS5A-specific kinase. As previously mentioned, NS5A has been shown to confer viral resistance to IFN, possibly through its association with PKR via the ISDR region of NS5A. This sequence contains two of three serine residues which contribute significantly to the hyperphosphorylation of NS5A (Tanji et al., 1995b). However, phosphorylation of NS5A by purified PKR was not observed *in vitro* (Gale et al., 1997) nor was it stimulated by PKR activators such as dsRNA and heparin (Reed and Rice, 1999). In addition, NS5A with mutation of Ser-2194 to alanine retained the ability to interact with PKR (Katze et al., 2000).

Table 1
Summary of properties of NS5A-associated protein kinase(s) (adapted from Reed et al., 1997)

pH	Neutral or slightly alkaline							
Divalent cation concentration	10 mM Mn^{2+} or 2.5 mM Mg^{2+} ($Mn^{2+} > Mg^{2+}$)							
Temperature and length of incubation	37 °C, 2 h or 30 °C 4 h							
Candidates (serine/threonine protein kinases)	CKII, CDKs, MAPKs, GSK3, MEK1, MEK6, MEK7, AKT, p70S6K, and CKI							
Inhibitors tested	Name		Bisindolyl maleimide I–HCl	Olomoucine	H-89 [a]	DRB [b]	Hypericin	A-3 [c]	Staurosporine
	In vitro	Concentration [d]	1 μM	1 mM	10 μM	100 μM	1 μM	200 μM	10 nM
		Inhibition	–	73%	–	67%	<25%	<25%	<30%
	In vivo	Concentration [d]	10 μM	1 mM	10 μM	100 μM	N/A	N/A	1 μM
		Inhibition	–	+(36%)	±(9%)	–	N/A	N/A	+(22%)

N/A, not tested.
[a] *N*-[2-(*p*-bromocinnamyl-amino) ethyl]-5-isoquinoline-sulfonamide dihydrochloride.
[b] 5,6-Dichloro-1-β-D-ribofuranosyl benzimidazol.
[c] *N*-(2-aminoethyl)-5-chloronaphthalene-1-sulfonamide.
[d] Maximum concentration tested.

Analysis of the effects of a panel of kinase inhibitors on NS5A phosphorylation has suggested that the protein kinase responsible for the basal NS5A phosphorylation may be a member of the CMGC family, which includes CKII and proline-directed kinases such as the cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs), and glycogen synthase kinase 3 (GSK3) (Reed et al., 1997). The nature of the aa sequence flanking the phospho-site Ser-2321 would support a role for a proline-directed kinase in HCV NS5A basal phosphorylation (Reed and Rice, 1999). NS5A in fact contains several phosphorylation sequence motifs (–[SP]–) for proline-directed protein kinases such as CDKs and MAPKs. In this regard, it is interesting that NS5A has been implicated in modulating MAPK signaling (He and Katze, 2002; Tan et al., 1999) and cell cycle control (Arima et al., 2001; Ghosh et al., 1999). With respect to CKII, it is noteworthy that this protein kinase has been shown to bind to the C-terminal portion of NS5A and phosphorylate it *in vitro* (Kim et al., 1999). However, it is not known whether CKII phosphorylates the sites previously identified on NS5A. Furthermore, deletion of all of potential CKII phosphorylation sites in NS5A (Huang et al., 2005) and small-molecule inhibition of CKII (Huang et al., 2006) did not significantly affect HCV RNA replication in replicon systems.

In another approach, Coito et al. (2004) screened 119 recombinant yeast serine/threonine protein kinases for their abilities to phosphorylate NS5A *in vitro*. In addition to confirming CKII as a potential NS5A kinase, several human counterparts of the identified yeast kinases, namely MEK1, MEK6, MEK7, AKT, p70S6K, and CKI were also found to be capable of phosphorylating NS5A in this study (Table 1). Notably, CKI was recently found to have a critical role in the hyperphosphorylation of NS5A in human hepatoma cell lines (see below). While follow-up experiments will be required to ascertain whether any other of these candidate kinases can phosphorylate NS5A in human liver cells, the identification of MEK family kinases supports a recent study that a protein kinase(s) in the Ras-MAPK pathway is involved in NS5A phosphorylation-dependent HCV RNA replication (Huang et al., 2006). Intriguingly, inhibition of MEK1/2 by small-molecule inhibitors also led to enhanced HCV replication, in line with the previously mentioned findings that suppression of NS5A hyperphosphorylation confers higher levels of HCV RNA replication. However, inhibition of MEK1/2 kinase activity did not lead to complete abrogation of NS5A phosphorylation, suggesting that basal phosphorylation of NS5A is carried by another protein kinase(s).

More recently, Quintavalle et al. (2006) used kinase inhibitors that specifically inhibit NS5A hyperphosphorylation to investigate which cellular kinases were specifically implicated in the formation of the NS5A p58 phospho-isoform. The CKI protein kinase family was thus identified as a major determinant of NS5A hyperphosphorylation. Interestingly, overexpression of either CKI-α, CKI-δ or CKI-ε gave rise to increased p58 levels in cultured hepatoma cells. Conversely, RNA interference of only CKI-α reduced NS5A hyperphosphorylation and rescue of inhibition of p58 formation was specifically achieved by CKI-α.

Although there is a possibility that NS5A is not a direct substrate of CKI and an enzyme downstream of CKI may be responsible for NS5A modification, the region around the putative NS5A hyperphosphorylation contains more than one consensus sequence for CKI recognition, suggesting that one or more of the serine residues situated in this region might indeed be a substrate for CKI. Indeed, NS5A was subsequently confirmed to be a direct substrate of CKI-α (Quintavalle et al., 2007), and thus may be one of the long-sought enzymes responsible for the hyperphosphorylation of NS5A and a potential drug target for anti-HCV therapy. It will be important to examine whether hyperphosphorylation of NS5A by CKI-α could modulate binding of NS5A to host cell proteins such as hVAP-A, which is critical for the formation of HCV replicase and viral RNA synthesis on ER membranes (see below). Antagonism of CKI-α, however, may pose potential tumorigenesis risk due to the negative role that it normally plays on the Wnt and Hedgehog signaling (Price, 2006).

An emerging model for NS5A-dependent modulation of HCV RNA replication

So how might differential phosphorylation of NS5A control HCV RNA replication? Both unphosphorylated and phosphorylated derivatives of NS5A appear to bind NS5B equally well (Huang et al., 2005), suggesting an indirect mechanism. A clue was provided by the finding that NS5A (as well as NS5B) can associate with the SNARE-like membrane protein hVAP-A via the region of NS5A involved in hyperphosphorylation (Tu et al., 1999). hVAP-A is a widely expressed, ER/Golgi-localized protein involved in intracellular vesicle trafficking. NS5A and NS5B bind different domains of hVAP-A, with NS5A binding to the C-terminus, and NS5B binding to the N-terminus of the cellular protein. Thus, hVAP-A could serve as a docking site for assembly and/or localization of HCV replicase on intracellular membranes. Consistent with the hypothesis, these three proteins colocalize significantly in cells, especially within the detergent-resistant membrane fraction or lipid rafts, the subcellular structure where HCV replicase is presumably located. Importantly, hVAP-A is critical for HCV RNA replication; expression of truncated, dominant-negative mutants or siRNA of hVAP-A decreased both HCV RNA and protein levels in HCV replicon cells (Gao et al., 2004). Finally, NS5A mutations that blocked interaction with hVAP-A also strongly reduced HCV RNA replication (Evans et al., 2004).

Intriguingly, a subset of the previously identified adaptive mutations suppressing NS5A hyperphosphorylation displayed increased binding capacity to hVAP-A (Evans et al., 2004). This inverse relationship between NS5A hyperphosphorylation and interaction with hVAP-A led the investigators to propose a model (Fig. 2). Basally phosphorylated NS5A normally binds hVAP-A which in turn may facilitate or stabilize NS5A inter-action with NS5B to regulate its polymerase activity. Hyperpho-sphorylation of NS5A prevents its binding to hVAP-A, disrupting the replicase complex and hence preventing viral RNA replication. Thus, NS5A phosphorylation likely functions as a regulatory switch by modulating the interaction of NS5A
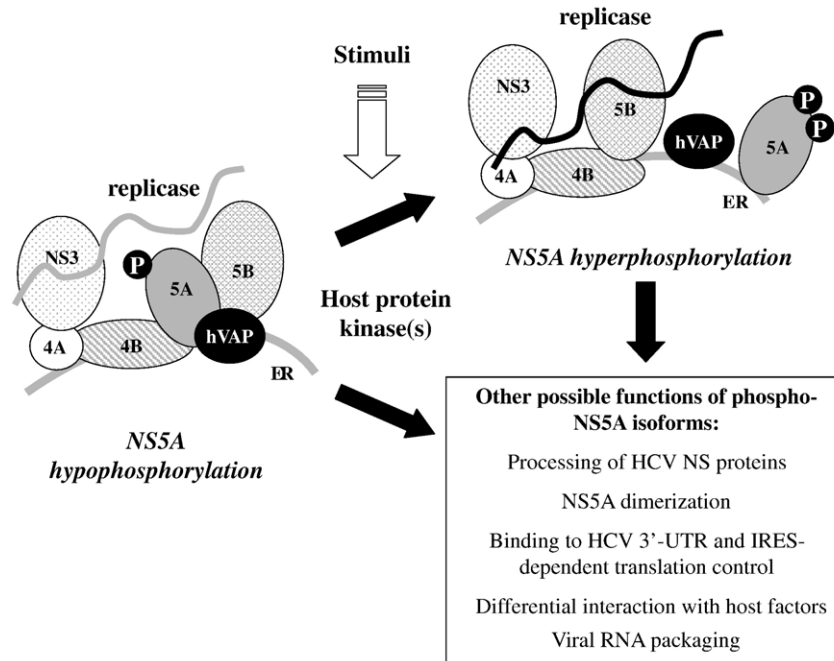
Fig. 2. Model describing of the role of NS5A kinase(s) in modulating HCV RNA replication. Schematic depicting the role of NS5A phosphorylation and its interaction with hVAP-A. NS5A is localized mostly to the ER membrane and is a part of HCV replicase, a multi-component replication complex comprising NS2 to NS5B and host cell factors. Basally phosphorylated NS5A binds hVAP-A which in turn may facilitate or stabilize NS5A interaction with NS5B to regulate its polymerase activity. Hyperphosphorylation prevents NS5A binding to hVAP-A, resulting in a disruption of the replicase complex and hence decreased viral RNA replication. Other possible functions for NS5A phosphorylation may include regulation of HCV nonstructural (NS) polypeptide processing, IRES-mediated translation and/or viral RNA packaging. Model is derived from Evans et al. (2004) and Gao et al. (2004).

with other host/viral components of the HCV replicase complex.

This picture, of course, is far from complete. If hyperphosphorylation of NS5A does indeed regulate viral RNA replication, one would predict the existence of extracellular and intracellular stimuli that are capable of altering the levels of NS5A hyperphosphorylation. In this regard, it is interesting that inhibition of basally activated Ras-MAPK pathway was sufficient to promote HCV RNA replication, whereas activation of the pathway suppressed viral RNA replication in a replicon system (Huang et al., 2006). Further analysis suggests that a Ras-MAPK pathway may normally serve to negatively control HCV RNA replication, at least in part through a direct or indirect modulation of NS5A protein phosphorylation. Moreover, HCV has been shown to possess mechanisms to down-modulate the MAPK signaling pathway (Tan et al., 1999; Macdonald et al., 2003, 2005), which includes NS5A interaction with upstream adaptor protein Grb2 (Tan et al., 1999) and Src tyrosine kinases (Macdonald et al., 2004). It is thus tempting to speculate that one function of non- or basally phosphorylated NS5A is to keep MAPK activation in check by binding to and sequestering/inhibiting Grb2 and/or Src kinases to allow HCV RNA replication. Activation of the Ras-MAPK pathway by external stimuli would disrupt this regulation through the induction of NS5A hyperphosphorylation and thus subsequent inhibition of viral RNA replication in cell culture. However, it is not known if the Grb2-NS5A interaction is influenced by NS5A phosphorylation or whether the PxxP

motifs of NS5A act as docking sites for SH3 domain-containing protein kinases.

Concluding remarks and future studies

Recent studies in the HCV replicon cell culture systems have suggested an important role for NS5A phosphorylation in HCV RNA replication. The major phosphorylation sites and several candidates of the responsible host cell kinases have been identified. However, since they were identified from *in vitro* replication systems and thus may be vulnerable to cell culture-adapted artifacts, they must be validated in the context of the complete viral life cycle using the recently developed infectious HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) as well as surrogate models such as DEN-2, YF and BVDV, and ultimately in the chimpanzee model (Table 2). Importantly, NS5A hyperphosphorylation appears to play different roles between *in vitro* and *in vivo* systems (Bukh et al., 2002) and the degree and requirements for hyperphosphorylation may vary between different HCV genotypes and isolates. It is also possible that NS5A phosphorylation contributes to a switch between viral RNA replication vs. RNA translation and/or packaging. The latter might also explain why hyperphosphorylation of NS5A is actively selected against in replicon-based systems. In this regard, a possible role for NS5A phosphorylation in modulating NS5A binding to the 3′ ends of HCV plus- and minus-strand RNAs (Huang et al., 2005) and modulation of IRES-dependent translation (He et al., 2003;

Table 2
Summary of reported NS5A mutations affecting HCV RNA replication

Mutation	HCV replicon RNA replication	HCV infectivity in chimpanzees	Notes
T1993A	N/A	N/A	Mutation was detected in infected chimpanzee (Thomson et al., 2001)
M2105V	N/A	N/A	Mutation was detected in infected chimpanzee (Thomson et al., 2001)
T2185A (genotype 1a)	↓ (Evans et al., 2004)	N/A	A2185T (genotype 1b), ↑ hVAP-A binding (Evans et al., 2004)
K2187G (genotype 1a)	↓ (Evans et al., 2004)	N/A	G2187K (genotype 1b), ↑ hVAP-A binding (Evans et al., 2004)
S2197P (S1172)	↑ (Blight et al., 2000)	↓ (Bukh et al., 2002)	↓ NS5A hyperphosphorylation (Blight et al., 2000)
A2199T	↑ (Blight et al., 2000)	N/A	No effect on hVAP-A binding (Evans et al., 2004)
S2204 (S1179) I/R	↑ (Blight et al., 2000)	N/A	↓ NS5A hyperphosphorylation (Blight et al., 2000), modulate hVAP-A binding (Evans et al., 2004)
P2209L	↑ (Kohashi et al., 2006)	N/A	Within ISDR, ↑ IFN response in patients (Watanabe et al., 2001)
Q2218H	↓ (Kohashi et al., 2006)	N/A	Most frequently detected within ISDR in patients (Watanabe et al., 2001)
E2356G	N/A	N/A	Mutation was detected in infected chimpanzee (Bukh et al., 2002)
S2362N	N/A	N/A	Mutation was detected in infected chimpanzee (Bukh et al., 2002)
D2375G	N/A	N/A	Mutation was detected in infected chimpanzee (Bukh et al., 2002)

Kalliampakou et al., 2005; Wang et al., 2003) has not been examined.

In addition, the questions as to how phosphorylation of NS5A affects its association with HCV NS4A, NS5B or ER membrane, NS5A dimerization, as well as its interactions with the plethora of host proteins need to be systematically addressed. While further understanding of the biology of NS5A phosphorylation will require further characterization of the protein kinases and phosphate acceptor sites involved in NS5A phosphorylation, it should be kept in mind that the association with NS5A with host cell kinases may in turn affect the physiological function of these kinases. In fact, NS5A can bind the PI3K kinase, and the interaction seems to stimulate the PI3K-AKT pathway, which would suppress cellular apoptosis, allowing viral persistence and/or malignant transformation (He et al., 2002; Street et al., 2004). In a more recent study, NS5A was found to interact with Raf-1 and the kinase activity was increased in NS5A-expressing or HCV replicon cells (Burckstummer et al., 2006). However, phosphorylation of NS5A by Raf-1 was not demonstrated in the study. Finally, CK1-α is known to regulate RNA-binding property of hnRNP C proteins (Kattapuram et al., 2005), which have been reported to bind the 3′-UTR of HCV genome (Gontarek et al., 1999). It may be interesting to examine whether the kinase activity of CK1α itself is modulated by NS5A as a potential mechanism by which NS5A regulates HCV RNA replication.

For many small RNA viruses such as HCV, it is imperative that their genomes encode proteins that are capable of performing more than one function, perhaps at different stages during the viral lifecycle and when necessary, depending on host cell biology. Phosphorylation (as well as other post-translational modifications) of a viral protein such as NS5A by a host cell protein kinase(s) to produce different biological functions is thus a clever and economic strategy. Understanding the process of NS5A phosphorylation, including the yet-to-be-identified cellular protein phosphatase(s) of NS5A, and its role in the HCV life cycle will not only reveal mechanisms of HCV RNA replication and/or pathogenesis but may also provide new avenues for the therapeutic intervention.

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