Review

Hepatitis C virus NS5A: enigmatic but still promiscuous 10 years on!

Douglas Ross-Thriepland† and Mark Harris

School of Molecular and Cellular Biology, Faculty of Biological Sciences, and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, UK

Since one of us co-authored a review on NS5A a decade ago, the hepatitis C virus (HCV) field has changed dramatically, primarily due to the advent of the JFH-1 cell culture infectious clone, which allowed the study of all aspects of the virus life cycle from entry to exit. This review will describe advances in our understanding of NS5A biology over the past decade, highlighting how the JFH-1 system has allowed us to determine that NS5A is essential not only in genome replication but also in the assembly of infectious virions. We shall review the recent structural insights – NS5A is predicted to comprise three domains; X-ray crystallography has revealed the structure of domain I but there is a lack of detailed structural information about the other two domains, which are predicted to be largely unstructured. Recent insights into the phosphorylation of NS5A will be discussed, and we shall highlight a few pertinent examples from the ever-expanding list of NS5A-binding partners identified over the past decade. Lastly, we shall review the literature showing that NS5A is a potential target for a new class of highly potent small molecules that function to inhibit virus replication. These direct-acting antivirals (DAAs) are now either licensed, or in the late stages of approval for clinical use both in the USA and in the UK/Europe. In combination with other DAAs targeting the viral protease (NS3) and polymerase (NS5B), they are revolutionizing treatment for HCV infection.

Introduction

The molecular biology of hepatitis C virus (HCV) has been the subject of many authoritative recent reviews and needs no detailed introduction here. However, it is important to note that since one of us co-authored a review on NS5A in this journal a decade ago (Macdonald & Harris, 2004), there has been an extraordinary step change in the field. This was brought about by the identification in 2005 of a cell culture infectious clone of the virus, a genotype 2a isolate termed Japanese fulminant hepatitis-1 (JFH-1), by the group led by Takaji Wakita in Tokyo (Wakita et al., 2005). For the first time it was possible to study the complete life cycle of the virus from entry to exit, and use reverse genetics to dissect and define the molecular requirements. Coupled with the subgenomic replicons (SGR) initially developed by Volker Lohmann and Ralf Bartenschlager in 1999 (Lohmann et al., 1999), these systems provided the molecular toolkit for a comprehensive analysis of HCV biology. As a result, we now have a much deeper understanding of the molecular and cellular aspects of HCV, but many questions remain. This review will focus on the function of NS5A, taking as its starting point the 2004 review, and highlighting how our understanding of this protein has advanced over the past decade.

In 2004, NS5A research focussed very much on the role of this protein in viral genome replication and interactions with cellular proteins. The use of the SGR system had established that NS5A participated in a multiprotein complex that replicated the viral genome, and further provided a physiologically relevant context for validation of the many interactions with cellular factors that had been described. However, nothing was known about the structure of the protein or its potential role in other stages of the virus life cycle. The past decade has filled in many of these gaps but has left many questions unanswered.

The structure of NS5A

The existence of a short (33 residue) amphipathic helix at the very N terminus of NS5A was well documented prior to 2004. The structure of a peptide spanning this sequence was solved by nuclear magnetic resonance (NMR) and the helix was shown to be both necessary and sufficient for membrane targeting in vivo, as well as essential for genome replication in the SGR system (Penin et al., 2004). The pioneering work of Tim Tellinghuisen provided the first insights into the structure of the remainder of NS5A. Using
a bioinformatics approach he first predicted that NS5A comprised three domains separated by short low-complexity sequences (LCSs) (Tellinghuisen et al., 2004) (Fig. 1). The first domain was further predicted to contain a conserved tetracysteine motif capable of coordinating a zinc atom. High-level expression of either full-length NS5A (genotype 1b) or domain I in E. coli allowed experimental validation of these predictions. First, full-length NS5A was shown to contain two trypsin-sensitive cleavage sites corresponding to the two LCSs – consistent with the prediction that these constituted flexible surface-exposed loops linking the domains. Atomic absorption spectroscopy confirmed that domain I bound a single atom of zinc; mutations in the tetracysteine motif abolished this interaction, and indeed abrogated genome replication in the SGR system, showing that zinc binding was essential for NS5A function. This study rapidly led to the publication of the structure of domain I at 2.5 Å resolution (Tellinghuisen et al., 2005). Intriguingly, this analysis revealed that, in addition to the zinc binding, domain I contained a disulfide bond towards the C terminus – both the zinc and disulfide contributing to the maintenance of the overall fold. As the reducing environment of the cytoplasm normally precludes the formation of disulfide bonds, this implies that, like the hepatitis B virus core protein, which has also been reported to contain a disulfide bond, NS5A would need to be protected from the reducing conditions, possibly by sequestration within the membranous structures that harbour the RNA replication machinery (Paul et al., 2013; Romero-Brey et al., 2012). The crystal structure revealed a

![Diagram](image-url)

**Fig. 1.** Summary of key structural features and dimeric conformations of NS5A domain I. (a) NS5A domain I from three different crystal structures (1ZH1, 3FQM and 4CL1) aligned by PyMOL. (b) Different dimeric or multimeric conformations observed by X-ray crystallography. Highlighted are the phosphorylation site at position 146 (red), disulfide bond (cyan), zinc-coordination motif (yellow) (Tellinghuisen et al., 2005) and lipid droplet-binding motif (purple) (Miyanari et al., 2007).
dimeric form of domain I with a basic groove between the dimers that was postulated to be an RNA-binding site. Subsequently, two other structural studies, one also of genotype 1b NS5A (Love et al., 2009), the other of genotype 1a (Lambert et al., 2014), revealed an identical monomeric structure, although these were configured in several alternative dimer conformations (see Fig. 1). There is some limited biochemical evidence for the formation of NS5A dimers in vitro, including a role for the zinc-coordinating cysteines (Liu et al., 2012), and some genetic evidence for trans-complementation of NS5A mutants, which has been interpreted as being consistent with dimerization in vivo. However, this is a controversial area and the jury is still out regarding the biological significance of NS5A dimerization.

In contrast to domain I, the other two domains are largely unstructured. NMR and other biophysical analyses of either domain II or domain III alone revealed an overall lack of secondary structure, consistent with the proposal that these domains are intrinsically disordered (Hanouelle et al., 2009a, 2010; Liang et al., 2006, 2007; Verdugem et al., 2011). However, a more recent NMR study, in which domain II was expressed with the flanking LCS1 and II, revealed evidence for the formation of three α-helical structures near the C terminus (Feuerstein et al., 2012). LCSII is proline rich and contains either one or two PxxP motifs, known to interact with cellular SH3 domains, Feuerstein and colleagues provide evidence for the additional involvement of these α-helical structures in SH3 domain binding.

A number of models for the structure of a membrane-associated NS5A dimer have been proposed but none is entirely satisfactory or convincing; for example we do not understand how the various dimers might be orientated relative to the membrane, nor how the individual domains might be orientated relative to each other. These models assume that domains II and III do not contribute to dimer formation; again this is conjecture. As discussed in the section ‘Phosphorylation of NS5A’, below, NS5A is a phosphoprotein and the potential role of phosphorylation in the structure is unknown, given that all the structural information has been derived from analysis of E. coli-expressed (and therefore unphosphorylated) protein. Many questions thus remain to be answered about the structure of this protein.

**Phosphorylation of NS5A**

NS5A is known to be extensively phosphorylated. Two forms of the protein with distinct apparent molecular masses can be observed by SDS-PAGE; these are referred to as the basal and hyperphosphorylated species. Data published over the past decade have shed light on the kinases involved, the residues phosphorylated and the functions of phosphorylation (Fig. 2, Table 1). Despite this, we are only really scratching the surface of this fascinating and complex topic and much of the data need clarification and corroboration. Whilst genetic analysis of putative phosphorylation sites can be informative, the only unambiguous method to identify these residues is MS analysis of protein purified from either SGR-harbouring or virus-infected cells. This analysis has been undertaken by three groups. First, the Tellinghuisen group analysed NS5A immunoprecipitated from SGR cells and identified a single phosphoserine (pS222) within a tryptic peptide corresponding to LCSI (LeMay et al., 2013). Secondly, our own study identified multiple phosphorylation sites within the same LCSI tryptic peptide, including pS222 (Ross-Thiepand & Harris, 2014). This tryptic peptide contains eight serines and we observed that it comprised a mixture of phosphospecies containing from one to seven phosphoserines. Thirdly, Masaki et al. (2014) confirmed the presence of phosphopeptides corresponding to LCSI. The results of mutagenic analysis of serines in LCSI are somewhat difficult to interpret, for example mutation of S229 to either alanine (phosphoablatant) or aspartate (phosphomimetic) was lethal for genome replication in genotype 1, suggesting that it is not simply phosphorylation of this residue that is important. Phosphoablatant mutations of S225, S232 and S235 were inhibitory, whereas the corresponding phosphomimetic mutations had no phenotype, suggesting a role for phosphorylation of these residues in genome replication. What is perhaps more intriguing is that all of these data were obtained in the context of JFH-1 and, although these residues are conserved in genotype 1b, the corresponding mutations in the Con1 SGR had opposite effects (Appel et al., 2005). Thus, the function of NS5A phosphorylation remains elusive. Early studies pointed to a negative effect of hyperphosphorylation on virus replication in genotype 1 (Evans et al., 2004); however, such a correlation cannot be observed in JFH-1. Indeed, a key question is the link between hyperphosphorylation and culture adaptation. The most robust culture-adaptive change is S2204I (corresponding to S232 in NS5A of genotype 1b). First identified in genotype 1, in which it confers a 20 000-fold increase in replicative efficiency (Blight et al., 2000), this mutation is adaptive for SGRs of all other genotypes tested (3a, 4a, 5a and 6a) (Kim et al., 2014; Saeed et al., 2012; Wose Kinge et al., 2014; Yu et al., 2014), apart from JFH-1. In genotype 1 the S2204I (S232I) mutation abrogates hyperphosphorylation (Blight et al., 2000), but this is not seen in JFH-1. These data point to fundamental differences between the genotypes with regard to the conformation of NS5A and/or the protein–protein interactions involved in genome replication. The availability of both SGRs and chimeric viruses containing the replicase of other genotypes provides tools to dissect these differences and ask the question: Which best represents the ‘normal’ NS5A – JFH-1 or genotype 1?
So which kinases phosphorylate NS5A? In pole position is casein kinase I (CKI), a family of kinases originally identified as being capable of phosphorylating casein in vitro and that are now known to be involved in a bewildering array of cellular processes (Cheong & Virshup, 2011). In 2006, an intriguing study showed that small-molecule inhibitors of NS5A hyperphosphorylation also inhibited CKI-α (Quintavalle et al., 2006). Subsequently, the same group demonstrated that these inhibitors bound to a range of kinases, one of which, CKI-α, was able to phosphorylate NS5A in vitro (Quintavalle et al., 2007). Peptide studies showed that CKI-α preferentially phosphorylated S232 after pre-phosphorylation of S232 and S238.

**Fig. 2.** Organization of NS5A: key protein–protein interaction and phosphorylation sites. Highlighted are the amphipathic helix (AH), tetracysteine zinc-coordination motif (Tellinghuisen et al., 2005), key residues implicated in daclatasvir (DCV) resistance (Fridell et al., 2010), the lipid droplet-binding motif (Miyani et al., 2007), PI4KIIIα-binding motif (Reiss et al., 2013), CypA-binding site (Hanouille et al., 2009b; Coelmont et al., 2010) and P2 polyproline SH3-binding motif (Hughes et al., 2009b). Also indicated are known phosphorylation sites that have been determined by mass spectrometry (red), and those for which there is genetic evidence but no biochemical data (blue). The sequences presented are from genotype 1b (J4 isolate, GenBank accession number AF054250, upper sequence) and genotype 2a (JFH-1 isolate, GenBank accession number AB047639, lower sequence).

**Table 1.** Summary of location and phenotype of NS5A phosphorylation sites

<table>
<thead>
<tr>
<th>Location; residue(s)</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sites identified by MS analysis of NS5A purified from cells infected with HCV or harbouring HCV SGR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domain I; 146</td>
<td>Phosphorylation reduces p58 and pS222</td>
<td>Ross-Thriepland &amp; Harris (2014)</td>
</tr>
<tr>
<td>LCSI; 222, 232</td>
<td>No phenotype; 228 and 238 not directly observed by MS, but share this phenotype</td>
<td>LeMay et al. (2013); Ross-Thriepland &amp; Harris (2014)</td>
</tr>
<tr>
<td>LCSI; 225, 229, 232, 235</td>
<td>Phosphorylation required for replication in JFH-1, impairs replication in Con1</td>
<td>Ross-Thriepland &amp; Harris (2014); Masaki et al. (2014)</td>
</tr>
<tr>
<td>LCSI; 348</td>
<td>No phenotype</td>
<td>Ross-Thriepland &amp; Harris (2014)</td>
</tr>
<tr>
<td>LCSI; 356</td>
<td>Phosphorylation required for replication and SH3 binding</td>
<td>Cordek et al. (2014)</td>
</tr>
<tr>
<td><strong>Putative phosphorylation sites supported by biochemical evidence (not observed by MS)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Domain III; 408, 412, 414, 415</td>
<td>Alanine mutation impaired virus assembly</td>
<td>Masaki et al. (2008)</td>
</tr>
<tr>
<td>Domain III; 452, 454, 457</td>
<td>Alanine mutation abrogated virus assembly and core interaction</td>
<td>Masaki et al. (2008)</td>
</tr>
<tr>
<td>Domain III; 457</td>
<td>Alanine mutation abrogated virus assembly, restored by aspartic acid mutation</td>
<td>Tellinghuisen et al. (2008a)</td>
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at S229, an observation that is consistent with our understanding of CKI-ż substrate preference, the critical role of S229 and our evidence for hierarchical phosphorylation. Recently, an siRNA screen also identified CKI-ż as a mediator of NS5A hyperphosphorylation (Masaki et al., 2014). This study also provided evidence that siRNA depletion of CKI-ż resulted in a loss of NS5A redistribution to lipid droplets. The case for phosphorylation of NS5A by CKI-ż is therefore compelling; however, it remains to be determined if a priming phosphorylation is required and, if so, which kinase(s) performs this function. Polo-like kinase I (Plk1) has also been shown to both interact with, and phosphorylate, NS5A (Chen et al., 2010). Plk1 knockdown or pharmacological inhibition resulted in a reduction of both hyperphosphorylation and RNA replication. Both Plk1 and a further kinase, TSSK2, were also identified by the recent siRNA screen (Masaki et al., 2014).

Very recently, the Cameron group identified a further phosphorylation site at the beginning of domain III. They reported that T356 (JFH-1 NS5A numbering; T2332 in the polyprotein) was phosphorylated by cAMP-dependent protein kinase (PKA) (Cordek et al., 2014). Alanine substitution of this residue had a modest (10-fold) effect on genotype 1b but was lethal in genotype 2a. This residue immediately follows a cluster of four basic residues and the polyproline motif (see Fig. 2 and ‘SH3 domain-mediated interactions’, below) and it was shown that phosphorylation altered the NMR spectrum of the prolines. It will thus be important to determine if phosphorylation of T356 modulates SH3 domain binding by NS5A (see ‘SH3 domain-mediated interactions’ below). Of note, in our hands alanine substitution of the four basic residues had no effect on JFH-1 genome replication or virus assembly (D. Ross-Thriepland, unpublished). As this alanine substitution abolishes the consensus PKA phosphorylation motif, this may allude to a role of T356 that is not dependent on PKA, although, intriguingly, pharmacological inhibition of PKA has been shown to reduce extracellular infectivity whilst having no effect on RNA replication or assembly of intracellular infectious particles (Farquhar et al., 2008). Clearly, further analysis will be required to determine whether PKA phosphorylation of NS5A at T356 is implicated in this phenotype.

Although MS did not identify any phosphopeptides within the C-terminal 80 residues of NS5A, most likely due to the absence of protease cleavage sites, both genetic and pharmacological approaches were used to identify a potential CKII phosphorylation site at serine 457 (10 residues from the C terminus of NS5A) (Tellinghuisen et al., 2008a). Alanine substitution or deletion of this serine had no effect on virus genome replication but reduced production of infectious virus by 4–5 logs, consistent with a role for the C terminus in this stage of the virus life cycle. In conclusion, NS5A phosphorylation is clearly functionally important, but the molecular mechanisms by which these post-translational modifications modulate the function of the protein remain to be elucidated. A key question will be: How does phosphorylation impact on the interactions of NS5A with other factors such as proteins, RNA and membranes?

**Functions of NS5A in the virus life cycle**

The advent of the JFH-1 infectious clone revealed that NS5A had direct roles in both virus genome replication and assembly. A strong body of evidence has shown that these two roles are independent and indeed map to different domains of the protein – domains I and II are required for genome replication, whereas domain III has a role in assembly. Domain III is in fact dispensable for RNA replication and, consistent with this, is able to tolerate large insertions (e.g. GFP) at several sites near the C terminus (Appel et al., 2005; Moradpour et al., 2004). Remarkably, some of these insertions are also viable within the context of the infectious clone and do not block the role of NS5A in particle assembly (Schaller et al., 2007). SGRs and viruses harbouring NS5A insertions have proven to be valuable tools enabling both imaging and proteomic studies of HCV replication.

The recent identification of hepacviruses closely related to HCV in a variety of both primate (Lauck et al., 2013) and non-primate species (Burbelo et al., 2012; Kapoor et al., 2011, 2013) has revealed a high degree of sequence homology within domain I of NS5A, including conservation of the tetracysteine zinc-binding motif. Furthermore, secondary structure prediction programs indicate that all hepacviruses likely share a very similar topology in this domain. In contrast, there is very low homology in domains II and III, exemplified by the Guevara hepacivirus (GHV), isolated from an Old World primate (the black and white colobus), which is predicted to have an 883 aa NS5A (Lauck et al., 2013); the N-terminal 175 aa aligned well with other NS5A sequences but the 690 aa C-terminal fragment did not align to anything in the GenBank databases. These observations suggest that domain I has critical and well conserved functions that are common to all hepacviruses, whereas the functions of the other two domains may be specific to individual viruses (perhaps reflecting different host species?). Consistent with this hypothesis, transposon mutagenesis demonstrated that very few insertion sites in domain I were tolerated (in comparison with domains II and III) (Arumugaswami et al., 2008). In agreement with the transposon study, saturation mutagenesis coupled with next-generation sequencing of virus pools derived from multiple rounds of passage has revealed that very few residues in the N terminus of domain I (aa 18–103) can tolerate substitutions (Qi et al., 2014). These data also confirmed the importance of the PTPPL sequence (residues 100–104 of NS5A), which was previously shown to be required for both association of NS5A with lipid droplets and release of infectious virus (Miyanari et al., 2007) (Fig. 2). This motif is situated just C-terminal to a phosphorylation site (S146; see ‘Phosphorylation of NS5A’, above) (Ross-Thriepland et al., 2014), although no effect of mutagenesis at this residue on either RNA replication or assembly and release of infectious
virus was observed. A corresponding mutagenic study of the C-terminal half of domain I (residues 103–213) has not yet been performed; however, given the high level of sequence conservation between HCV isolates and the fact that the three-dimensional structure of this part of the protein is known, this could shed light on the functions of this domain. A notable exception is the identification of the PI4KIIIα-binding site (Reiss et al., 2013), as discussed in ‘Phosphorylation of NS5A’, above. Thus, although this could shed light on the functions of this domain.

In contrast, domain II has been extensively studied both by deletion analysis and by alanine scanning mutagenesis. Apart from the N-terminal 30 residues of this domain, residues spanning the remainder were shown to be essential for genome replication in both genotypes 1b (Con1) (Tellinghuisen et al., 2008b) and 2a (JFH-1) (Ross-Thriepland et al., 2014). Intriguingly, there were a number of residues towards the C terminus of domain II that were conserved between the two genotypes yet when mutated exhibited different phenotypes, e.g. Y317A was lethal in Con1 but had a null phenotype in JFH-1. This observation suggests that it is not possible to extrapolate between the different genotypes and may perhaps reflect differences in protein–protein interactions.

As mentioned, domain III is largely dispensable for genome replication, and surprisingly a large part of this domain could also be deleted whilst wild-type levels of virus assembly and release were retained (Appel et al., 2008). This analysis revealed that the C-terminal 38 residues of domain III harboured the major determinant of virus assembly. However, within JFH-1 (and other genotype 2 isolates) this region contains an 18 amino acid insertion relative to other genotypes. This insertion was shown to be dispensable for virus assembly and release, although a deletion of this sequence had a modest effect on genome replication (Hughes et al., 2009a). Combining these two sets of data suggests that only the C-terminal 15 residues are in fact required for assembly. Within this sequence is the CKII phosphorylation site S457 (see ‘Phosphorylation of NS5A’, above). This serine, and two others (S452, S454), were also shown to be required for the interaction of NS5A with core protein (Masaki et al., 2008). Further to this, a number of studies have shown that core protein and NS5A colocalize on the surface of lipid droplets (Miyarani et al., 2007), and this localization of NS5A was dependent on the activity of the cellular enzyme diacylglycerol acyltransferase-1 (Camus et al., 2013). Intriguingly, treatment with a CKII inhibitor (DMAT) had no effect on NS5A colocalization with lipid droplets, in contrast to the depletion of CKI-z, which phosphorylates NS5A in the serine-rich LCSI (see ‘Phosphorylation of NS5A’, above). Thus, although both CKI-z and CKII phosphorylation of NS5A have been implicated in virus assembly, they likely act at different stages in this process.

Taken together, these data have led to a model whereby NS5A recruits nascent genomes from endoplasmic-reticulum-associated replication complexes to lipid droplets where they interact with core protein and proceed to become encapsidated into new virus particles. However, this model is highly speculative and there are significant caveats — for example it is unclear how NS5A associated with nascent genomic RNA within the membrane-bound replication complexes could re-localize and interact with the cytoplasmic face of lipid droplets. In this regard, dynamic imaging of NS5A-GFP in SGR-harbouring cells demonstrated the presence of two populations of NS5A-positive foci: large structures exhibiting restricted mobility, and small fast-moving structures (Wolk et al., 2008). More recently, imaging of virus-infected cells confirmed the existence of both populations and further showed that NS5A-GFP-positive foci can move around the cell in a dynein-dependent fashion, providing a potential mechanism for the relocalization (Eyre et al., 2014).

NS5A-interacting partners

Since the last review, the list of host-cell proteins that have been shown to interact with NS5A has greatly expanded and now comprises some 130 interactors. A detailed discussion of all of these interacting partners is beyond the scope of this short review, so we have cherry picked a limited number of NS5A interactions that illustrate the various roles of NS5A in virus genome replication, virus assembly and manipulation of the host-cell environment.

To complement this section we also provide a supplementary table of all the interacting proteins (Table S1, available in the online Supplementary Material).

As one viral protein cannot interact simultaneously with 130 cellular proteins, the data may therefore point to different pools of NS5A, separated both spatially and temporally, that have different roles and interact with different subsets of cellular factors. How such complexity is controlled remains a key question, concerning which the potential involvement of phosphorylation may provide some answers (see ‘Phosphorylation of NS5A’, above). In this regard, as discussed above (see ‘The structure of NS5A’), it has recently been proposed that domains II and III of NS5A belong to the class of intrinsically disordered proteins, which can adopt a range of conformations, potentially therefore allowing the protein to interact with a range of partners (Feuerstein et al., 2012). Furthermore, it may be that some of these interactions only have roles under certain conditions or in response to specific stimuli.

SH3-domain-mediated interactions

To illustrate the last point we should like to first consider the binding of NS5A to cellular SH3-domain-containing proteins via a polyproline (PxxPxR) motif present in LCSII (Fig. 2). Although the PxxPxR motif is absolutely conserved in all HCV isolates, alanine substitutions of these prolines (a mutant termed PA2), which abolished SH3 domain interactions, had no effect on either SGR replication or infectious virus production (Hughes et al., 2009b). However, we
demonstrated that this motif was required for inhibition of oxidative-stress-mediated activation of a pro-apoptotic potassium channel, Kv2.1 (Mankouri et al., 2009), such that cells infected with a JFH-1 PA2 mutant were more sensitive to the induction of apoptosis by an exogenous oxidant. This observation is consistent with the suggestion that one function of the PxxPxxR motif is to prevent the apoptotic response to oxidative stress, allowing the virus to persist. In the highly transformed human hepatoma cell line Huh7, this is potentially a superfluous function as these cells are derived from a liver cancer and are inherently resistant to oxidative stress; however, it might be important in the infected liver, and this is supported by the observation that a PA2 mutant virus did not establish infection in the chimpanzee model (Nanda et al., 2006).

Mechanistically, we demonstrated that NS5A interacted with the SH3-domain-containing kinase mixed-lineage kinase 3 (MLK3) (Amako et al., 2013). MLK3 is upstream of p38MAPK, which is activated by oxidative stress and subsequently phosphorylates and activates Kv2.1. The MLK3 SH3 domain was identified as an NS5A interactor from a phage display library of the human SH3-ome (Kärkkäinen et al., 2006). This analysis also confirmed that NS5A bound to the SH3 domain of amphiphysin II (BinI), as previously documented (Zech et al., 2003), and furthermore highlighted an interaction with the SH3 domains of the adaptor protein CMS, which has provided some mechanistic insights into the effects of NS5A on epidermal growth factor receptor trafficking (Mankouri et al., 2008; Z. Igloi, A. Kazlauskas, K. Saksela, A. Macdonald, J. Mankouri & M. Harris, unpublished).

Phosphatidylinositol 4-kinase (PI4K)
This lipid kinase phosphorylates phosphoinositide lipids on position 4 of the inositol ring. The product is phosphatidylinositol 4-phosphate (PI4P), which then recruits effector proteins such as small GTPases to cellular membranes. PI4K and PI4P have been shown to play roles in the replication of a number of viruses (Delang et al., 2012), including HCV. There are four PI4K isoforms in mammals – types II and III and α/β isoforms, based on size, catalytic properties and domain structure. A number of studies have demonstrated that NS5A specifically interacts with, and activates, the endoplasmic-reticulum-resident isoform PI4KIIIz, leading to an increase in cellular PI4P lipids (Reiss et al., 2011). This is required for the changes in cytoplasmic membranes to form the so-called membranous web – the location for HCV genome replication. Binding to PI4KIIIz mapped to a short (seven-residue) sequence near the C terminus of domain I (Reiss et al., 2013) (Fig. 2), although mutagenesis of this sequence did not completely abolish the PI4KIIIz interaction, suggesting that multiple binding sites might exist. However, mutations in this sequence, in common with PI4KIIIz siRNA silencing, impaired RNA replication and disrupted the membranous web. A negative effect of PI4KIIIz binding on NS5A hyperphosphorylation was reported, and it was further shown that a kinase-inactive PI4KIIIz mutant bound to NS5A but had no effect on phosphorylation (Reiss et al., 2013). However, there is a caveat in that these experiments were conducted in cells constitutively expressing T7 polymerase and transfected with a plasmid vector for NS3-5B polyprotein expression. It will be important to confirm these data in cells harbouring actively replicating HCV.

Cyclophilin A (CypA)
The cyclophilins are a family of peptidyl-prolyl isomerasases (PPIase), which catalyse the cis–trans isomerization of the peptide bond preceding a proline residue to facilitate protein folding. CypA has been known for many years to interact with the human immunodeficiency virus 1 (HIV-1) Gag protein and its incorporation into HIV particles is essential for infectivity (Luban et al., 1993). Over the past decade, cyclophilin inhibitors such as cyclosporin A (CsA) have been shown to inhibit HCV replication (Nakagawa et al., 2004, 2005; Watashi et al., 2003), and more recently it has been shown that NS5A directly interacts with CypA (Chatterji et al., 2010; Hanouille et al., 2009b). These studies have shown that the C terminus of domain II binds to the active site of CypA (Fig. 2). NS5A is proline rich and there is in vitro evidence for CypA-mediated cis–trans isomerization in domain II (Hanouille et al., 2009b). This is consistent with the identification of CsA-resistance mutations within this domain (the most notable being D316E, Y317N or a combination of the two – termed DEYN). Paradoxically these mutations did not affect the binding of NS5A to CypA, and did not alter the disruption of that binding by CsA (Coelmont et al., 2010). However, they did confer on HCV the ability to replicate in cells in which CypA expression had been suppressed by siRNA, suggesting that they reduce the CypA dependence of HCV replication. This raises the bigger question as to the role of CypA in HCV replication. Presumably CypA binding and isomerization of domain II of NS5A favour folding into a specific structure, thereby presenting a defined interacting surface. It follows that mutations such as DEYN must also be compatible with such a structure. As domain II is inherently unstructured and flexible, CypA might provide another mechanism for switching between different functions of NS5A. To further complicate the story, CsA resistance has also been mapped to domain III and in vitro experiments have shown that CypA both interacts with, and isomerizes, this domain (Verdegem et al., 2011).

CypA also plays a role in the immune response, interacting with Ca++/calcineurin to activate the transcription factor NFAT-1, and indeed CsA is a well characterized immune suppressor. However, the identification of CsA analogues such as alisporivir and NIM811 that lack the immune-suppressive activity but retain the ability to block HCV replication suggested that only CypA PPIase activity was required to effectively block HCV replication. More recently, the story has been complicated by the observation...
that CypA inhibition can also block HCV replication by reversing the attenuation of the IFN response that is mediated by NS5A inhibition of protein kinase R (PKR) and eIF2-α phosphorylation (Daito et al., 2014). The exact interplay between PKR, CypA, NS5A and the IFN response requires further investigation; in particular it is pertinent to note that both CypA and PKR interact with domain II of NS5A, albeit in adjacent and not overlapping regions.

**RNA**

Both our laboratory and that of Craig Cameron (Huang et al., 2005) have shown that NS5A binds RNA in vitro. We demonstrated that each of the three domains possesses the ability to bind the HCV 3′ untranslated region, with full-length NS5A (lacking the N-terminal amphipathic helix) exhibiting a higher affinity for the poly(U/UC) region than for the X-region (Foster et al., 2010). CypA stimulated the RNA-binding activity of domain II (but not domains I and III) in a PPIase-dependent fashion (Foster et al., 2011). Intriguingly, RNA binding by the DEYN mutant was unaffected by CypA, providing further evidence that this mutation might favour a specific conformation of domain II. Detailed biochemical characterization of NS5A–RNA binding revealed that it was inhibited by a high concentration of EDTA, implying a requirement for Zn²⁺ or other divalent cations, and that NS5A bound preferentially to short (5–6 nt) uracil-rich RNAs (Hwang et al., 2010). MS of NS5A cross-linked to polyU agarose and digested with trypsin revealed interacting sites in both domains I and II but not domain III; however, this could be explained by the lack of tryptic cleavage sites in domain III. As yet there are no published data pertaining to NS5A–RNA binding in infected or SGR-harbouring cells, although we have recently used two complementary methods to demonstrate that this does occur. First, both cellular and viral RNA are associated with NS5A purified from HCV-infected cells by using a Strep-tag system (Z. Igloi & M. Harris, unpublished). Secondly, we have used an approach where nascent viral RNA is labelled with 5-ethynyl-uridine in the presence of actinomycin D and subsequently covalently linked to biotin-azide via a bio-orthogonal ‘Click’ chemistry reaction. 5-Ethynyl-uridine-labelled RNA can then be purified by streptavidin-agarose chromatography and NS5A is associated with this RNA (Walter & Harris, unpublished). It will be important to determine the specific RNA sequences that are bound by NS5A in infected cells, as well as to define the RNA-binding motifs within the protein and investigate how this binding might be modulated by phosphorylation or CypA interactions. It is tempting to speculate that RNA binding by NS5A could underpin its roles in different stages of the virus life cycle, i.e. genome replication and assembly. Further to this, an attractive hypothesis is that domain I interactions with RNA might be involved in genome replication, with domain II/III interactions then orchestrating the transport of nascent genomes to sites of assembly. These two functions could conceivably be regulated by a phosphorylation- or CypA-mediated conformational switch. However, as yet there is no biochemical evidence to either support or refute this hypothesis.

**NS5A as a target for direct-acting antivirals (DAAs)**

Over the past few years there have been extraordinary advances in the therapy for HCV infection – the standard IFN and ribavirin therapy has been rapidly superseded by combination therapy with a range of DAAs targeted not only to the viral enzymes – NS3 protease and NS5B RNA-dependent RNA polymerase – but also to NS5A. The first of these putative NS5A inhibitors was developed by Bristol-Myers Squibb; originally called BMS-790052, it has been renamed daclatasvir (DCV) (Gao et al., 2010). DCV was identified by a high-throughput screen for compounds that inhibited SGR replication and has an extraordinary potency and specificity: half maximal effective concentration, EC₅₀ <100 pM against a range of HCV genotypes; therapeutic index (CC₅₀/EC₅₀, where CC₅₀ is the 50% cytotoxic concentration) >100 000; and EC₅₀ >10 μM against other viruses. DCV was defined as an NS5A inhibitor by virtue of its lack of effect on NS3 or NS5B in *in vitro* enzymatic assays, and, more importantly, the observation that the development of DCV resistance was associated with specific mutations in the amphipathic helix and/or domain I of NS5A. These are commonly at positions 28, 30, 31 or 93 (Fig. 2) – with the most potent resistance mutations giving up to 10 000-fold increases in EC₅₀ values (Fridell et al., 2010). It should be noted that DCV retains nanomolar potency against all single resistance mutations, suggesting that multiple mutations would be required to give significant resistance in patients. The potency of DCV has been extrapolated to the clinical situation, resulting in an accelerated process of approval for treatment of HCV infection and impressive outcomes for patients – this aspect of DCV has been exhaustively reviewed recently (Pawlotsky, 2014). It has been followed by a number of alternative molecules produced by other companies, notably ledipasvir/GS-5885 (Gilead) and ombitasvir/ABT-267 (Abbott), both of which are in phase 3 clinical trials.

Despite this clinical success, the mechanism of action of DCV remains obscure. It is a large (700 Da) dimeric molecule, leading to the proposal that it could bind the dimeric form of domain I, perhaps stabilizing the dimer. This proposal was supported by data published in the first DCV paper (Gao et al., 2010), in which it was shown that a biotin-tagged derivative of DCV bound to NS5A in lysates of SGR-harbouring cells. More recently, further biochemical evidence has corroborated this initial observation. Berger et al. (2014) also showed binding of NS5A expressed in Mch7 cells to a biotinylated DCV derivative. Interestingly, in this study the Y93H resistance mutation showed only a modest (30%) reduction in binding of NS5A to the inhibitor, although a proviso is that in these experiments...
the NS3-5B polyprotein was expressed from a plasmid and thus not in the context of active RNA replication. An alternative in vitro approach used the technique of microscale thermophoresis to show direct binding of DCV to NS5A domain I with a $K_D$ in the nanomolar range, a loss of binding to the Y93H resistance mutant and an inhibitory effect of DCV on binding of domain I to short (5 nt) polyU RNA (Ascher et al., 2014). As DCV did not affect dimerization of domain I, the authors proposed that it did indeed stabilize a dimeric form of NS5A that was unable to bind RNA. It has been estimated that at the EC50 of DCV there are only six molecules of the drug per cell, whereas the precise quantitative analysis of SGR-harbouring cells conducted by Quinkert et al. (2005) revealed a total of approximately 1 million non-structural proteins per cell, a 1000–10 000-fold excess over negative-strand RNA. Although only $<5\%$ of these non-structural proteins were present in a protease-resistant fraction (i.e. in membrane-bound replication complexes), this would imply that DCV was able to target the subpopulation of NS5A that was actively involved in RNA replication and had presumably adopted a specific conformation. Not only does this imply an extraordinary level of selectivity, but it also requires that DCV precisely targets an ‘active’ population of NS5A only present within membrane-bound replication complexes, bringing to mind the old adage ‘a needle in a haystack’. Although, given that it has been estimated that eukaryotic cells contain between $10^9$ and $10^{10}$ protein molecules (Milo, 2013), perhaps ‘a needle in a field of haystacks’ would be a more appropriate analogy.

Paradoxically it has been demonstrated that DCV treatment has global effects on NS5A, for example altering the subcellular localization and fractionation patterns (Lee et al., 2011), or reducing hyperphosphorylation (Fridell et al., 2011; Qiu et al., 2011) However, the caveat relating to these data is that they were obtained following plasmid transfection, thus again not in the context of active RNA replication. More recently, the picture has been complicated by the observation that DCV treatment rapidly blocked assembly and release of infectious virus particles, well before any measurable effects on RNA replication, and indeed had no effect on pre-existing replication complexes (McGivern et al., 2014). Furthermore, DCV-related compounds have been reported to block HCV-induced membrane rearrangements, independently of RNA replication (Berger et al., 2014). On reflection, these data are consistent with the notion that NS5A has multiple roles in both genome replication and virion assembly. They also support the existence of multiple pools of NS5A: first, one that is involved in virion assembly and is perhaps located in the cytosol and thus easily accessible to DCV; secondly, one in pre-existing replication complexes that is refractory to DCV, with the implication that NS5A plays no role in ongoing RNA replication; thirdly, a pool of NS5A that is orchestrating the assembly of active replication complexes and is sensitive to DCV, albeit over a longer timescale. Although it is clear that much remains to be elucidated about the mode of action of DCV (and related compounds), these studies highlight the potential utility of NS5A inhibitors in understanding the many functions of the protein.

Concluding remarks

This review did not set out to be comprehensive, and we apologize in advance to those colleagues whose work has not been represented here. It represents a personal perspective on the last decade of NS5A research – the controversies, dogmas and remaining questions. We hope that it will be thought-provoking and stimulate some debate in the field, and particularly that it will contribute to the discussion about the future of basic research into HCV. The landscape is changing rapidly; the advent of highly effective DAA therapy means that the translational agenda will no longer be a tool to justify basic research. However, patients will be treated with drugs that have no defined mode of action and are proposed to target a protein that remains enigmatic to say the least. The drugs have shown us that NS5A is a valid target; however, when resistance emerges – and it will, as this is the most variable virus known to man and is now about to be subject to selective pressure – then a better understanding of the biology of this protein will be essential to enable the development of new classes of inhibitors. We must not forget that it was the advances in the molecular biology of HCV that enabled the development of DAAs. We abandon that translational pipeline at our peril.

Acknowledgements

We thank Andrew Macdonald and Nicola Stonehouse for critical comments on this manuscript. Work in the M.H. laboratory is funded by a Wellcome Trust Senior Investigator Award (096670MA).

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