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The Nonstructural NS5A Protein of Hepatitis C Virus: An Expanding, Multifunctional Role in Enhancing Hepatitis C Virus Pathogenesis

Gregory R. Reyes

Infectious Diseases and Oncology, Schering-Plough Research Institute, Kenilworth, N.J., USA

Key Words

Hepatitis C virus · NS5A · Interferon-α · PKR · Interferon sensitivity-determining region · Viral pathogenesis

Abstract

The hepatitis C virus (HCV) NS5A gene product is a phosphorylated 56- to 58-kD nonstructural protein that displays a multitude of activities related to enhancement of viral pathogenesis. Although associated with other viral encoded proteins as part of the viral replicase complex positioned on the cytoplasmic side of the endoplasmic reticulum, a role for NS5A in viral replication has not been defined. Post-translational modifications of NS5A include phosphorylation and potential proteolytic processing to smaller molecular weight forms able to translocate to the nucleus. Both the identification of a putative interferon (IFN) sensitivity-determining region within NS5A, as well as the direct interaction with and inhibition of the IFN-induced double-stranded RNA-dependent protein kinase (PKR) by NS5A remain controversial. Truncated versions of NS5A can act as transcriptional activators, while other recently characterized interactions of NS5A with cellular proteins indicate its pleiotropic role in HCV-host interactions. NS5A itself has no direct effect on IFN-α signaling or activation, but other abundant interactions with members of the cellular signaling apparatus, transcription activation machinery and cell cycle-regulatory kinases have been described (e.g. growth factor receptor-bound protein 2, p53, p21/waf and cyclins). Many of these interactions block the apoptotic cellular response to persistent HCV infection. More recently, another altogether different mechanism attenuating the IFN-α response was reported based on induction of interleukin (IL)-8. IL-8, in model systems, potentiates viral replication and mutes the nonspecific intracellular IFN antiviral response. Evidence supporting a complex multimechanistic role of NS5A in promoting viral persistence, pathogenesis and, indirectly, viral-related hepatocarcinogenesis indicates its key role in HCV pathobiology.

Introduction

Hepatitis C virus (HCV) causes chronic hepatitis in 50–84% of infected individuals. Although difficult to recognize and relatively asymptomatic in the majority of individuals, chronic HCV infection is associated with high rates of progression (approximately 30%) to chronic liver disease, cirrhosis and hepatocellular carcinoma [1]. The factors that dictate progression versus those that result in an asymptomatic carrier state are unknown. The

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Fax + 41 61 306 12 34
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Gregory R. Reyes, MD, PhD
Infectious Diseases and Oncology
Schering-Plough Research Institute
2015 Galloping Hill Road, Kenilworth, NJ 07033 (USA)
Tel. +1 908 740 3200, Fax +1 908 740 7164, E-Mail gregory.reyes@spcorp.com

complications of end-stage liver disease in HCV infection are the major causes of liver transplantation in the USA [72]. Available serologic and nucleic acid diagnostic tests have extended our understanding of the natural history of HCV infection, and the former were instrumental in reducing the incidence of transfusion-transmitted hepatitis. Even so, about 3.9 million people are infected in the USA, and the seroprevalence (indicative of chronic infection), although variable, is generally in the range of 3%, indicating that as many as 170 million people are chronically infected worldwide [75].

HCV is a linear, positive-sense, single-stranded RNA virus of approximately 9.6 kb in length that was molecularly characterized in the late 1980s [6]. It is a distinct genus (hepacivirus) within the family Flaviviridae. Other members of this family include the genus *Flavivirus* (e.g. dengue virus, yellow fever virus and West Nile virus) and members of the genus *Pestivirus* (e.g. bovine viral diarrhea virus, hog cholera virus [48]). Common genomic features of the Flaviviridae include expression of viral genes from a single large open reading frame with co- or post-translational polyprotein processing by a combination of host and viral proteases to yield individual structural and nonstructural (NS) gene products. For HCV, the viral polyprotein precursor of about 3,000 amino acids yields 10 proteins, including the amino terminal structural proteins (C, E1, E2 and p7) and the NS proteins encoded in the carboxy 2/3 of the genome (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [reviewed in ref. 36]. A very recently discovered new viral encoded protein expressed in an alternative open reading frame by ribosomal frame shifting may add an eleventh protein to the viral repertoire [74, 76]. Known as ARFP or F, its function is unknown, but its expression in infected individuals has been confirmed by its demonstrated immunoreactivity with patient sera.

The putative HCV gene products were originally assigned functions based on the identification of homologous counterparts in the genomes of previously characterized Flaviviridae members. Although these related viruses may serve as useful surrogates in assigning certain NS gene functions related to processing, replication and viral maturation, investigation into the pathogenic roles of the various individual HCV gene products has been limited, since robust in vitro infection with HCV has not been achieved. The recent development, however, of a subgenomic replicon [45] overcomes some limitations, but progress in the development of an authentic in vitro system for serial viral propagation is still needed.

Therapies for HCV currently approved by the United States Food and Drug Administration include interferon (IFN)-α and pegylated (polyethylene glycol conjugated) IFN-α, either alone or in combination with ribavirin [47]. Sustained virological response (SVR) is defined by a reduction in serum viral RNA below the limits of detection (by reverse transcriptase polymerase chain reaction) for a 6-month period beyond completion of therapy. Optimal therapy with pegylated IFN-α in combination with ribavirin is achieved in HCV genotypes 2 and 3, where an SVR of approximately 80% is possible in previously untreated patients; response rates with HCV type 1 genotypes are less remarkable, with an SVR of only approximately 40% after 1 year of combination therapy. It is well established, but equally enigmatic, that sustained response to IFN-α-containing regimens strongly correlates with viral genotype (2 and 3) and other factors such as circulating viral load [28]. While the viral and/or host factors that influence the differing response rates have not been fully delineated, the roles of certain virus encoded proteins in promoting/enhancing viral replication and establishing a persistently infected state are beginning to be defined. In this regard, it is clear that viruses have evolved elaborate strategies that minimize the impact of the host defense and adaptive immune responses to viral infection.

IFN Sensitivity-Determining Region and PKR Modulation of the IFN-α Response

Clearly, HCV genotype is one factor that determines the likelihood of achieving an SVR with IFN-α-containing regimens, and this observation led to an investigation of potential viral factors that might be responsible. Complete HCV genomic sequences from IFN-α responder and nonresponder patients identified sequence heterogeneity in NS protein NS5A and the definition of what was described as an IFN sensitivity-determining region (ISDR) (fig. 1) [9, 10]. The observation correlated an increased number of mutations (equal to or greater than 4) within the ISDR of NS5A with an improved IFN-α-induced SVR, thereby focusing attention on the possible role of NS5A in conferring sensitivity (or resistance) to IFN-α therapy. The existence of the so-called ISDR within NS5A has, however, been controversial, since support for initial findings has been variable in numerous subsequent reports [25, reviewed in ref. 55].

Following the description of the putative ISDR, NS5A was shown to directly bind and inhibit the activity of cel-

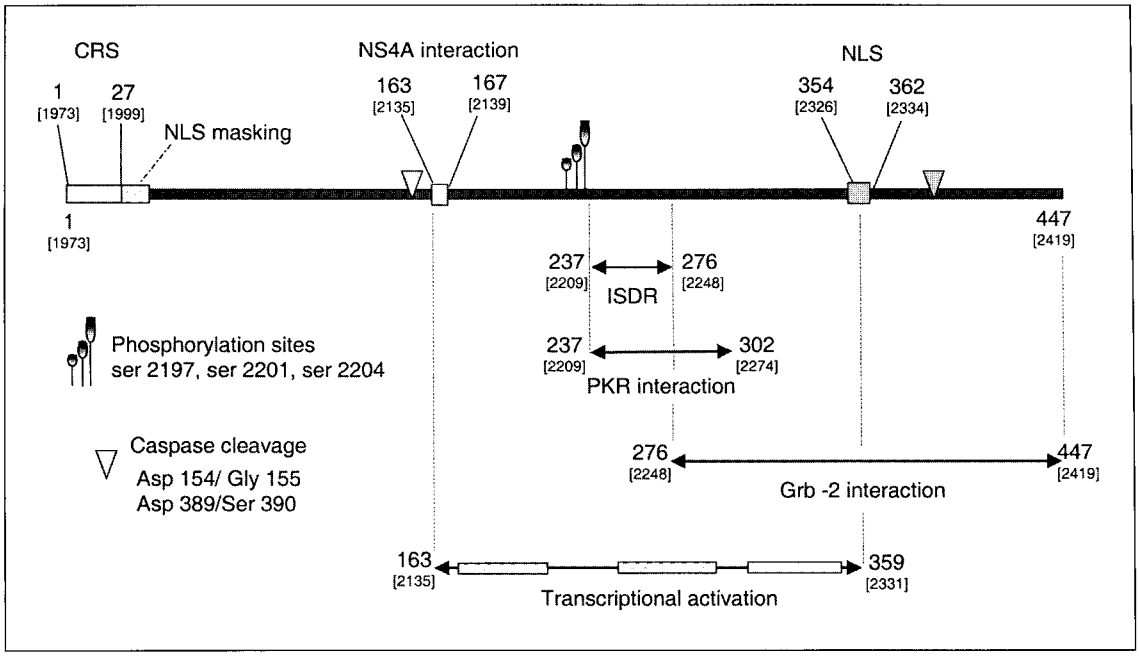

Fig. 1. The figure summarizes the various functional and interactive domains identified for the NS5A gene of HCV genotype 1b. The numbers indicate the amino acids in serine protease (NS3/4A)-processed NS5A, as well as the amino acid positions (in square brackets) in the full-length 1b polyprotein. The NS5A region important for NS4A-dependent hyperphosphorylation is indicated (NS4A interaction), followed by the serine cluster that undergoes NS4A-dependent phosphorylation to the 'hyperphosphorylated' 58-kD form of NS5A. The putative caspase cleavage sites that flank this region have been shown to release a 31-kD product that both localizes to the nucleus and has transcriptional activator activity [61]. The location of the CRS, NLS and the sequence capable of masking the NLS (NLS masking) are indicated. The region identified as interacting with PKR and its associated ISDR phenotype are indicated. Transcriptional activation is mapped to the indicated region, overlapping PKR and ISDR, with two acidic regions (hatched boxes) upstream of the proline-rich region (solid box). The Grb-2-interacting region is mapped to the C-terminus of NS5A.

lular encoded protein kinase R (PKR), and thus a potential mechanism of action for the overlapping ISDR activity was identified [15]. IFN-induced double-stranded RNA-activated PKR is one of the prominent, well-characterized mechanisms by which IFN-α mediates antiviral activity. Once activated by IFN-α, PKR undergoes autophosphorylation and inhibits initiation of translation by phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2-α). eIF2-α is the GTP-binding initiation factor that is responsible for facilitating met-tRNA binding to the 40S ribosomal subunit to initiate protein translation. The end result of interferon activation of PKR is a generalized nonspecific inhibition of cellular protein translation resulting in decreased viral replication. In essence, NS5A interacting with PKR may work, in part, to counteract the antiviral activity of IFN-α and sustain viral protein expression. Although substantial supportive biochemical data have been generated [16, 23], once again, controversy exists in that other groups have not replicated these results [56, 70, reviewed in ref. 66]. In addition to NS5A, the HCV structural protein E2 has also been identified as having structural elements that interact with and inhibit PKR [71].

The role of NS5A as a prognostic molecular marker for IFN-α response, via mutation of ISDR and inhibition of PKR, continues to be debated (see Conclusion). However, a number of other characterized associations between NS5A and cellular proteins have been described, with potential roles in enhancing HCV pathogenesis. The key to whether these other interactions are important may rest on how their activity relates to the post-translational modifications of NS5A.

Post-Translational Modification of NS5A

NS5A is a 56-kD phosphoprotein with a 58-kD hyperphosphorylated form [34, 69]. The regions of the protein that undergo basal phosphorylation to yield the 56-kD form are located between amino acids (aa) 2200–2250 and 2351–2419 and are independent of NS4A-modulated hyperphosphorylation of NS5A to the slower migrating 58-kD form [3, 69]. It should also be noted that hyperphosphorylation is inhibited in part by the N-terminal region of NS5A, such that deletion of the amino terminal aa 102–162 promotes hyperphosphorylation [3]. The major sites of NS5A hyperphosphorylation are serine residues immediately N-terminal to the ISDR in the central region of the protein (fig. 1). The colinear (in cis) expression of other upstream NS proteins (NS3, NS4A, NS4B) is apparently required for processing to the hyperphosphorylated 58-kD form, illustrating the tight control of HCV-1a NS5A post-translational processing [52]. Indeed, mutations of the NS3 protease that abrogate enzymatic activity effectively blocked phosphorylation of NS5A [52]. The requirement for colinear expression of amino terminal NS proteins for hyperphosphorylation has not been reported by others [3], but may indicate strain differences between these studies. Conversely, the absence of detectable NS5A hyperphosphorylation in HCV genotype 2a may have been due to a need for upstream coexpression of other NS proteins rather than true genotype differences in hyperphosphorylation based on inability to complex with NS4A [27]. Could this be a tantalizing clue regarding the differential response rates seen in HCV genotypes 1 and 2 to IFN-α therapy? The functional consequences of this differential phosphorylation have not been determined; however, it is provocative that several serine sites for phosphorylation are located adjacent to the ISDR. At least two major serine phosphorylation sites have been identified, including the highly conserved serine 2194 [38] as well as a site that may be specific to genotype 1a at serine 2321 [59].

The kinase responsible for NS5A phosphorylation has not been definitively identified, although early studies had suggested that a member of the so-called CMGC group of serine-threonine kinases might be responsible [60]. Further evidence has been discovered (molecular weight, substrate specificity, heparin inhibition) that NS5A is phosphorylated in a non-cell cycle-dependent manner by casein kinase II, a member of the CMGC kinase group [41]. Substrates of the CMGC family are believed to include many transcription factors, including NF-κB, making casein kinase II a regulator of transcription factor activity (see below). An earlier report of an NS5A-bound serine-threonine protein kinase may in fact describe the same CMGC kinase family member [30]. It should be noted that NS5A is not phosphorylated by PKR.

As already noted, the role that phosphorylation plays in the various activities identified for NS5A has not been elucidated. Phosphorylation of NS5 in dengue virus type 2 appears to regulate its trafficking from cytoplasmic replicase complexes to the nucleus, in the process disrupting replicase interactions. If parallels are drawn to dengue virus, phosphorylation of NS5A may play a similar role in regulating viral protein interactions and cytoplasmic/nuclear partitioning. As shown for dengue, NS3 (serine protease) and NS5 (RDRP) interact in the perinuclear space, but phosphorylated NS5 shows preferential nuclear localization [35]. The fact that deletion of the amino terminal cytoplasmic retention signal (CRS) leads to both hyperphosphorylation and nuclear localization supports the potential for nuclear partitioning of NS5A p58; however, further experimental validation is needed (see below).

NS5A has a perinuclear cytoplasmic location consistent with association with the endoplasmic reticulum (ER). Recently, NS5A together with NS5B have both been found to associate with the recently described human homologue of the 33-kD *Aplysia californica* vesicle-associated protein, hVAP33, which has been shown to be predominantly associated with the ER by cell fractionation studies [17]. NS5A and NS5B colocalized to the cytoplasmic side of the ER through distinct and separate interactions with amino and carboxy terminal regions, respectively, of hVAP30. It has previously been shown that the major NS proteins of HCV were membrane associated [26], similar to what has been described for the ER association of other flaviviruses such as dengue and West Nile virus. Whether hVAP33 acts only as a scaffold protein or has some other function requires further study.

Other possible reported post-translational events include proteolytic processing to activate (or unmask) a functionally active nuclear localization signal (NLS) at aa position 354–362 (fig. 1) [31]. As already noted, uncleaved intact NS5A is a likely member of the multiprotein replicase complex located on the cytoplasmic side of the ER. Cell-based cleavage and its regulation of NS5A trafficking have been investigated, and it was shown that selected N- or C-terminal truncations of NS5A were sufficient to permit its migration into the nucleus after transient transfection [61]. Proteolytic processing in both the N and C regions of the molecule was also seen after tran-

sient transfection of full-length NS5A, and this processing was enhanced by apoptotic stimuli. Inhibition by the caspase inhibitor Z-VAD-FMK suggested that the protease activity was due to a caspase-like protease. Modification of potential cleavage sites confirmed their utilization and the generation by caspase cleavage of a nuclear-localized product (aa 155–389). This 31-kD form of NS5A demonstrated transcriptional activation (see below) with apparent regulation (enhancement) through phosphorylation by the alpha catalytic subunit of PKA [30]. Other experiments have indicated the presence of a 27-aa CRS capable of sequestering NS5A in the cytoplasm with dominant activity over the NLS (fig. 1) [61]. Similar results obtained by Song et al. [62] demonstrated not only an N-terminal CRS but another C-terminal CRS (aa 353–447) and a separate region (aa 27–38) functional in masking NLS activity [62].

Interactions between NS5A and Growth Factor Receptor-Bound Protein 2

Growth factor receptor-bound protein 2 (Grb-2) is an adaptor protein that mediates signal transduction between cell surface receptors and downstream proteins in the signal transduction cascade [13]. Interactions between NS5A and Grb-2 have been revealed by immunoprecipitation and confirmed by immunoblot analysis using anti-Grb-2 antisera and cell lysates from recombinant NS5A containing vaccinia virus-infected HeLa cells [67]. Interactions with Grb-2 were localized to a highly conserved proline-rich SH3 binding motif (PXXP) present near the carboxy terminus of NS5A, with indications of cooperative Grb-2 binding between the N- and C-terminal SH3 domains. NS5A-Grb-2 binding inhibited phosphorylation of extracellular signal-regulated kinases (ERK) 1 and 2 and blocked signaling by exogenous epidermal growth factor [67]. The most direct interpretation of these studies is that NS5A disrupts Grb-2-mediated signaling pathways to inhibit mitogenic signaling. It is of interest, however, that NS5A did not affect Grb-2/Sos levels. Rather than perturbing the mitogenic pathways, it is also possible that NS5A disrupts the role of Grb-2 in promoting apoptosis [11]. Furthermore, it has also been shown that the ERK cascade has a role in the induction of IFN-α gene expression, whereby ERK1/2 translocates to the nucleus, where it activates transcription factors, including the STATs [8]. It is therefore possible that NS5A may disrupt IFN-α-mediated antiviral effects in part through its interaction with Grb-2; however, it is unclear how ER-associated NS5A might mediate these effects.

NS5A Modulation of Intracellular Calcium

More recently, HCV NS5A was shown to alter intracellular calcium levels, thus inducing oxidative stress and activating STAT-3 and NF-κB [21]. STAT-3 was constitutively activated by NS5A and antioxidant treatment countered this effect. The proposed mechanistic pathway inducing these effects postulates that NS5A, localized to the ER, leads to the release of calcium from ER stores, making it available to mitochondria, where it induces the formation of reactive oxygen species, thereby leading to NF-κB activation. Activation of NF-κB was abrogated by treatment with calcium chelators (e.g. EGTA-AM) as well as antioxidants (e.g. N-acetyl *L*-cysteine). NS5A was directly responsible for the generation of reactive oxygen species after transfection of either NS5A or the entire NS region of the HCV genome as detected by FACS analysis. This new activity of NS5A in activating NF-κB could play an antiapoptotic role in cellular physiology and may indirectly predispose hepatocytes to cellular transformation. It is therefore perhaps significant that elevated levels of NF-κB have been detected in chronic hepatitis C liver [64]. Free radicals have also been shown to play a role in the development of carcinogenesis [63]. Transgenic studies with HCV in mice have shown that all develop steatosis, with a subset developing hepatocellular carcinoma [43]. Reactive oxygen species are also elevated in the livers of these HCV transgenics and might therefore play a role in the etiology of hepatocellular carcinoma [43].

Transcriptional Activation and Repression by NS5A

NS5A has been shown to interact with Snf2-related CREB-binding protein activator protein (SRCAP) by yeast two-hybrid screening and in the process modulate transcription [19]. SRCAP has been identified as a novel ATPase that enhanced the ability of CREB-binding protein to function as a coactivator for a number of transcription factors [33]. Interestingly, it was found that adenoviral E1A could also disrupt the interaction between CREB-binding protein and SRCAP to effect transcriptional repression. A similar viral-mediated inhibition of cellular transcription is possible through the interaction of NS5A

with SRCAP, and in fact, downregulation of p21/waf1 was observed in these studies.

A more direct role as a transcriptional activator in yeast and HuH-7 cells has been described for NS5A deletion mutants after fusion to the GAL4 DNA-binding domain [7, 37, 68]. The most potent transcriptional activation was seen with amino terminal deletions of NS5A with transcriptional activation mapping to the central ISDR. Full-length NS5A was inactive. Apart from the ISDR, two separate acidic regions and one proline-rich region were present within the minimally defined region and all are considered consensus motifs for transcriptional activation (fig. 1). The region was further refined by deletion analysis to encompass the ISDR and short flanking regions (aa 228–284) [14]. The transcriptional activity of an ISDR from an IFN-sensitive phenotype (i.e. containing 6 aa changes in the ISDR) was enhanced 90-fold compared to that from a nonmutated ISDR (resistant phenotype). The greatest activity was seen when the proline-rich region was included. Mutation of a single amino acid from the normally occurring alanine residue at position 252 to phenylalanine had a pronounced effect on increasing basal activity, with even greater activity seen with this mutation in the context of other NS5A mutations [14]. Although the appropriate 'machinery' exists for transcriptional activation, it remains to be determined whether it is functional, since all studies have utilized NS5A constructs without associated NS proteins. Recent data from Satoh et al. [61], however, are perhaps the most compelling in that they demonstrate that nuclear localization is not only possible but enhanced by apoptotic stimuli [61].

NS5A Perturbation of Apoptosis and Growth Control

The p53 tumor suppressor protein is a key cellular regulator of cell growth and programmed cell death (apoptosis) following exposure to various genotoxic stimuli. Its pivotal role is illustrated by the frequency with which it is inactivated in human malignancies [73]. Modulation of p53 activity by direct interaction with a viral gene product is a strategy used by many transforming and chronically infecting viruses. Examples include the SV40 large T antigen, human papilloma virus E6, adenovirus E1B, human cytomegalovirus IE84 and vIRF (K9 open reading frame) of Kaposi's sarcoma-associated herpesvirus [51]. Regulation of p53 occurs both at the level of protein stability (turnover) as well as through modulation of its biological activities. One of the effector genes for p53 is the cyclin-dependent kinase inhibitor p21/waf1, which regulates Rb phosphorylation and G1-S cell cycle progression and whose expression (p21 promoter activity) had previously shown to be downregulated by NS5A [20]. More recently, the direct physical association between p53 and NS5A was shown in vivo by coimmunoprecipitation with colocalization to a perinuclear location by confocal microscopy. Therefore, NS5A transcriptional repression of p21/waf1 probably results from a direct interaction between NS5A and the amino terminus of p53, where other negative p53 regulators such as mdm2 bind. NS5A interaction with the p53 consensus DNA-binding site has been shown to result in global repression of p53 transcriptional activation [46]. The X protein of another chronically infecting, transforming hepatitis virus, hepatitis B virus, has also been shown to interact with and sequester p53 in the cytoplasm [65]. Whether this is a general attribute of chronically infecting hepatitis viruses is unknown. Sequestration of p53 by NS5A may have direct effects in attenuating the innate endogenous cellular antiviral response, in that it has been shown that double-stranded RNA is reduced in its ability to induce expression of IFN-stimulated genes (ISG 15) in the absence of p53. p53 might therefore act independently of IFN to amplify the antiviral response [29]. It is also possible that this interaction may indirectly promote HCV-associated hepatocarcinogenesis by blocking the p53-mediated apoptotic program. Similar antiapoptotic effects have been shown for NS5A in protecting against tumor necrosis factor (TNF)-α-mediated cell death by blocking caspase-3 activation and cleavage of poly (ADP-ribose) polymerase [18].

NS5A, after either induction or constitutive expression in stably transfected lines, has also been shown to interact specifically with Cdk1/2-cyclin complexes with subsequent *inhibition* of cell growth [2]. Accumulation of cells in G0/G1 or G2/M was dependent on the cellular background as well as whether expression was constitutive or induced consistent with an *induction* of p21/waf1. Direct interaction of NS5A with Cdk1 was shown both in vitro and in vivo. This interaction disrupted cyclin-dependent activation, whereas an inhibition of Cdk2 activity was believed to be mediated by induction of p21/waf1. It is unclear how to reconcile these results with the NS5A-p53-mediated repression of p21/waf1 noted above.

NS5A Induction of Interleukin-8: Inhibition of IFN-α Antiviral Responses

Interleukin (IL)-8 is a proinflammatory chemokine induced by certain viral infections. It belongs to the CXC chemokine family and is active in inducing neutrophil chemotaxis, angiogenesis and hematopoiesis [49]. IL-8 has been found to enhance, in a dose-dependent manner, the cytopathic effect and virus yield of encephalomyocarditis virus (EMCV; a positive-stranded RNA virus), but not vesicular stomatitis virus (a negative-stranded RNA virus) [40]. Anti-IL-8-specific monoclonal antibodies were able to abrogate the effect. The IL-8-associated enhancement of EMCV infection also correlated with decreased 2′-5′-oligo adenylate synthase (OAS) activity independently of an effect on OAS gene expression [40]. IL-8 has also been shown to enhance replication of the double-stranded DNA virus cytomegalovirus [50].

A direct inhibitory effect of IL-8 on IFN-α activity has been demonstrated [39]. IL-8 inhibited IFN-α-mediated anti-ECMV activity in a dose-dependent manner with an ED_{50} of 150 pg/ml (maximum inhibition of 10 ng/ml). The effect was reversible by anti-IL-8 antibody. Interestingly, the IL-8 effect was evident as early as 20 h before or 20 h after IFN-α treatment, indicating that a later step in the IFN-α pathway was affected. It is puzzling that the inhibitory effect on IFN-α was only manifest in the presence of poliovirus and EMCV (positive-stranded RNA viruses) and herpes simplex type 1 (double-stranded DNA virus), but not the negative-stranded vesicular stomatitis virus. OAS activity was similarly unaffected in vesicular stomatitis virus-infected cells but was substantially reduced in EMCV-infected cells. Recently, an IL-8 homolog (vIL-8) was identified in the genome of Marek's disease virus, a lymphotropic herpesvirus responsible for a contagious lymphoproliferative disease in chickens. Recombinant Marek's disease virus lacking vIL-8 showed a decrease in lytic infection when compared to parental virus [54].

While NS5A has no direct effects on IFN-α-stimulated gene factor 3 (ISGF-3), STAT-1 phosphorylation, upregulation of PKR or MHC class I, it has been shown to be capable of inducing (by at least 8-fold) the expression of IL-8 (both mRNA and protein) [57]. Various deletion mutants of NS5A were tested and it was shown that amino terminal deletions enhanced IL-8 induction, presumably through better nuclear localization. Results were similar for NS5A genes isolated from either IFN-α responder or nonresponder patients or using an NS5A with a deleted ISDR region. Rather than direct interaction of NS5A with the IL-8 promoter, it was determined that NF-κB as well as AP-1 were critical to IL-8 induction in the presence of TNF-α. Furthermore, IL-8 induction resulted in an approximately 12-fold inhibition of IFN-α antiviral effects in an in vitro bioassay. These experiments established that not only did NS5A induce the expression of IL-8, but that IL-8 was capable of inhibiting IFN-α activity.

Do these observations with IL-8 have some clinical correlate or relevance? IL-8 levels were found to be elevated in 132 HCV-infected patients compared to uninfected healthy control individuals (n = 32) [58]. The mean IL-8 level in HCV-infected individuals was 1,731 ± 290 pg/ml, versus 12.35 pg/ml in uninfected controls. It was also demonstrated that pretreatment levels of IL-8 were associated with responders (1,606 ± 773 pg/ml) versus nonresponders (2,727 ± 951 pg/ml), with partial responders being intermediate (2,409 ± 986 pg/ml). TNF-α, a potent inducer of IL-8, was also found to be significantly elevated in HCV infection (12.46 ± 1.4 vs. 6.11 ± 3.3 pg/ml in normals). A recent analysis of cytokine profiles (TNF-α, IL-6, IL-8 and IL-12) as possible predictors of sustained response in chronic hepatitis C (noncirrhotic patients) found higher basal (pretherapy) cytokine levels of TNF-α and IL-8 in chronic hepatitis C [53]. Among these markers, TNF-α was much lower in sustained responders compared to patients who relapsed after therapy or nonresponders [53]. TNF-α, IL-6 and IL-12 all dropped in the population with a sustained response with no apparent correlation of cytokine profile with a particular therapeutic regimen.

Finally, it should be noted that IL-8 and Gro-α, ligands of the chemokine receptors CXCR1 and CXCR2, are both produced upon HIV infection and stimulate HIV-1 replication. As in HCV infection, elevated levels of IL-8 were found in the lymphoid tissue of patients with AIDS. The effect of blocking IL-8 in HIV infection was investigated using a small molecule and it was shown that replication could be inhibited using a compound that blocked the interaction of IL-8 with its receptor [42].

Conclusion

In the course of HCV infection, complex extracellular and intracellular interactions occur soon after exposure that in all likelihood determine the natural course of disease and disease progression. The result of these biological interactions between virus and host is the establishment of a persistent infection in the majority of exposed individuals that, although asymptomatic in most, has the

potential to evolve into chronic hepatitis and associated life-threatening sequelae. Of the various post-translational events documented for NS5A, the key questions are which (if any) of these occur and what is their timing and relation to the ever-growing list of NS5A-host protein interactions. Indeed, what are the pathobiological consequences of these myriad interactions? If colinear expression of upstream NS proteins is critical to appropriate post-translational processing, for example the generation of hyperphosphorylated p58, then results obtained to date by transfection of single genes may lack relevance to actual events in the viral life cycle.

Both in vitro and in vivo evidence supports the notion of complex pathobiological interplay between HCV and host. In the recently described subgenomic HCV replicon system, only low-level viral replication is initially observed upon establishing cell lines containing self-replicating HCV RNAs. Higher replicon levels (copy numbers) were obtained once cell culture-adaptive mutations occurred [5, 44]. These mutations were dispersed throughout the NS region of the subgenomic replicon. An increased intracellular copy number was due to enhanced replication, and the most effective mutations could be synergistic, as seen with NS3 and NS5A (hyperphosphorylation at serine 2197). Similar NS5A mutations were seen in culture-adapted HCV replicons by Blight et al. [5], and most were localized in the NS5A region, again affecting serine 2197 as well as serine 2204, with the latter most effective at enhancing viral replication. It would therefore appear that complete hyperphosphorylation of NS5A is not required for high-level intracellular copy number, replication or persistence. These adaptive mutations for enhanced replication do indicate, however, the important role of NS5A in modulating host-viral interactions.

The effectiveness of IFN-α has been tested in the HCV replicon system, and while it is effective in reducing the cell-associated copy number by 10- to 20-fold in a dose-dependent manner, there was no apparent increased sensitivity or resistance when replicons contained NS5A derived from either IFN-α-sensitive or -resistant individuals [22]. Furthermore, IFN-α could not 'clear' virus from cells after prolonged treatment, and stepwise increases in IFN-α dose did not yield an overt IFN-α-resistant phenotype. Once again, the critical adaptive mutation for high intracellular viral copy number was the NS5A serine 2197 hyperphosphorylation site reported in previous studies [22].

An important recent observation made regarding IFN-α response rates in early infection (early HCV seroconversion) illustrates that the ongoing dynamic between host and virus can be interrupted and shifted in favor of viral eradication [32]. Intensive treatment with IFN-α (5 million U/day s.c. for 4 weeks, followed by 5 million U 3 times per week for 20 weeks), begun on average 89 days after presumed primary infection, yielded an astonishing response rate of 98% (43 patients). HCV RNA became negative on average 3.2 weeks after beginning therapy. In effect, the development of chronic hepatitis C was prevented and the course of natural infection altered. What are the differences in early versus late infection that might account for this improvement in response rates? The extracellular events are very likely driven by humoral and cellular immune responses to virus infection as well as by innate/nonspecific host defenses. Humoral immunity may not have a significant role in clearing virus since mutation under selective pressure effectively evades neutralizing antibodies. Of note is that these remarkable response rates in early treatment were apparently not genotype dependent.

There is evidence that NS5A does not have direct effects on STAT/JAK signaling via ISGF-3 induction. It is, however, quite clear that regulated expression of the *entire* HCV polyprotein effectively blocks IFN-α-induced signaling via the Jak-STAT pathway and could therefore contribute to the reduction in IFN-α efficacy [24]. The time course for HCV protein induction as well as the level of HCV protein expression was inversely correlated with the ISGF-3 induction. Analysis showed that the block was downstream of STAT phosphorylation, homo- or heterodimerization, interaction with ISGF-3γ-p48 (to form ISGF-3) or nuclear translocation of STAT complexes. Therefore, the block is likely to occur at the level of STAT binding to DNA, and indeed, IFN-α target gene upregulation was blocked [24]. When the activity of PKR was determined for the same inducible cell line, no inhibition of PKR activity, nor of its substrate eIF2-α, was detected, leading to the suggestion that the biological 'context' of viral protein expression (as a polyprotein with post-translational processing), may be critical to the authentic role played by NS5A [12].

The colocalization of NS5A and NS5B on the cytoplasmic side of the ER through interaction with hVAP30 would support the role of NS5A in HCV replication. Mutations within NS5A and other NS regions of HCV can enhance viral expression in subgenomic replicon systems, therefore indicating some role for NS5A in modulating viral expression and replication apart from IFN-α-mediated processes. Many of the recently described interactions of NS5A with cellular proteins result in an anti-

apoptotic response. It is of interest that activated PKR can also play a proapoptotic role through the FADD/caspase-8 pathway [4].

The molecular pathogenesis underlying HCV persistence and chronicity is at best poorly understood. Eradication of viral infections is a complex interplay between the virus replication strategy and its underlying pathobiology, balanced against the host's susceptibility and immune response. The recent insightful observations on the efficacy of early IFN-α therapy are, however, mitigated somewhat by the difficulty in recognizing and diagnosing acute HCV infection. It is therefore imperative that alternative therapies be developed for the pool of chronically infected individuals who do not respond to current therapies. It will be of interest to determine if interruption of any of the described functions of NS5A translates into improved therapeutic outcomes for HCV infection.

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