

Review

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Hepatitis C virus NS5A: tales of a promiscuous protein

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The non-structural 5A (NS5A) protein of hepatitis C virus (HCV) has been the subject of intensive research over the last decade. It is generally accepted that NS5A is a pleiotropic protein with key roles in both viral RNA replication and modulation of the physiology of the host cell. Our understanding of the role of NS5A in the virus life cycle has been hampered by the lack of a robust *in vitro* system for the study of HCV replication, although the recent development of the subgenomic replicon has at least allowed us to begin to dissect the involvement of NS5A in the process of viral RNA replication. Early studies into the effects of NS5A on cell physiology relied on expression of NS5A either alone or in the context of other non-structural proteins; the advent of the replicon system has allowed the extrapolation of these studies to a more physiologically relevant cellular context. Despite recent progress, this field is controversial, and there is much work to be accomplished before we fully understand the many functions of this protein. In this article, the current state of our knowledge of NS5A, discussing in detail its direct involvement in virus replication, together with its role in modulating the cellular environment to favour virus replication and persistence, are reviewed. The effects of NS5A on interferon signalling, and the regulation of cell growth and apoptosis are highlighted, demonstrating that this protein is indeed of critical importance for HCV and is worthy of further investigation.

Hepatitis C virus

Hepatitis C virus (HCV) is the only member of the genus *Hepacivirus*, within the family *Flaviviridae*. Viral isolates are further classified into six genotypes (1–6) and 30 subtypes, the most intensively studied of which are genotypes 1a and 1b. It is estimated that 170 million individuals are infected with this virus (Anonymous, 1999), and in 80% of cases the virus establishes a chronic infection, resulting in fibrosis, cirrhosis and, increasingly, hepatocellular carcinoma (HCC). Current therapy, which consists of a combination of pegylated alpha interferon (IFN- α) and ribavirin, gives a response rate of between 48% (genotypes 1, 4, 5 and 6) and 88% (genotypes 2 and 3) (Poynard *et al.*, 2003). The genome is a positive-sense 9.6 kb RNA molecule, comprising 5' and 3' untranslated regions (UTRs) flanking a single open reading frame encoding a polyprotein of ~3000 aa (Fig. 1). A second translation product, the F protein, is produced by ribosomal frameshift (Xu *et al.*, 2003). Little is known about the function of this protein, although it has been shown to localize to the endoplasmic reticulum (ER). The 5' UTR contains an internal ribosome entry site

(IRES), allowing cap-independent initiation of translation. The polyprotein is cleaved into 10 polypeptides by cellular and viral proteinases. At the N terminus are three structural proteins – Core and the glycoproteins E1 and E2. Following E2 is p7, a protein that oligomerizes to form cation channels (Griffin *et al.*, 2003) and has been speculated to be incorporated into virus particles. The C-terminal two-thirds of the polyprotein comprise the six non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B. Two of these are functionally well characterized: NS3 complexed with NS4A is a proteinase that cleaves between the non-structural proteins (it also has helicase activity); NS5B is the viral RNA-dependent RNA polymerase. The others presumably play as yet undefined roles in virus replication; however, an increasing body of literature points to a role for these proteins in perturbing cell signalling and mediating immune evasion. In this regard, NS5A is of great interest, and here we will review our current knowledge of this protein.

Structure of the NS5A protein

A major limitation in our understanding of NS5A is the paucity of structural information. The protein is predicted to be predominantly hydrophilic and to contain no *trans*-membrane helices. A number of structural features of the protein have, however, been derived experimentally.

Published online ahead of print on 8 June 2004 as DOI 10.1099/vir.0.80204-0.

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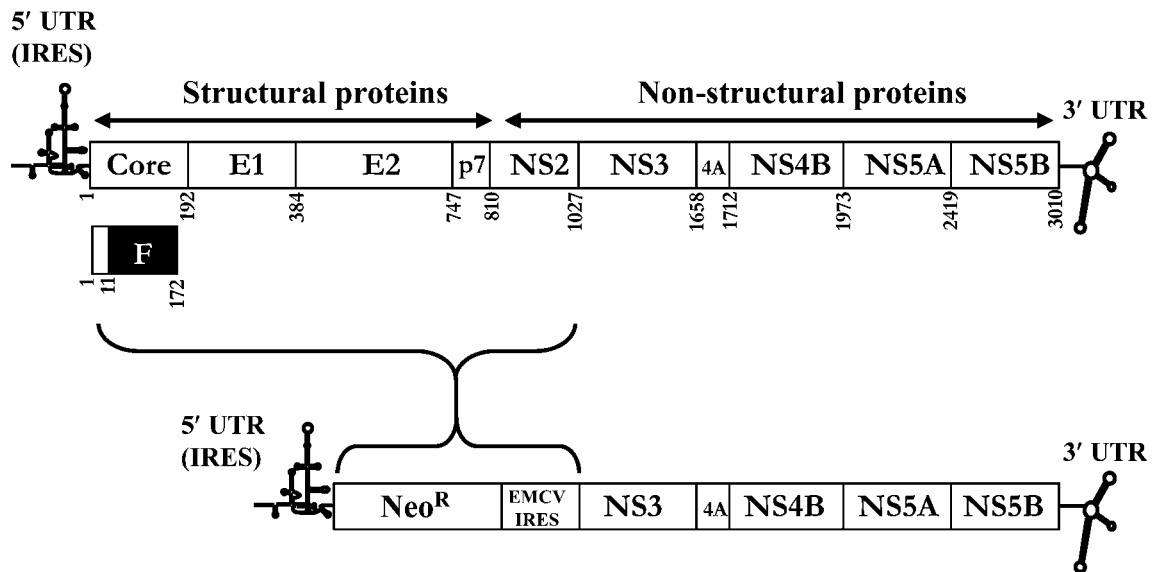


Fig. 1. Structure of the HCV genome and a typical subgenomic replicon. The genome of HCV consists of a single open reading frame encoding a polyprotein of approximately 3010 aa, flanked by UTRs. The 5' UTR contains an IRES. In addition, both UTRs are highly structured (predicted structures are depicted) and are involved in initiation of RNA synthesis. The F protein is generated by a ribosomal frameshift ($-2/+1$) at codon 11 of the Core sequence, thus F and Core share the N-terminal 10 aa (Xu *et al.*, 2003). The typical subgenomic replicon was constructed by replacing the Core through to NS2 region with the gene encoding neomycin phosphotransferase and a second IRES, derived from the picornavirus EMCV (Lohmann *et al.*, 1999). The numbers underneath the genome represent polyprotein amino-acid sequence numbers of the genotype 1b infectious clone (J4 isolate, GenBank accession no. AF054247).

N-terminal amphipathic helix

The N-terminal 30 aa of NS5A were predicted to form a highly conserved amphipathic alpha helix (Brass *et al.*, 2002) (Fig. 2a), and were shown to be both necessary and sufficient to mediate association of NS5A (or GFP) with the ER membrane. By a combination of *in vitro* biochemical assays and comparison with other, similarly membrane-anchored, proteins, it was postulated that the N terminus of NS5A lies parallel to the membrane such that the hydrophobic face of the helix was buried within the bilayer; this interaction appeared to confer upon NS5A the properties of an integral membrane protein. Subsequently, this structure was shown to be essential for HCV RNA replication.

NS5A phosphorylation

Two phosphorylated forms of NS5A, termed p56 and p58, can be distinguished by their electrophoretic mobility. The p56 form of NS5A is a basally phosphorylated protein, modified by phosphorylation in the centre and near the C terminus. Although the sites of basal phosphorylation have not been identified, data suggest that it is mediated by members of the CMGC kinase family – including casein kinase II (CKII), mitogen-activated protein kinases (MAPKs) and glycogen synthase kinase 3 (GSK-3). Inhibitor studies failed to define the kinase(s) involved (Reed *et al.*, 1997); however, other studies showed that NS5A stably associated

with both CKII (Kim *et al.*, 1999) and cAMP-dependent protein kinase (Ide *et al.*, 1997), both of which were able to phosphorylate a GST–NS5A fusion protein *in vitro*. Coito *et al.* (2004) screened the yeast 'kinome' for the ability to phosphorylate baculovirus-expressed NS5A *in vitro* and identified eight candidate kinases. NS5A was phosphorylated by the human homologues of these kinases, including CKI and II, MAPK kinases MEK1, MKK6 and MKK7 β 1 and, lastly, Akt and p70S6K. Interestingly, two of these (MEK1 and Akt) are indirect targets for regulation by NS5A itself.

The p58 form of NS5A is hyperphosphorylated within a serine-rich region in the centre of the protein (Fig. 2b), although there is little consensus as to the identity of the phosphorylated serine residues. Katze *et al.* (2000) demonstrated that Ser2194 (polyprotein numbering, residue 221 of NS5A) was the major phosphorylation site in an HCV1b isolate of NS5A expressed in insect or COS cells. In contrast, Tanji *et al.* (1995) showed that Ser2197, Ser2201 and Ser2204 were required for hyperphosphorylation of HCV1b NS5A, although they did not conclusively show that these residues were phosphorylation sites. This discrepancy may be explained by the fact that, as discussed below, the presence of the other non-structural proteins modulates hyperphosphorylation; whereas Tanji and co-workers utilized a construct expressing NS2 to NS5A, Katze and co-workers expressed NS5A alone. Reed & Rice (1999) showed that Ser2321 (residue 348 in NS5A), although poorly conserved throughout different genotypes of HCV,

most efficient in Mn^{2+} -containing buffers (Reed *et al.*, 1997). Thus, the precise identity of the kinase(s) responsible for NS5A phosphorylation remains to be elucidated.

Hyperphosphorylation is dependent on the presence of other non-structural proteins. NS4A (expressed *in trans*) was required for the production of the p58 NS5A form of an HCV1b isolate (HCV-J) (Kaneko *et al.*, 1994; Asabe *et al.*, 1997). Furthermore, residues 162–166 of NS5A were required for NS4A-dependent phosphorylation. Interestingly, deletion of the N-terminal 162 residues of NS5A resulted in NS4A-independent hyperphosphorylation. The reason for this observation is unclear. Perhaps binding of NS4A might induce a conformational change in NS5A, facilitating hyperphosphorylation? Deletion of the N terminus of NS5A could thus alter the overall conformation of the protein allowing access by the kinase(s). Liu *et al.* (1999), using another HCV1b isolate (HCV-BK), demonstrated a requirement for NS2 generated by auto-proteolysis from the NS2–3 precursor. Two groups (Neddermann *et al.*, 1999; Koch & Bartenschlager, 1999) showed that hyperphosphorylation of an HCV1b (Con1) NS5A required its expression as part of a continuous polyprotein with NS3, NS4A and NS4B, and required the protease activity of NS3. These studies were performed following transient transfection: it will be important to verify such data using the subgenomic replicon system in which NS5A will be involved in replication of the viral RNA. Interestingly, in replicon cells, the p58 form of NS5A was less stable than p56 (half-life ~ 7 h compared to 16 h) (Pietschmann *et al.*, 2001), suggesting that the two forms of the protein play distinct roles in the virus replication cycle.

Other structural features of NS5A

NS5A contains three consensus ProXaaXaaPro motifs: these are present in many signalling proteins, form extended alpha helices and bind to SH3 domains. Two closely spaced motifs near the C terminus (residues 343–356) (Tan *et al.*, 1999; Macdonald *et al.*, 2004) (Fig. 2a and d) are predicted to be surface-exposed, consistent with the ability of NS5A to bind tightly to SH3 domains. In contrast, an N-terminal motif (residues 26–32) is dispensable for SH3 binding and is predicted to lie within the previously discussed amphipathic helix and is thus unlikely to be available for binding to other proteins.

The second C-terminal motif overlaps with a putative nuclear localization signal (Ide *et al.*, 1996). The physiological significance of this signal is unclear, however, as native NS5A is entirely cytoplasmic, being associated with the ER membrane via the N-terminal amphipathic helix. Consistent with this, Satoh *et al.* (2000) showed that N-terminal truncations of NS5A localized to the nucleus; they also reported that NS5A could be cleaved by a caspase-like protease and that one of the cleavage products lacked both N and C termini and was located within the nucleus. However, these observations were made in cells expressing

NS5A alone and have not been documented in a more physiologically relevant environment such as replicon cells; indeed in the latter NS5A is exclusively cytoplasmic (Mottola *et al.*, 2002; Shi *et al.*, 2003).

Functions of NS5A in virus replication

Information derived mainly from the subgenomic replicon system, originally described by Lohmann *et al.* (1999), suggests that NS5A is involved in genomic RNA replication. Replicons consist of bicistronic RNA transcripts in which the HCV IRES drives translation of neomycin phosphotransferase; a second IRES [from encephalomyocarditis virus (EMCV)] drives translation of NS3–NS5B (Fig. 1). *In vitro* transcripts are transfected into the human hepatoma cell line Huh7 and stable cell clones selected with G418. These cells then harbour a cytoplasmic, autonomously replicating RNA and express large amounts of NS3–NS5B. Although not without its drawbacks, for example the rapid mutation rate due to the error-prone nature of NS5B and the limited range of cells that support replicon replication, the system has allowed the investigation of the biochemistry of HCV RNA replication and points to a key role for NS5A in this process.

NS5A is part of a multi-protein, membrane-bound replication complex

By analogy to other RNA viruses, it has been hypothesized that the HCV non-structural proteins form a complex located on cytoplasmic membranes that catalyses replication of the viral genome. The replicon system has confirmed this, and shown that this complex contains NS5A. Together with the other non-structural proteins, NS5A co-localized with viral RNA in a cytoplasmic membrane structure termed the ‘membranous web’ (Egger *et al.*, 2002; Mottola *et al.*, 2002), the generation of which required the NS4B protein. Replicon cell membrane fractions isolated by differential centrifugation contained both p56 and p58 forms of NS5A and were competent for synthesis of HCV RNA *in vitro* (Hardy *et al.*, 2003). An RNA–protein complex purified from replicon cells by a two-step affinity procedure with biotin/digoxigenin-labelled oligonucleotides (Waris *et al.*, 2004) contained all of the non-structural proteins, including NS5A, although hyperphosphorylated NS5A appeared not to be present in this complex. Two studies from Michael Lai’s group (Shi *et al.*, 2003; Gao *et al.*, 2004) analysed the location of HCV RNA replication by membrane flotation and showed that almost all NS5A (although not the hyperphosphorylated form), and all the viral RNA, co-localized with caveolin-2 in an NP-40-resistant fashion. They proposed, therefore, that viral RNA replication occurred within a detergent-resistant fraction of cellular membranes – most likely representing lipid rafts. Further evidence for a critical role of NS5A came from experiments in which the amphipathic membrane-targeting helix was mutated in the context of the replicon (Elazar *et al.*, 2003). Introduction of three helix-disrupting

mutations (Fig. 2a) completely abrogated the ability of the replicon to establish G418-resistant colonies, implying that NS5A membrane association is an indispensable event during HCV RNA replication.

Evidence for direct interactions between NS5A and other non-structural proteins

The formation of a multi-protein complex requires specific interactions between the individual components. In this regard, NS5A was shown to bind to NS5B, both *in vitro* and in transiently transfected cells. This binding required residues 105–162 and 277–334 of NS5A (Shirota *et al.*, 2002) and four discontinuous regions of NS5B (Qin *et al.*, 2001). However, somewhat paradoxically, when present at a molar ratio of 0.1 or below, NS5A modestly stimulated NS5B polymerase activity, but at higher ratios NS5A inhibited NS5B activity. It is unclear why NS5A should inhibit NS5B; however, it may be that the results obtained *in vitro* using purified NS5A and NS5B fusion proteins do not accurately reflect the interaction between these two proteins *in vivo*, when other viral and cellular factors may be involved. It was shown that deletions within NS5A that abrogated the interaction with NS5B also rendered the subgenomic replicon non-functional (Shimakami *et al.*, 2004), whereas a deletion that had no effect on NS5B binding was replication-competent. The authors conclude that NS5A–NS5B interactions are necessary for HCV RNA replication; however, an alternative interpretation – that these deletions perturb the structure of NS5A, thereby abrogating another function associated with genomic replication – should also be considered.

Dimitrova *et al.* (2003), using a combination of standard biochemical and genetic protein–protein interaction assays, demonstrated that NS5A was able to interact independently with all the non-structural proteins, including itself. Taken together with the observation that NS5A hyperphosphorylation required the presence of the other non-structural proteins, it is clear that NS5A does indeed participate in a multi-protein replication complex. However, the molecular details of the structure and function of this complex remain major challenges for the future.

Culture adaptation of the replicon provides further evidence of a role for NS5A in RNA replication

With the development of the replicon system a key question was asked: why was the relative efficiency of establishment of G418-resistant colonies so low, when the level of RNA replication in selected cells was high? The answer lay in the ability of the replicon to undergo mutation, leading to increases in the RNA replication efficiency (Lohmann *et al.*, 2001; Krieger *et al.*, 2001; Blight *et al.*, 2000). Thus, although transfection of the replicon only gave rise to G418-resistant colonies at a frequency of 20–40 per μg of RNA, RNA extracted from replicon cells and re-transfected into naïve Huh7 cells gave a much higher efficiency of

colony formation (Lohmann *et al.*, 2001). After cDNA cloning of RNA derived from replicon cells, sequencing revealed changes throughout the non-structural region (although interestingly not in the UTRs). These cloned replicons exhibited a much higher efficiency of colony formation compared with the original replicon RNA. When engineered back into the parental replicon, single amino-acid substitutions dramatically enhanced the efficiency of colony formation – the most effective was an Arg2884Gly substitution in NS5B (500-fold increase). In NS5A, Glu2163Gly enhanced colony formation 70-fold, whereas Lys2330Glu had no effect. Interestingly, combination of Glu2163Gly (NS5A) with Arg2884Gly (NS5B) rendered the replicon non-viable, whereas Lys2330Glu (NS5A) with Arg2884Gly (NS5B) retained the 400-fold enhancement of colony formation: the explanation for this remains obscure as these residues map outwith the NS5A–NS5B interaction sites (Qin *et al.*, 2001; Shirota *et al.*, 2002).

Krieger *et al.* (2001) confirmed that these culture-adaptive mutations increased the efficiency of colony formation by enhancing RNA replication, and they identified a further single amino-acid substitution in NS5A (Ser2197Pro) that enhanced colony formation 1000-fold. Blight *et al.* (2000) identified a cluster of mutations in NS5A between residues 2177 and 2204, as well as a deletion of 47 aa (2207–2254), that each stimulated colony formation. The most effective of these (20 000-fold stimulation) was Ser2204Ile. The most interesting observation from these studies is that both Ser2197 and Ser2204 were shown previously to be required for hyperphosphorylation (Fig. 2b); indeed, reduced levels of the p58 form of NS5A were observed in cells harbouring replicons with mutations at these residues (Blight *et al.*, 2000). The implication of these results is that NS5A hyperphosphorylation is dispensable for viral RNA replication and, together with the observations that hyperphosphorylated NS5A is not a component of the membrane-bound RNA replication complex (Shi *et al.*, 2003; Waris *et al.*, 2004), these data suggest that this form of NS5A may play a distinct role in the virus life cycle, perhaps during particle assembly. This suggestion is further supported by the observation that, after introduction into a chimpanzee of an RNA transcript derived from an infectious clone containing the Ser2197Pro mutation, within 7 days the virus reverted to the parental sequence at that position (Bukh *et al.*, 2002). A further possibility might be that hyperphosphorylated NS5A functions during translation – perhaps it acts directly to modulate IRES-mediated translation, for example by interacting with ribosomal components or even the IRES itself? In this regard, NS5A has been shown to stimulate IRES-mediated translation (He *et al.*, 2003) and interact with RNA-binding proteins such as the La antigen (Houshmand & Bergqvist, 2003) that itself stimulates the IRES. Furthermore, IRES activity is regulated by the cell cycle (Honda *et al.*, 2000), and the CMGC family of kinases that have been reported to hyperphosphorylate NS5A include the cyclin-dependent kinases whose activity is very tightly linked to the cell cycle.

It is tempting to speculate that NS5A hyperphosphorylation might also be linked to the cell cycle; however, this hypothesis remains to be tested.

NS5A and cellular signalling pathways

The interactions between NS5A and cellular signalling pathways have been the subject of intense investigation, a trend that started with the observation that HCV resistance to IFN could be explained by the ability of NS5A to bind to and inhibit the dsRNA-activated, IFN-induced kinase PKR (Gale *et al.*, 1997). Since then NS5A has been shown to bind to a range of cellular signalling molecules (Table 1), somewhat reminiscent of the Nef protein from human immunodeficiency virus type 1 (HIV-1) (Harris, 1996; Renkema & Saksela, 2000).

As with Nef, the challenge in understanding these interactions is to prove their physiological significance. In the following section we will concentrate on three key

physiological processes within the cell that have been shown to be perturbed by NS5A, namely, the response of virus-infected cells to IFN signalling, the modulation of cell growth, and the multiple mechanisms by which NS5A has been reported to inhibit the cellular apoptotic response. We will finish the section by briefly discussing other NS5A targets.

NS5A and the IFN response – the role of PKR

The links between NS5A and the IFN response have been reviewed recently (Tan & Katze, 2001) so this section will be brief and highlight more recent observations. NS5A was first linked to IFN-responsiveness in patients by molecular epidemiological studies (Enomoto *et al.*, 1995, 1996) that identified a stretch of 40 aa in the centre of NS5A conserved in IFN-resistant HCV isolates (Fig. 2c). HCV variants with mutations within this region appeared to be more sensitive to therapy, suggesting that NS5A played a role in conferring IFN resistance, thus this region was

Table 1. Cellular proteins shown to interact with NS5A

Protein	Interacting sequences (if known)	References
IFN-induced dsRNA activated protein kinase (PKR)	ISDR, polyprotein residues 2209–2248 (+26 residues C-terminal to ISDR), residues 244–296 within the catalytic domain of PKR	Gale <i>et al.</i> (1997, 1998)
Growth factor receptor-binding protein 2 (Grb2)	C-terminal Class II polyproline motif in NS5A (350–356, polyprotein 2322–2328), N-terminal SH3 domain of Grb2	Tan <i>et al.</i> (1999); Macdonald <i>et al.</i> (2004)
Phosphoinositide-3-kinase p85 subunit	N-terminal 110 residues of NS5A or residues 270–300 (polyprotein 2243–2272), SH3 domain of p85	He <i>et al.</i> (2002); Street <i>et al.</i> (2004)
Amphiphysin II	C-terminal Class II polyproline motif in NS5A (350–356, polyprotein 2322–2328), SH3 domain of amphiphysin II	Zech <i>et al.</i> (2003)
Src-family kinases Fyn, Lck and Hck	C-terminal Class II polyproline motif in NS5A (350–356, polyprotein 2322–2328), SH3 domain of Src kinases	Macdonald <i>et al.</i> (2004)
Src-family kinase Lyn	Two C-terminal Class II polyproline motifs in NS5A (342–356, polyprotein 2315–2328), SH3 domain of Lyn	Macdonald <i>et al.</i> (2004)
Transcription factor SRCAP	C-terminal 62 residues of SRCAP, NS5A site undefined	Ghosh <i>et al.</i> (2000b)
Karyopherin β 3	N-terminal 200 residues of NS5A (polyprotein 1973–2172), C-terminal 90 residues of karyopherin β 3	Chung <i>et al.</i> (2000)
Apolipoprotein A1 (ApoA1)	N-terminal 224 residues of NS5A, middle 'domain' of ApoA2 (residues 67–167)	Shi <i>et al.</i> (2002)
hVAP-33 (SNARE-like protein)	C terminus of hVAP-33, NS5A site undefined	Tu <i>et al.</i> (1999); Gao <i>et al.</i> (2004)
p53	N-terminal 150 or C-terminal 88 residues of NS5A, residues 33–88 of p53	Majumder <i>et al.</i> (2001); Qadri <i>et al.</i> (2002); Lan <i>et al.</i> (2002)
Bax	Residues 262–277 of NS5A (polyprotein 2232–2247), Bax site undefined	Chung <i>et al.</i> (2003)
TNF-R1-associated death domain protein (TRADD)	Not defined	Majumder <i>et al.</i> (2002)
Cyclin-dependent kinase 1 (Cdk1)	Not defined	Arima <i>et al.</i> (2001)
Homeodomain protein PTX1	Residues 133–220 of NS5A, PTX1 site undefined	Ghosh <i>et al.</i> (2003)
TNF-R-associated factor 2 (TRAF2)	Residues 148–301 of NS5A, TRAF domain of TRAF2	Park <i>et al.</i> (2002)
hTAF _{II} 32 (component of TFIID)	FXX ϕ motif, residues 175–179 in NS5A (polyprotein 2147–2151)	Lan <i>et al.</i> (2002)
TATA-box binding protein (TBP)	Residues 210–281 of TBP, NS5A site undefined	Qadri <i>et al.</i> (2002)
La autoantigen	N-terminal 83 residues of NS5A, La site undefined	Houshmand & Bergqvist (2003)

termed the IFN sensitivity determining region (ISDR). Other groups confirmed these data, showing that expression of NS5A from IFN-resistant HCV1b isolates rendered cells partially resistant to the effects of IFN and allowed growth of either vesicular stomatitis virus (VSV) (Noguchi *et al.*, 2001) or EMCV (Polyak *et al.*, 1999). NS5A isolates from genotype 1a or 2a IFN-responders or 1b ISDR-deleted mutants did not inhibit IFN activity in these assays. However, two studies (Paterson *et al.*, 1999; Aizaki *et al.*, 2000) failed to find a correlation between ISDR sequence and ability to inhibit IFN activity, and subsequently it has been suggested that sequences towards the C terminus of NS5A, outwith the ISDR, are required to inhibit IFN activity (Nousbaum *et al.*, 2000).

Biochemical data have shown that NS5A bound to and inactivated PKR (Gale *et al.*, 1997), an IFN-induced gene product that is activated by binding to dsRNA, commonly produced during RNA virus genome replication. PKR phosphorylates the translation initiation factor eIF-2 α , shutting down protein translation (Williams, 2001). The interaction of NS5A with PKR required the ISDR and an additional 26 residues C-terminal to the ISDR (Gale *et al.*, 1998). Within PKR, the binding site was identified as the dimerization domain. Binding of NS5A to PKR resulted in inhibition of both PKR autophosphorylation and phosphorylation of an exogenous substrate. However, more recently, conflicting results have been presented. Podevin *et al.* (2001) and Ezelle *et al.* (2001) were unable to observe any effect of NS5A (from either IFN-responders or non-responders) on the activity of PKR in either Huh7 or HeLa cells, although Podevin *et al.* (2001) did show that all isolates of NS5A inhibited IFN activity (using both EMCV and VSV). Furthermore, Podevin *et al.* (2001) were also unable to detect an NS5A–PKR interaction either by co-immunoprecipitation or by co-immunofluorescence.

PKR-independent effects of NS5A on the IFN response

As a result of the controversy surrounding the link between NS5A and PKR, a number of groups have sought evidence for effects of NS5A on IFN activity that are independent of PKR. In this regard, it has been reported that NS5A is able to upregulate production of interleukin (IL) 8. This cytokine can attenuate the antiviral properties of IFN (Khabar *et al.*, 1997), suggesting that endogenously produced IL8 may facilitate virus infection. Polyak *et al.* (2001a) demonstrated that NS5A upregulated the IL8 promoter, thereby increasing the levels of IL8, an observation confirmed by microarray analysis (Girard *et al.*, 2002). Importantly, this observation has been verified *in vivo* as HCV-infected patients showed higher levels of serum IL8 compared with controls; in addition, levels were higher in IFN non-responders compared to responders, suggesting a further mechanism for inhibition of this antiviral pathway (Polyak *et al.*, 2001b).

Many recent studies have analysed the effects of IFN on the

subgenomic replicon (space constraints preclude a detailed review of this literature here). However, it is important to point out that, although IFN- α has been shown to inhibit replicon function at the level of both RNA replication (Frese *et al.*, 2001; Guo *et al.*, 2001) and IRES-mediated translation (Wang *et al.*, 2003), this inhibition appeared not to depend on whether the replicons were derived from IFN-resistant or -sensitive HCV isolates. Wang *et al.* (2003) suggested that the interaction of NS5A with PKR was associated with a partial rescue of replicon replication. Clearly, further analysis of the effect of NS5A on the IFN response in the context of the replicon is called for. However, such analysis is complicated by the observations that some studies have shown activation of the dsRNA response by replicons (Pflugheber *et al.*, 2002; McCormick *et al.*, 2004). In addition, there is evidence that Huh7 cells are defective in some aspects of the dsRNA response (Lanford *et al.*, 2003).

Expression of the whole HCV genome in an osteosarcoma cell line resulted in an inhibition of IFN signalling through the Jak/Stat pathway (Heim *et al.*, 1999). This inhibition resulted in reductions in both the expression levels and DNA-binding activity of the IFN- α -stimulated transcription factor ISGF3, consistent with reduced activity of the ISGF3 components Stat1 and IRF9 (p48). The mechanisms and HCV proteins involved in this inhibition were not clarified, although Stat1 tyrosine phosphorylation and nuclear translocation were not impaired. This study was supported by *in vivo* data (Blindenbacher *et al.*, 2003), showing reduced ISGF3 DNA-binding activity and enhanced susceptibility to infection with lymphochoriomeningitis virus in transgenic mice expressing the complete HCV genome. Although not analysed in these studies, there is growing evidence that serine phosphorylation of Stats plays a pivotal role in the regulation of IFN signalling (Shuai, 2003). MAPK family members have been shown to serine phosphorylate Stat1 and this has been linked with maximal transcriptional activation (Song *et al.*, 2002). As discussed below, NS5A inhibits the extracellular signal-regulated kinase (ERK) MAPK, possibly via an interaction with the adaptor protein Grb2 (Tan *et al.*, 1999; Macdonald *et al.*, 2003). By inhibiting ERK, NS5A may reduce levels of Stat1 serine phosphorylation and in doing so impair the cellular response to IFN- α (Tan *et al.*, 1999); however, this hypothesis is not supported by the observation that NS5A from IFN-resistant and IFN-sensitive genotypes of HCV (1a, 1b and 2a) was able to bind Grb2 (Macdonald *et al.*, 2004).

NS5A, mitogenic signalling pathways and cell growth

To establish a chronic infection, viruses must modulate host cell mitogenic signalling pathways to regulate cell growth and activation. Mitogenic signalling is mediated, for the most part, by the MAPK, a family of serine/threonine kinases that mediate the cellular response to a variety of extracellular stimuli. Three main pathways have been characterized (Chang & Karin, 2001): these are the previously mentioned ERK pathway, the stress-activated

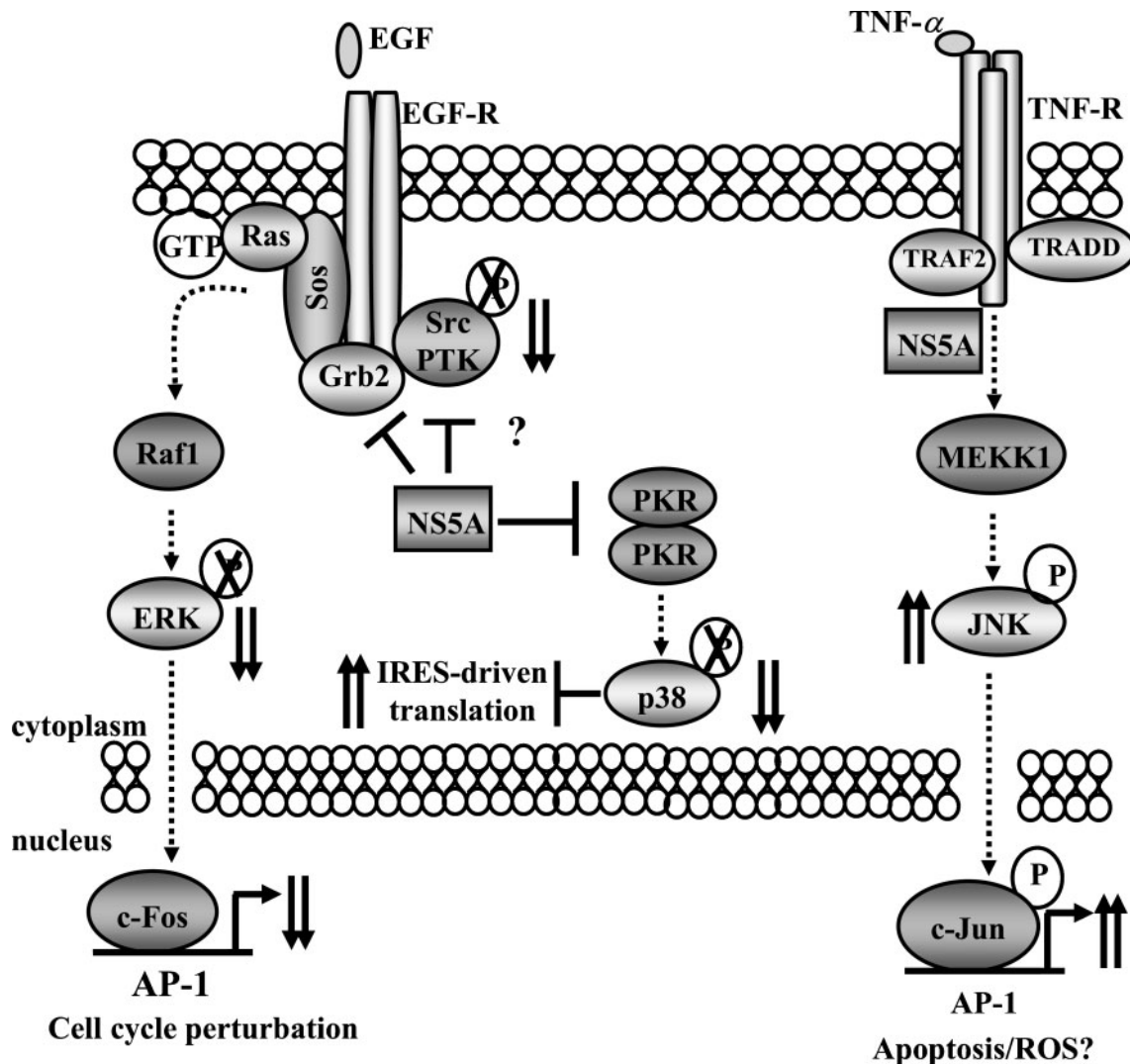


Fig. 3. NS5A modulation of MAPK signalling pathways. NS5A is able to regulate all three classes of MAPK, with varying cellular effects. Stimulation of cells with growth factors leads to the formation of a Grb2–Sos complex at cell membrane and GTP exchange on Ras, which activates the ERK signalling pathway. Binding of NS5A to Grb2 or members of the Src family of tyrosine kinases, mediated via a PxxP–SH3 domain interaction, subverts this pathway and reduces ERK signalling. NS5A is also able to bind adaptor proteins recruited to the TNF receptor, and increases activation of the stress-related JNK MAPK pathway upon stimulation with TNF- α . Virus replication gives rise to the formation of dsRNA, which stimulates the activation of PKR. NS5A binds to and inhibits PKR, which in turn leads to reduced activation of the p38 MAPK. One of the downstream targets of p38, Mnk1, targets the cell translation machinery and promotes IRES-driven translation. Events stimulated by NS5A are denoted by $\uparrow\uparrow$, those inhibited by NS5A denoted by $\downarrow\downarrow$.

protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway, and the less well understood p38MAPK pathway (Fig. 3). All of these are based around the principle of a triple-kinase signalling module consisting of a MAPK that is itself phosphorylated on serine and threonine residues by a MAPK kinase (MAPKK), which in turn is activated by a variety of upstream MAPK kinase kinases (MAPKKK) (Chang & Karin, 2001).

Recent data suggest that NS5A modulates all three MAPK signalling pathways, potentially with opposing cellular

functions. NS5A was able to inhibit the ERK MAPK pathway (Tan *et al.*, 1999; Macdonald *et al.*, 2003; Georgopoulou *et al.*, 2003). A detailed analysis using dominant active variants of components of the ERK pathway and pharmacological inhibitors demonstrated that this inhibition occurred between the receptor and Ras–GTPase activity, and resulted in a downregulation of the activity of the AP-1 transcription factor (Macdonald *et al.*, 2003). The mechanism(s) by which NS5A is able to inhibit ERK activation are as yet unknown, although data from *in vitro* binding assays have shown that NS5A is able to bind Grb2 (Tan *et al.*, 1999;

Macdonald *et al.*, 2004). Grb2 binds to phosphorylated tyrosine residues on the activated EGF receptor via an SH2 domain (Tari & Lopez-Berestein, 2001), recruits the guanine nucleotide exchange factor Sos to the plasma membrane, which then catalyses Ras GDP–GTP exchange, subsequently activating the ERK pathway. It is conceivable that by binding Grb2 NS5A inhibits the formation of the Grb2–Sos complex or subverts the subcellular localization of Grb2 to reduce signals emanating from the activated EGF receptor. However, it has been reported that NS5A does not prevent the formation of a Grb2–Sos complex (Tan *et al.*, 1999), and NS5A is thus reminiscent of the Sprouty proteins (Hanafusa *et al.*, 2002) which bind to the Grb2 SH2 domain and inhibit recruitment of Grb2–Sos to growth factor receptors without inhibiting Grb2 binding to Sos. Further speculation must await a more-detailed investigation of the composition of the protein complex recruited to the EGF receptor, although our recent data suggest that NS5A may interact with other proteins that are implicated in EGF signalling, including the Src family of tyrosine kinases (Macdonald *et al.*, 2004).

It is interesting to note that NS5A inhibits ERK signalling, whereas both hepatitis B virus X protein (Klein & Schneider, 1997) and hepatitis E virus ORF3 (Korkaya *et al.*, 2001) activate this signalling cascade. Why there should be a disparity between these three hepatotropic viruses is not understood, although the need for differential activation of ERK may reflect differences in the life cycle of each virus. Indeed, data suggest that activation of ERK by IL1 may inhibit HCV subgenomic replicon replication (Zhu & Liu, 2003). In this context, it is pertinent to note that poly(I)•poly(C)-mediated induction of IRF1 activation is profoundly inhibited in cells treated with antagonists of ERK (Harcourt & Offermann, 2001); therefore, an inhibition of ERK may perturb the innate immune response. However, a causative link between NS5A-mediated ERK inhibition and aberrant IFN signalling remains to be proven, and the observation of an antagonistic effect of ERK on RNA replication still requires further verification.

It has also been demonstrated that NS5A is able to inhibit the EGF-stimulated activation of p38MAPK (He *et al.*, 2001). This pathway plays a pivotal role in control of protein translation via a downstream target, Mnk-1, which in turn phosphorylates and regulates the activity of the translation initiation factor eIF4E (Wang *et al.*, 1998). NS5A expression resulted in decreased eIF4E phosphorylation (He *et al.*, 2001). Although the mechanism by which NS5A inhibits p38 is unclear, p38 is a substrate for PKR, itself known to be downregulated by NS5A. Phosphorylation of eIF4E increases cap-dependent translation, and NS5A-mediated inhibition of this event might result in a cellular environment that favoured cap-independent (IRES-mediated) initiation of translation, a hypothesis supported by evidence showing that NS5A stimulates HCV IRES-driven translation (He *et al.*, 2003).

Thirdly, NS5A has also been shown to activate the

stress-activated JNK MAPK signalling pathway (Park *et al.*, 2003). At first glance this may seem at odds with the data of Macdonald *et al.* (2003), who found no modulation of JNK activity in replicon cells. However, on closer inspection this discrepancy can be reconciled. NS5A was shown to bind to the adaptor protein TRAF2, which is recruited to the tumour necrosis factor (TNF) receptor upon ligand binding and is involved in activation of JNK (Liu *et al.*, 1996). NS5A does not directly activate JNK, it merely potentiates activation mediated via TNF- α (Park *et al.*, 2003): basal levels of JNK activity were unaffected by NS5A. Activation of JNK by NS5A was mediated by the interaction with TRAF2, and overexpression of TRAF2 was sufficient to induce NS5A-mediated activation of JNK in the absence of TNF- α stimulation. Taken together, these data show that NS5A is able to differentially regulate members of the MAPK family in a stimulus-dependent manner. It is pertinent to note that, as discussed above, three MAPKKs (MEK1, MKK6 and MKK7 β 1) have all been shown to phosphorylate NS5A (Coito *et al.*, 2004). These three MAPKKs phosphorylate ERK, p38MAPK and JNK, respectively, suggesting that the interaction between NS5A and MAPK signalling may occur at multiple stages.

One of the many effects of the deregulation of MAPK signalling is perturbation of host cell cycle control; indeed, activation of the ERK pathway by growth factors is required for hepatocytes to progress through a restriction point in the late G₁ phase (Talarmin *et al.*, 1999). Many viruses perturb the cell cycle in order to facilitate maximal virus replication, in some cases causing a block in G₁, for example reovirus HA3 (Saragovi *et al.*, 1999). Interestingly, a recent study of liver biopsies from HCV-infected patients observed that the proportion of cells in G₁ was significantly increased in comparison to samples from patients with alcohol-induced liver injury (Freeman *et al.*, 2003). In this regard, NS5A has been shown to regulate the cell cycle. However, the results of these studies are ambiguous. For example, a reduction in expression of the cyclin-dependent kinase inhibitor p21^{WAF1} by NS5A was observed (Ghosh *et al.*, 1999), and was shown to be due to a direct interaction between NS5A and p53, leading to cytoplasmic sequestration of the latter (Majumder *et al.*, 2001). Interestingly, NS5A was shown to bind to the amino terminus of p53, which is the region bound by host cellular inhibitors of p53 such as MDM2 (Michael & Oren, 2003). By reducing p21^{WAF1} levels NS5A promoted cell growth, suggesting that NS5A was able to subvert cell cycle regulation and bypass checkpoint controls, one outcome of which could be tumour formation: indeed, when NS5A was expressed in nude mice all of the animals developed tumours within 2 weeks (Ghosh *et al.*, 1999). Many viral proteins, including human papillomavirus 16 E6, hepatitis B virus pX, adenovirus E1B/55 kDa and the simian virus 40 large T antigen (Munger & Howley, 2002; Murakami, 2001; Burgert *et al.*, 2002; Chen & Hahn, 2003), inhibit p53 and p21^{WAF1} activity. All of these viruses establish chronic infections and correlate with oncogenesis, and it is interesting to speculate

that by subverting cellular growth NS5A may also facilitate the development of HCC, increasingly cited as a long-term consequence of HCV infection. A note of caution must be raised, however, as it has been observed that in a variety of stable cell lines NS5A causes an increase in the G₂/M phase of the cell cycle, resulting in growth retardation (Arima *et al.*, 2001). This was caused by a direct interaction between NS5A and the cyclin-dependent kinase Cdk1 and resulted in the activation of p21^{WAF1}. It is difficult to find consensus between these opposing observations; however, these experiments were to a large degree performed in transformed cell lines of non-hepatic origin. To clarify the effects of NS5A on the cell cycle it is vital that experiments are conducted in primary human hepatocytes; however, such experiments are technically challenging.

NS5A and apoptosis

Apoptosis is an important process for the elimination of virus-infected cells and, consequently, many viruses that establish a chronic infection have evolved mechanisms to evade this response to ensure their persistence. Over recent years there have been numerous publications demonstrating reduced apoptosis in cells expressing various HCV proteins, including NS5A, and data from these studies suggest that NS5A may utilize multiple mechanisms to inhibit both extrinsic and intrinsic apoptotic stimuli.

TNF- α is a potent extrinsic apoptotic stimulus (Gaur & Aggarwal, 2003), and it has been demonstrated that cells expressing NS5A are refractile to TNF- α treatment, undergoing significantly less apoptosis than control cells (Ghosh *et al.*, 2000a; Miyasaka *et al.*, 2003). These findings have been confirmed using a transgenic mouse model in which NS5A was expressed from a liver-specific promoter (Majumder *et al.*, 2002). Biochemical analysis revealed that the inhibition of TNF- α signalling was mediated by an interaction between NS5A and the TNF- α -responsive adaptor protein TRADD. TRADD is recruited to the activated TNF receptor upon ligand binding and forms a multimeric complex with the TNF receptor, a second death domain containing adaptor protein, FADD, and TRAF2. This complex then signals to a variety of downstream effectors, including caspases and NF- κ B. By binding to TRADD, NS5A was able to block these signals. However, this study also found that, unlike TNF- α -mediated signalling, NS5A was unable to inhibit apoptosis stimulated by FasL (Majumder *et al.*, 2002). This may be because NS5A is unable to bind FADD, the death domain adaptor protein recruited to the Fas receptor upon ligand binding.

Intrinsic apoptosis is regulated by a balance between pro- and anti-apoptotic signals, for example the Bcl2 family of proteins contain pro- and anti-apoptotic members that localize to the mitochondrial and nuclear membranes, where they form heterodimers, driven by interactions between Bcl-2 homology (BH) domains. The balance between various members of the family dictates the apoptotic response (Cory & Adams, 2002). One recent study

noted that sequences within the ISDR share homology with the BH domains of anti-apoptotic members of the Bcl2 family (Chung *et al.*, 2003). The putative BH domain within NS5A allowed it to bind to the pro-apoptotic Bcl2 protein, Bax, rendering cells refractile to certain pro-apoptotic agonists (Chung *et al.*, 2003). An important caveat to this work is the requirement for NS5A to co-localize with Bax within the nucleus. However, intact NS5A resides exclusively within the cytoplasm, although truncated forms have been shown to localize to the nucleus (Satoh *et al.*, 2000). NS5A also targets another pro-apoptotic Bcl2 family member, Bad (He *et al.*, 2002; Street *et al.*, 2004), by a different mechanism (see below).

As discussed previously, NS5A is able to bind to and sequester p53 in the cytoplasm. As well as its role in regulating cell growth, p53 also acts as an intrinsic apoptotic signal following DNA damage or unscheduled DNA synthesis (Fridman & Lowe, 2003). Most viruses that target p53 have tumorigenic properties, highlighting the importance of this protein for cell survival. The ability of NS5A to bind p53 and induce cytosolic sequestration may prevent DNA binding and transactivation of target genes, resulting in impaired apoptosis (Lan *et al.*, 2002). This may have implications for hepatocarcinogenesis, as any adverse effects on p53 activity could lead to compromised DNA repair, creating a pool of hepatocytes containing unstable genetic material with increased probability of neoplastic transformation. The varied effects of NS5A on apoptosis are illustrated in Fig. 4.

NS5A perturbation of phosphatidylinositol 3-kinase-mediated signalling pathways

Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric enzyme consisting of a p85 regulatory subunit and a p110 catalytic subunit; the latter phosphorylates the 3' carbon of the inositol ring of membrane phosphatidylinositol lipids to produce phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Cantley, 2002). PIP3 is bound by pleckstrin homology (PH) domains, recruiting PH domain-containing proteins to the plasma membrane. A key downstream effector of PI3K is the serine kinase Akt/PKB that is phosphorylated and activated by another PH domain-containing kinase, PDK1 (Fig. 5). NS5A bound to the PI3K p85 subunit (He *et al.*, 2002), and Street *et al.* (2004) further showed that this involved the p85 SH3 domain and a novel motif within NS5A. NS5A formed a stable complex with PI3K in Huh7 cells harbouring a subgenomic HCV replicon, and this interaction augmented the lipid kinase activity of PI3K tenfold both *in vivo* and *in vitro* (Street *et al.*, 2004), resulting in a concomitant increase in Akt activity. As Akt is a pivotal link in a survival signalling cascade mediated by PI3K, activation of this kinase is effected by many viruses (Cooray, 2004). Akt activation leads to changes in glucose transport and synthesis, as well as translation and cell survival. Many targets of Akt are pro-apoptotic cellular

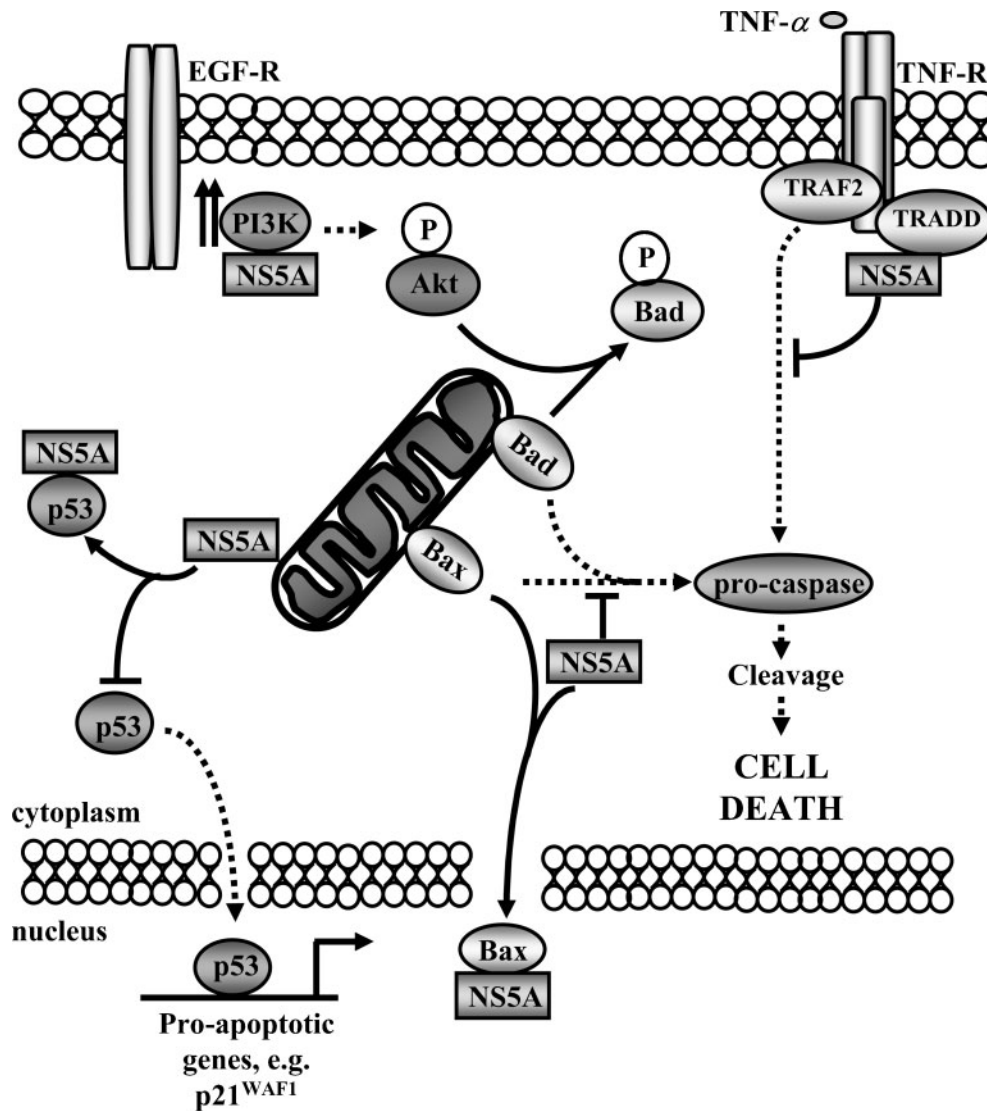


Fig. 4. NS5A mediates extrinsic and intrinsic stimulated apoptosis. Cellular apoptosis pathways can be activated by external and internal stimuli, including binding of stress cytokines (TNF- α) to cell surface receptors or DNA damage within the cell. NS5A binds the TNF- α adaptor proteins TRADD and TRAF2 and inhibits activation of cellular caspases, the executioners of apoptosis. In addition, NS5A is able to divert at least two pro-apoptotic Bcl2 proteins from the mitochondria to the nucleus (Bax) or the cytosol (Bad). NS5A also prevents nuclear translocation of the tumour suppressor protein p53 by cytoplasmic sequestration. In all cases, NS5A-mediated signalling subversion is indicated by continuous arrows, whereas 'normal' host signalling is denoted by dotted arrows.

proteins, including the Forkhead transcription factor family (FKHR) (Tang *et al.*, 1999), the pro-apoptotic Bcl2 family protein Bad (Cory & Adams, 2002), caspase-9 and p21^{WAF1} (Coffer *et al.*, 1998). Phosphorylation of these targets by Akt results in cytosolic sequestration and inactivation. NS5A-mediated activation of Akt resulted in the phosphorylation and inhibition of Bad (Street *et al.*, 2004), and also correlated with resistance to pro-apoptotic stimuli. Furthermore, our more recent data suggest that NS5A-mediated upregulation of PI3K may also have much wider significance for the survival of the host cell

and for tumorigenesis. Another Akt target is GSK-3 β , the dysregulation of which is often associated with HCC (Desbois-Mouthon *et al.*, 2002). Interestingly, NS5A expression increases Akt-dependent phosphorylation of GSK-3 β and thereby stabilizes a key downstream target of GSK-3 β , the proto-oncogene β -catenin (Street *et al.*, unpublished data). Stabilized β -catenin has been associated with the inhibition of TNF- α -induced apoptosis within hepatocytes (Shang *et al.*, 2004) and is increasingly implicated in a range of tumours, particularly HCC (Cui *et al.*, 2003; Edamoto *et al.*, 2003; Prange *et al.*, 2003; Ban

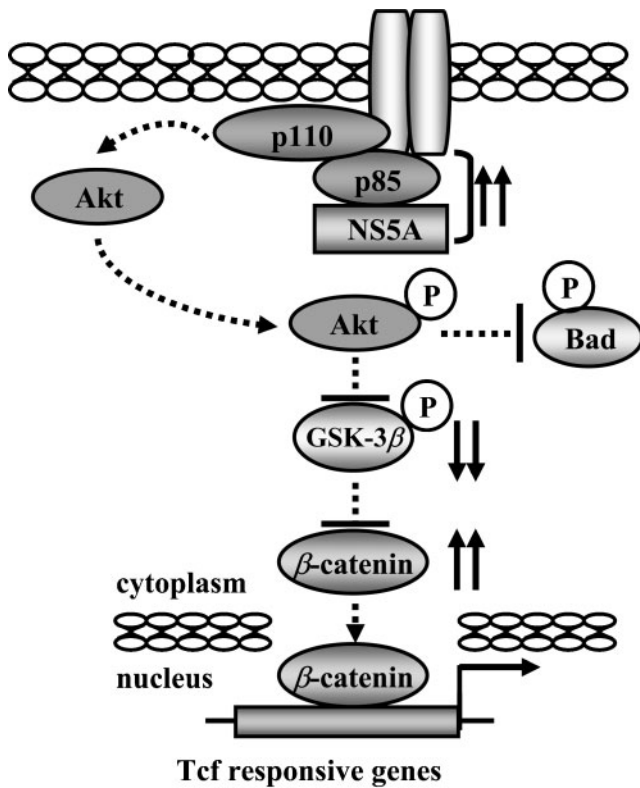


Fig. 5. Activation of PI3K-mediated survival pathways by NS5A. Binding of NS5A to the SH3 domain of the p85 regulatory subunit of PI3K recruits the p110 catalytic subunit and activates lipid kinase activity. In turn this recruits and activates the Akt kinase, which is a master control switch within the cell and inhibits several pro-apoptotic proteins, including the Bcl2 homologue Bad. Akt phosphorylates the GSK-3 β kinase, a negative regulator of β -catenin, and in turn activates β -catenin which can translocate to the nucleus and bind to promoter sequences within genes associated with cell survival. In all cases, NS5A-mediated subversion of signalling is indicated by continuous arrows, whereas 'normal' host signalling is denoted by dotted arrows; events stimulated by NS5A are denoted by $\uparrow\uparrow$, those inhibited by NS5A denoted by $\downarrow\downarrow$.

et al., 2003). These exciting data may, therefore, have profound implications for the association of HCV with the development of HCC.

NS5A and calcium/reactive oxygen signalling

NS5A has been shown to alter the intracellular balance of calcium signalling and induce the formation of reactive oxygen species (ROS) (Gong *et al.*, 2001) (Fig. 6), the outcome of which is an upregulation of both NF- κ B and Stat3. NF- κ B regulates expression of many proteins, such as cytokines, IFNs and adhesion molecules, and plays a key role in the host apoptotic process (Kucharczak *et al.*, 2003). The mechanism of NF- κ B activation was shown to involve the efflux of calcium from the ER to mitochondria,

the activation of tyrosine kinases, including Zap70, and the phosphorylation of the NF- κ B inhibitor I κ B α , thus allowing translocation of NF- κ B to the nucleus (Waris *et al.*, 2003). These results were confirmed in cells expressing NS5A alone or harbouring the subgenomic replicon. Cells isolated from HCV-infected livers also exhibited increased levels of nuclear NF- κ B (Tai *et al.*, 2000), providing evidence for the physiological importance of this effect of NS5A. It is therefore likely that, in the context of an HCV infection, one of the key roles of NS5A is to increase ROS production and activate the anti-apoptotic properties of NF- κ B, which may indirectly predispose hepatocytes to cellular transformation.

ROS activation by NS5A also upregulates the proto-oncogene Stat3 and, as with NF- κ B, this is likely to involve a calcium-dependent signalling pathway (Gong *et al.*, 2001). Studies using anti-oxidants demonstrated reduced Stat3 tyrosine phosphorylation and transactivation compared to controls, which is entirely consistent with the well characterized ability of ROS to activate Stat3 (Simon *et al.*, 1998). At this time, the identity of the NS5A-activated tyrosine kinase responsible for phosphorylating Stat3 is not known; however, studies on HIV-1 Nef showed that activation of Stat3 in monocyte-derived cells was mediated by upregulation of the Src-family kinase Hck (Briggs *et al.*, 2001). Like Nef, NS5A is able to interact with and modulate the activity of Src-family kinases such as Fyn (Macdonald *et al.*, 2004) – NS5A activated Fyn in replicon cells and Fyn subsequently phosphorylated Stat3. However, it is likely that there are several kinases that may fulfil this role *in vivo*, and preliminary data suggest that Fyn may not be the only NS5A-activated kinase that can phosphorylate Stat3 (Macdonald *et al.*, unpublished observations). The precise role played by Stat3 in HCV-induced pathogenesis is at this stage unknown, although Stat3 dysregulation has been implicated in numerous carcinomas, including HCC (Yoshikawa *et al.*, 2001). One could speculate that activation of Stat3 by NS5A is yet another mechanism by which HCV promotes survival signalling within infected hepatocytes.

Subgenomic replicons have been used to ask whether NS5A-mediated elevation of ROS might affect HCV replication. Controversially, two studies showed that ROS either suppressed (Choi *et al.*, 2004) or stimulated (Qadri *et al.*, 2004) replicon replication; the former study showed that treatment of cells with anti-oxidants restored levels of replicon replication. Further study is clearly warranted to develop our understanding of the role of ROS production in HCV replication.

Other NS5A targets

There are many other NS5A-interacting partners (Table 1), unfortunately space constraints preclude the detailed discussion of these here. However, a number of them are worthy of brief mention as they are involved in protein trafficking and membrane morphology, and thus may have relevance for the formation and function of the

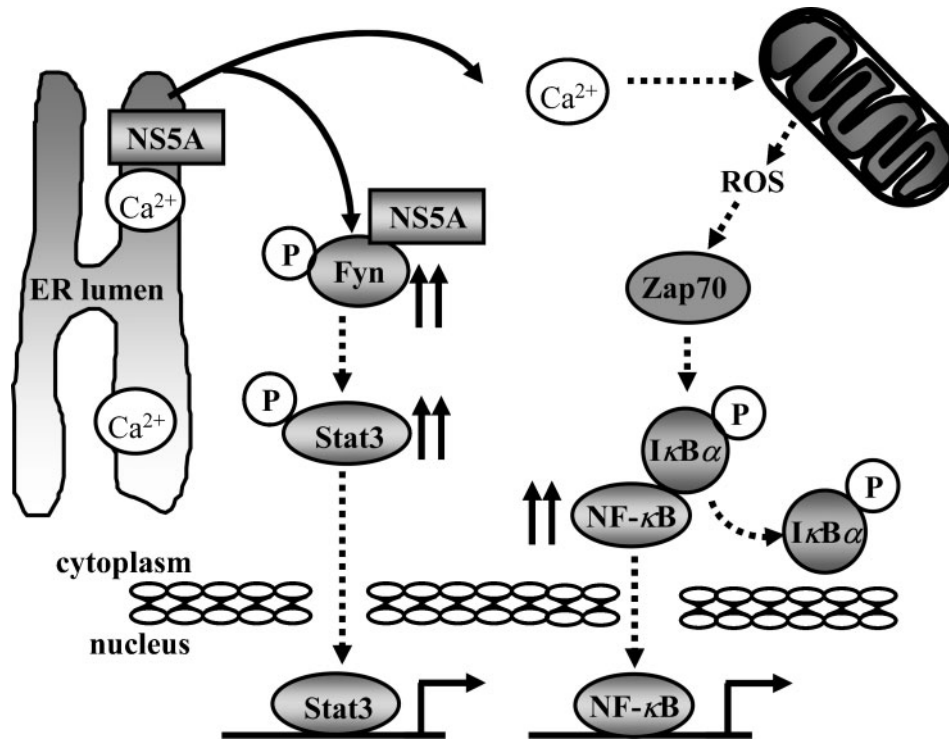


Fig. 6. NS5A and calcium signalling. By a mechanism that is not well understood NS5A causes efflux from the ER of calcium, which is subsequently taken up by mitochondria and induces the release of ROS. Increased ROS levels activate the tyrosine kinase Zap70, which in turn phosphorylates the NF- κ B inhibitor protein I κ B α and targets it for degradation. Free from inhibition, NF- κ B translocates to the nucleus where it binds to promoter-specific sequences. Calcium efflux activates Stat3 via Fyn-mediated tyrosine phosphorylation, which leads to nuclear accumulation and transactivation of specific genes. NS5A also activates Fyn tyrosine kinase activity, stimulating Stat3 activity further.

membrane-bound replication complex. These include karyopherin β 3 (Chung *et al.*, 2000), the SNARE-like protein hVAP-33 (Tu *et al.*, 1999; Gao *et al.*, 2004), apolipoprotein A1 (Shi *et al.*, 2002) and amphiphysin II (Zech *et al.*, 2003). It is tempting to speculate that, whilst at the ER, NS5A may interact with many of these proteins to aid in the formation of a membrane-bound replication complex. This idea is supported by recent data (Gao *et al.*, 2004) showing that siRNA or dominant negative mutants of hVAP-33 resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes and reduced levels of HCV RNA and proteins in replicon cells. Over the next few years the use of the replicon system should strengthen our understanding of many of these interactions, and determine whether they play a role in the formation of a replication complex.

Concluding remarks

Although there is much conflicting experimental data in the literature, it is generally accepted that NS5A plays a critical role in the replication of HCV, both directly, with regard to viral RNA replication, and indirectly, by modulating the host cell environment to favour the virus. The molecular details of the involvement of NS5A in the

process of HCV genomic RNA replication remain to be characterized. NS5A is clearly associated with replicating RNA in a cytoplasmic replication complex, but we currently have little or no information about precisely what function it is performing. NS5A also perturbs signalling pathways within the cell and there are many convincing arguments to explain why such perturbations would benefit the virus.

What direction should research into NS5A take? A key goal must be to elucidate the three-dimensional structure of the protein. This would provide a rational framework for mutagenesis and facilitate a better understanding of the protein-protein interactions participated in by NS5A. It is certain that the list of NS5A-interacting proteins, together with the list of NS5A functions, will grow; however, the challenge will be to 'validate' these findings, linking laboratory observations to the pathogenesis of HCV infection, at both the cellular and organismal levels. Our understanding of the role of NS5A in virus replication would be greatly aided by the development of more-robust replication systems for HCV, ideally involving productive infection of primary human hepatocytes, allowing the function of NS5A to be analysed in a truly physiologically relevant setting. The problems of cultivating such cells in the laboratory would seem to preclude such experiments

in the conceivable future; however, developments such as the 'mosaic mouse' model (Mercer *et al.*, 2001), full-length replicons (Ikeda *et al.*, 2002), and the use of replicons in cells other than Huh7 (Zhu *et al.*, 2003; Ali *et al.*, 2004) provide optimism for the future.

Will NS5A ever be a target for antiviral therapy? As a protein with no known enzymic activity it is certainly not amenable to current drug screening protocols. However, as inhibitors of the HCV replicative enzymes (NS3 and NS5B) are still in development, attention is being drawn to other potential targets. We believe that NS5A is such a target, and that the challenge ahead is to prove that specific NS5A-cellular protein interactions are critical for virus replication. Strategies to disrupt these interactions would then provide the basis for novel, much-needed therapies for HCV. It seems likely that NS5A will continue to keep us busy for many years into the future!

Acknowledgements

We thank Nicola Stonehouse and David Rowlands for critical reading of this manuscript. Work in the authors' laboratory is supported by the Medical Research Council, The Wellcome Trust and the Biotechnology and Biological Sciences Research Council.

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