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MINIREVIEW

How Hepatitis C Virus Counteracts the Interferon Response: The Jury Is Still out on NS5A

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Interferons (IFNs) induce an antiviral state in the cell through complex and indirect mechanisms, which culminate in a direct inhibition of viral replication and stimulation of the host adaptive responses. Viruses often counteract with elaborate strategies to interfere with the induction as well as action of IFN effector molecules. This evolutionary battle between viruses and IFN components is a subject of intense research aimed at understanding the immunopathogenesis of viruses and the molecular basis of IFN signaling and action. In the case with hepatitis C virus (HCV), this may have profound implications for the therapeutic use of recombinant IFN in treating chronic hepatitis C. Depending on the subtype of HCV, current IFN-based treatment regimens are effective for only a small subset of chronic hepatitis C patients. Thus, one of the Holy Grails in HCV research is to understand the mechanisms by which the virus may evade IFN antiviral surveillance and establish persistent infection, which may eventually provide insights into new avenues for better antiviral therapy. Despite the lack of an efficient tissue culture system and an appropriate animal model for HCV infection, several mechanisms have been proposed based on clinical studies and *in vitro* experiments. This minireview focuses on the HCV NS5A nonstructural protein, which is implicated in playing a role in HCV tolerance to IFN treatment, possibly in part through its ability to inhibit the cellular IFN-induced PKR protein kinase.

Key Words: hepatitis C virus; interferon; E2; NS5A; PKR; JAK-STAT pathway.

INTRODUCTION

Not since the scientific community discovered the human immunodeficiency virus (HIV), has there been such an explosion of basic and clinical research activities directed toward a pandemic virus. A hepacivirus belonging to the *Flaviviridae* family, hepatitis C virus (HCV) is responsible for chronic liver disease that often leads to liver cirrhosis, hepatic failure and hepatocellular carcinoma (Di Bisceglie, 1998). Although hepatocytes are the major target site of HCV infection, viral replication has also been detected in other organs and cell types, in particular lymphoid cells (Manns and Rambusch, 1999). Therefore, apart from being the leading indication for liver transplantation in the United States, chronic HCV infection is also a major risk factor for the development of autoimmune diseases, including mixed cryoglobulinemia and glomerulonephritis. There is currently no vaccine for HCV, which infects approximately 2.0% of the world population, including 4.0 million Americans (Alter *et al.,* 1999). With up to 10,000 deaths each year in the United States and an estimated 230,000 new HCV infections annually worldwide, chronic hepatitis C is poised to become a serious global medical problem with considerable burden on the health care system.

More than two decades ago, intense research was focused on the potential therapeutic use of interferons (IFNs). IFNs are a family of cytokines secreted in eukaryotic cells early in response to various stimuli, particularly viral infection (Vilcek and Sen, 1996; Biron, 1998). Upon binding to their specific cell surface receptors of target cells, IFNs trigger a cascade of intracellular reactions that culminate in the induction of a large number of genes whose protein products mediate the pleiotropic multiple effects of IFN. These include induction of an antiviral state in their target cell, immune stimulation, and cell growth and differentiation. Because of these properties, recombinant forms of IFNs were then touted as a panacea for a wide range of illnesses, including cancers and viral disease (Borden and Parkinson, 1998). However, IFNs have not produced the results the public had hoped for, although they have been used with some success in the treatment of a number of malignancies, including hairy cell leukemia, metastasizing renal carcinoma, AIDS-associated Kaposi sarcomas, as well as viral infections such as hepatitis B and human papillomavirus. Perhaps the therapeutic use of IFNs has had

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the most profound impact on the treatment of chronic hepatitis C. First approved by the Food and Drug Administration in 1991, recombinant IFN-α, alone or in combination with the synthetic guanosine analogue ribavirin, remains the treatment of choice for chronic HCV infection today, despite the severe side effects associated with its use in the clinic.

However, IFN-α monotherapy leads to sustained viral clearance in only 10-15% of HCV-infected patients (Hoofnagle, 1999). The recent introduction of ribavirin has resulted in only moderate improvement, with 30% of patients achieving a sustained virologic response to the combination therapy (McHutchinson *et al.,* 1998; Battaglia and Hagmeyer, 2000). Therefore, overcoming IFN resistance remains a major challenge for effective IFN-based therapy and future management of the HCV pandemic. In view of that, there is tremendous interest to determine the molecular basis for HCV nonresponsiveness to IFN therapy, which may provide insights into improved therapeutic modalities. This remains a difficult and challenging task, as neither an efficient *in vitro* culture system nor a small animal model is available for HCV infection and propagation (Gale and Beard, 2001). The first hint of a potential molecular mechanism for HCV evasion of IFN response was suggested when clinical observations showed a substantial correlation between mutations in the viral *NS5A* gene from certain HCV genotypes and response to IFN-α treatment in HCV-infected patients.

HCV RESISTANCE TO IFN THERAPY: THE ISDR STORY

Despite the lack of an adequate tissue culture system for HCV infection and replication, viral proteins have been identified and characterized by the use of *in vitro* transcription/translation systems and the transfection of recombinant clones (Reed and Rice, 2000). The HCV genome is a 9.6-kb positive-sense single-stranded RNA containing a 5′ nontranslated region (NTR), a single open reading frame that encodes a polyprotein of ~3000 amino acids, and a short 3′ NTR (for reviews, see Bartenschlager and Lohman, 2000). The 5′ NTR, which is the most highly conserved region among HCV isolates, contains an internal ribosome entry site that directs the translation of the viral polyprotein. The HCV polyprotein precursor is co- and post-translationally cleaved by viral proteases and host cell signal peptidases, resulting in at least three structural (core, E1, and E2) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Early research efforts have focused on the viral enzymes essential for HCV replication, including the NS3 serine protease/helicase and the NS5B RNA-dependent RNA polymerase. The NS5A nonstructural protein, which has no recognized sequence motif or known enzymatic activity, has received little attention until 1995 when it was first linked to IFN responsiveness in HCV-infected patients.

HCV resistance to IFN therapy is loosely defined as the continual presence of HCV RNA in patient serum after treatment (Pawlotsky, 2000). As with many RNA viruses, HCV circulates in the host as a population of quasispecies, most likely selected from mutations accumulated in the HCV genome due to infidelity of the NS5B RNA polymerase during viral replication (Bukh *et al.,* 1995). Different HCV isolates or genotypes display different levels of sensitivity to IFN treatment. HCV genotypes 1 and 4 are particularly less sensitive to IFN therapy than are HCV genotypes 2 and 3; the latter groups display up to 85% response to peglyated IFN and ribavirin combination therapy. This is problematic in the United States where HCV genotype 1 is the predominant form. Since HCV genome differences may affect the structure and function of viral genome and proteins, these modifications may in turn affect their interactions with numerous host cell functions, including those involved in the antiviral action of IFN in infected cells. To begin to elucidate the mechanisms by which select HCV variants escape the antiviral effects of IFN, Enomoto and colleagues compared full-length sequences of IFN-α-responsive and -nonresponsive viruses from HCV-infected patients (Enomoto *et al.,* 1995, 1996). Patients who completely responded to IFN therapy were found to carry HCV 1b genotype isolates that contained recurring multiple mutations ($\geq$4) within a discrete region of 40 amino acids in the carboxyl half of NS5A, which corresponds to residues 2209 to 2248 of the HCV polyprotein (Fig. 1). These observations suggest that this NS5A region, termed the IFN sensitivity-determining region (ISDR), may play a role in HCV resistance to IFN treatment.

However, the predictive value of ISDR in IFN therapeutic outcome, especially for European and North American HCV isolates, has been questioned by other studies (reviewed by Herion and Hoofnagle, 1997; Korth and Katze, 2000). These apparently discrepant results are often difficult to compare or interpret considering the small numbers of patients examined in each study and different IFN treatment regimens, as well as insufficient data on important parameters such as time of viral exposure, the host immune response, and the severity of HCV-related disease. Furthermore, methodologic biases in the definition and selection of sensitive and resistant HCV isolates as well as in the generation of NS5A sequences may also account for the conflicting results. Nevertheless, a recent study based on a new statistical analysis of a database of 675 published individual ISDR sequences strongly support the correlation of NS5A ISDR with the IFN response (Witherell and Beineke, 2001).

Despite this controversy, the ISDR provides the first hint of a potential molecular mechanism by which specific HCV genotypes might escape the IFN response,

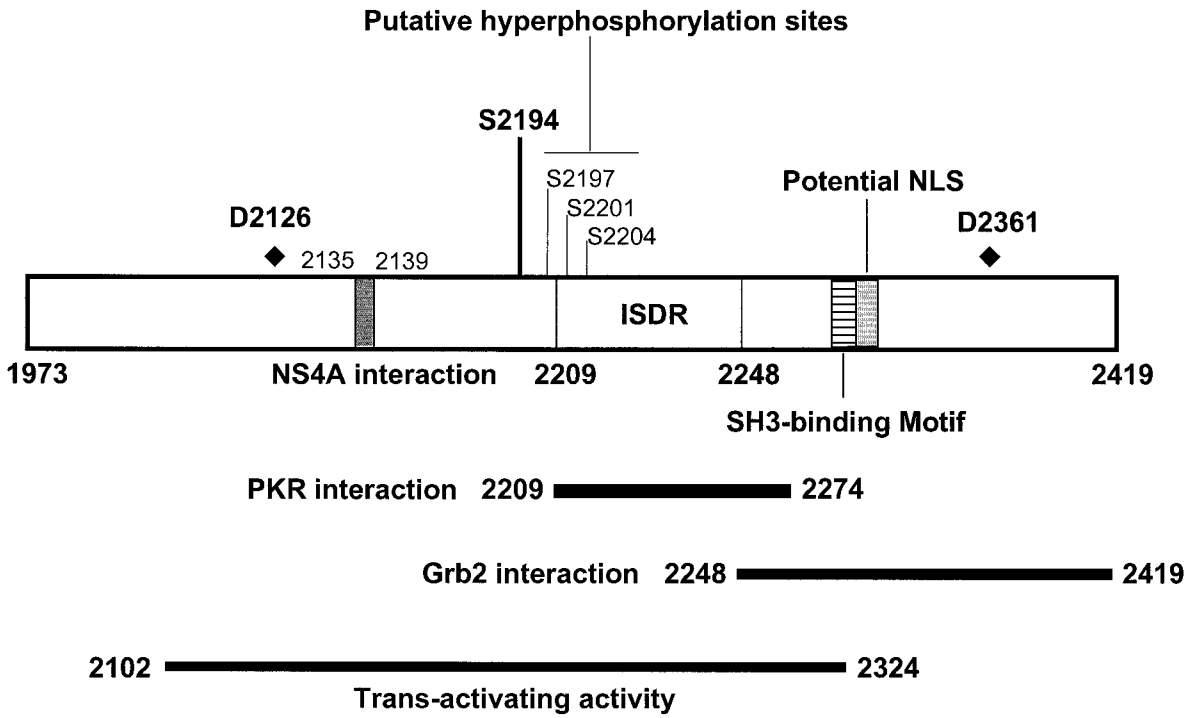

FIG. 1. Structural and functional domains of NS5A. NS5A contains a highly conserved proline-rich region (amino acids 2322–2328) that is homologous to SH3-binding motifs and a potential nuclear localization signal (amino acids 2328–2336). The locations of the conserved phosphorylation site, serine 2194, putative hyperphosphorylation sites, caspase-mediated cleavage sites (◆), NS4A interaction region, and the interferon sensitivity-determining region (ISDR) are also indicated. The region that mediates interaction with the IFN-induced PKR protein kinase or the cellular Grb2 adaptor protein or exerts trans-activating activity is indicated by a solid bar. Amino acid numbers represent positions in the HCV polyprotein. See text for further details.

thereby contributing to the low response rate of HCV to IFN therapy. Enomoto *et al.* (1996) proposed that HCV viruses that are resistant to IFN therapy contain multiple mutations in the ISDR, which may affect NS5A interaction with one or more IFN-induced antiviral effectors. In support of this hypothesis, we have demonstrated that NS5A is able to interact with and inhibit the IFN-induced PKR protein kinase, a primary mediator of the IFN-induced antiviral response.

HCV NS5A INHIBITION OF PKR: A NEW FOE FOR AN OLD FRIEND

PKR is one of the few known intracellular enzymes that are induced by IFNs and which have been well characterized in terms of their antiviral properties in the cell (reviewed by Kaufman, 2000). The PKR protein kinase can be activated by a variety of stress signals, including the presence of viral specific double-stranded (ds) RNA in the cell. The host presumably uses dsRNA as a critical signal to mobilize the innate system because dsRNA or RNA with similar secondary structures is a common replicative intermediate generated by a large number of viruses. Upon activation via a dsRNA-binding induced protein conformational change, PKR phosphorylates the GTP-binding eukaryotic initiation factor 2 (eIF2). The eIF2 facilitates binding of the initiator Met-tRNA$_i^{Met}$ to the 40S ribosomal subunit during translation initiation. Phosphorylation of the α subunit of eIF2 (eIF2α) on Ser51 by PKR converts eIF2 into a competitive inhibitor of its guanine nucleotide exchange factor eIF2B, resulting in the inhibition of general cellular protein synthesis and hence virus replication. Viruses must therefore abrogate PKR action to avoid the detrimental effects associated with a PKR-mediated translational block.

Different viruses use distinct strategies to work against PKR function depending on their viral replication cycle and genome size (gene-coding capacity) (reviewed by Gale and Katze, 1998b, 2000). Adenovirus and Epstein–Barr virus counteract by encoding small RNA transcripts that form an inhibitory complex with PKR, thereby functioning as a competitive inhibitor of dsRNA binding. Reovirus, vaccinia virus (VV), and influenza virus utilize viral dsRNA-binding proteins, σ3, E3L and NS1, respectively, to sequester dsRNA activators of PKR. Some viruses employ more than one tactic to neutralize PKR. Influenza virus also encodes a novel mechanism to repress PKR activity by activating the cellular P58^{IPK} inhibitor, which binds to and possibly blocks PKR from dimer formation. VV directs another viral protein, K3L, to act as a pseudosubstrate of PKR, competing with eFI2α for phosphorylation. In the case of VV, mutant viruses lacking K3L display an IFN-sensitive phenotype partly be-

cause of the failure to inactivate PKR. A more direct mechanism for counteracting PKR is employed by herpes simplex virus (HSV), which encodes the γ_1ICP34.5 protein to recruit the cellular protein phosphatase 1 catalytic subunit to dephosphorylate eIF2α.

The NS5A 56/58-kDa phosphoprotein is believed to be part of the viral replicase complex, as it can be coimmunoprecipitated with other viral nonstructural proteins (Hijikata *et al.,* 1993). Based on heterologous expression of NS5A in cell culture and *in vitro* systems, we demonstrated NS5A is capable of interacting with and inhibiting PKR (Gale *et al.,* 1996, 1997). The ability of NS5A to bind PKR apparently requires the ISDR as well as adjacent 26 amino acids downstream (Gale *et al.,* 1998a; Fig. 1). Consistent with the idea that NS5A inhibits PKR in an ISDR-dependent mechanism, the introduction of specific mutations within the ISDR identical to those in IFN-responsive strains of HCV abolished the ability of NS5A to bind to PKR or inhibit PKR function and eIF2α phosphorylation in a yeast system. These results suggest that mutations in the ISDR can disrupt the NS5A-PKR interaction and possibly render HCV isolates containing such mutations susceptible to IFN therapy. Additional *in vitro* studies revealed that NS5A may function in a fashion analogous to the cellular $P58^{IPK}$ inhibitor of PKR in that it also binds to a region of PKR previously shown to mediate kinase dimerization (Gale *et al.,* 1998a). However, this has not yet been validated by *in vivo* experiments and it is not known whether mutations in the ISDR or the adjacent downstream sequence can disrupt PKR dimer formation.

Recently PKR has been established as an apoptotic effector capable of mediating apoptosis in response to stress signals (Tan and Katze, 1999). We found that NS5A could interfere with the ability of PKR to mediate the dsRNA-dependent apoptotic pathway (Gale *et al.,* 1999). NIH 3T3 cell lines that constitutively expressed wild-type NS5A, but not an ISDR deletion mutant, significantly decreased the number of apoptotic cells in response to dsRNA treatment, as by DNA fragmentation using the TUNEL assay. In a separate study by Ghosh *et al.* (2000), both the MTT assay and Annexin V staining were used to demonstrate an inhibition of TNF-α-induced apoptosis by NS5A expression in hepatoma HepG2 cells, although in this case NS5A may function by blocking the activation of caspase-3. Interestingly, PKR has been shown to potentiate apoptosis through activation of the caspase-mediated death pathway (Tan and Katze, 1999; Balachandran *et al.,* 2000; Gil and Esteban, 2000). Moreover, caspase 3 has been demonstrated to modulate the efficiency of protein synthesis by cleaving eIF2α (Satoh *et al.,* 1999). Taken together, these studies suggest NS5A may possess antiapoptotic properties, at least in part due to its ability to neutralize PKR function(s). Inhibition of PKR and caspase 3, or other related caspases, may therefore enable HCV to avoid specific host apoptotic pathways and thus contribute to the antiviral actions of IFN. In addition, since apoptosis is a common cellular response to virus infection, it is enticing to speculate that the antiapoptotic properties of NS5A may play a role in the ability of HCV to develop a persistent infection. However, this notion is challenged when a recent study found NS5A expression in HeLa cells did not inhibit dsRNA-induced apoptosis, which was scored only on the basis of cell viability assayed by trypan blue exclusion (Ezelle *et al.,* 2001). Whether this discrepancy is due to the difference in assays and/or cell lines used in these studies remains to be clarified.

The PKR-dependent antiapoptotic properties of NS5A may also contribute to the mechanism by which HCV infection leads to hepatocellular carcinoma. In support of this view, constitutive expression of NS5A could induce a transformed phenotype in murine NIH 3T3 fibroblasts (Gale *et al.,* 1999; Ghosh *et al.,* 1999). Not surprisingly, injection of these transformed cells into nude mice resulted in tumor formation (Gale *et al.,* 1999). Although cells expressing an ISDR deletion mutant also exhibited a growth-stimulatory phenotype, they did not induce tumor formation when injected into nude mice. Thus, while the mechanism by which NS5A transforms cells is not fully understood, it is likely that the oncogenic properties of NS5A are partly related to its ability to inhibit PKR. However, it remains to be seen whether the expression of the NS5A protein alone or in combination with an established oncoprotein(s) is sufficient to immortalize primary fibroblasts. Moreover, caution should be taken when interpreting results obtained from overexpression studies in nonphysiologically relevant systems. On this note, there is some conflicting data in that constitutive expression of NS5A in human osteosarcoma (U2-OS) cell lines resulted in a cytopathic effect and severely reduced proliferation (Polyak *et al.,* 1999). More recently, expression of NS5A in Chang human liver cells has been shown to result in reduced cell proliferation and colony formation efficiency (Arima *et al.,* 2001). In this system, NS5A appears to act by targeting the CDK1/2-cyclin complex, leading to a delay in cell cycle progression, although the exact mechanisms are not known.

NS5A SUPPRESSION OF IFN-INDUCED ANTIVIRAL ACTIVITY

Despite the demonstration that NS5A inhibits PKR, direct examination of the role of NS5A in mediating IFN resistance is limited by the inability to propagate HCV in cell culture system. We and others have thus examined whether expression of NS5A could at least reduce the IFN-induced antiviral effects in cells and therefore allow viral protein synthesis and replication in infected cells. In the absence of NS5A expression, IFN treatment significantly inhibited the protein synthesis of normally IFN-sensitive viruses, including vesicular stomatitis virus

(VSV) (Gale *et al.,* 1999) and VV (He *et al.,* 2001) in infected HeLa cells. In contrast, viral protein synthesis was partially restored in HeLa cells stably expressing NS5A or cells infected with recombinant VV expressing NS5A. Importantly, these results correlated with the phosphorylation levels of PKR and eIF2α in the infected cells. Similarly, other investigators have demonstrated that NS5A expression alone partially inhibited IFN antiviral action in other mammalian cell lines, including U5OS, HepG2 cells, and L929 murine fibroblasts, allowing replication of encephalomyocarditis virus (EMCV) and VSV during IFN challenge (Paterson *et al.,* 1999; Polyak *et al.,* 1999; Song *et al.,* 1999; Aizaki *et al.,* 2000).

While the above studies cumulatively suggest that NS5A expression alone confers partial resistance to the antiviral effects of IFN against IFN-sensitive viruses, the involvement of the ISDR is less clear. Expression of NS5A containing substitutions in the ISDR resembling those from an IFN-sensitive HCV 1b genotype strain is unable to rescue VSV protein synthesis in HeLa cells (Gale *et al.,* 1999) during IFN challenge. Furthermore, NS5A from a HCV 1b genotype strain is more efficient in suppressing the IFN antiviral affects than that from a HCV 1a genotype strain in HepG2 cells (Polyak *et al.,* 1999). Song *et al.* (1999) also showed that expression of the NS5A protein derived from an IFN resistant, but not an IFN sensitive, HCV 1b subtype strain can block the IFN action against VSV in L929 cells, although the effect against Japanese encephalitis virus is marginal. This inhibition is significantly weaker when a short stretch of the IFN-resistant NS5A, including the ISDR, is replaced with the corresponding fragment from the IFN sensitive HCV strain.

On the contrary, human amnion-derived FL cell lines expressing an NS5A containing a mutated ISDR sequence corresponding to that of an IFN-resistant HCV isolate are still able to rescue EMCV replication when challenged with IFN (Aizaki *et al.,* 2000). In HepG2 cells, expression of an NS5A form devoid of the ISDR also retains the ability to suppress the IFN-induced antiviral activity against EMCV (Polyak *et al.,* 1999). Another study actually found NS5A clones derived from an IFN responsive patient were much more potent inhibitors of IFN antiviral effects than those derived from a nonresponder when expressed in human fibroblast cells (Paterson *et al.,* 1999). When Francois *et al.* (2000) analyzed the IFN response of a human cell line UHCV-11 engineered to inducibly express the entire HCV genotype 1a polyprotein, they found that expression of HCV proteins could also support EMCV replication better than vector control cells during IFN challenge. The resistance of UHCV-11 cells to IFN appeared independent of inhibition of PKR in that there was no detectable colocalization of PKR and HCV proteins or change in either PKR activity or eIF2α phosphorylation in these cell lines. However, this study did not determine which HCV protein(s) is responsible for conferring the IFN resistance to EMCV. Taken together, these studies suggest that NS5A may also counteract the antiviral effects of IFN via other mechanisms independent of the ISDR or its interaction with PKR.

A significant advancement in HCV research came with the development of a subgenomic biscistronic HCV replicon DNA construct containing a selectable marker gene (neomycin phosphotransferase) and genes encoding the viral nonstructural proteins (Lohman *et al.,* 1999). In this system, the HCV IRES directs the translation of the *neo* gene, whereas the expression of the HCV nonstructural genes is mediated by the IRES of EMCV. Transfection of RNA transcribed from this construct into the hepatoma cell line Huh-7 cells resulted in selectable autonomous replicating HCV subgenomic RNAs, providing for the first time a tool to investigate HCV replication in cell culture and the molecular interactions between HCV and its host. However, despite the relatively high replication levels of the HCV subgenomic RNAs within selected cells, the number of G418-resistant colonies is too low for robust analysis. More recently, several investigators identified and characterized a number of adaptive mutations that enhanced the initiation of the replication of the HCV replicon, as scored by increased efficiency of colony formation by several orders of magnitude (Blight *et al.,* 2000; Lohmann *et al.,* 2001; Pietschmann *et al.,* 2001). Intriguingly, in one study, a majority of these adaptive mutations were localized within NS5A, including a deletion of the entire ISDR (Blight *et al.,* 2000). In addition, the HCV replicons were highly sensitive to IFN-α treatment in that replication of the adapted HCV subgenomic RNA could readily be blocked by treatment of cells with IFN-α (Blight *et al.,* 2000; Frese *et al.,* 2001). The absence of the ISDR did not appear to significantly affect the sensitivity of the replicon to the IFN treatment, suggesting that the ISDR is nonessential for HCV replication and IFN resistance (Blight *et al.,* 2000). However, deletion of the ISDR rendered the lowest transduction efficiency among all of the culture-adaptive mutations identified. Thus, the ISDR may contribute to the overall efficiency of HCV RNA replication, perhaps through the regulation of PKR. However, the level and activity of endogenous IFN-inducible genes, including PKR, were not examined in this study. Very recent work suggests that specific dsRNA structures within the HCV replicon RNA are sufficient to activate PKR *in vivo,* and that efficient HCV RNA replication may correspond with a block in PKR-dependent signaling pathways of the host cell (M. J. Gale, Jr., personal communication).

It is still not clear how IFN-α inhibits HCV subgenomic replication in the replicon: by preventing the accumulation of RNA at the transcription or degradation step, or by blocking synthesis or function of viral proteins. At any rate, results obtained from these studies must be interpreted with caution, as the replicon is still by no means a viral infection system; there is no virion propagation

and assembly due to the lack of structural proteins. Furthermore, there are recent reports suggesting that Huh-7 cells may be inherently defective in IFN response mechanism (Keskinen *et al.,* 1999; Melen *et al.,* 2000). The definition of replicon sensitivity to IFN is not clear; there is no systematic analysis of the quantitative effect of different doses of IFN-α on HCV subgenomic RNA replication over a time course to determine the kinetics of the IFN action. Moreover, the possibility that the observed inhibition of HCV subgenomic RNA replication after IFN-α treatment is due to a block in EMCV IRES activity, which could result in a reduction of HCV protein synthesis and consequently RNA replication, has not been excluded.

HCV May Encode a Two-Pronged Approach to Inhibit PKR

The E2 glycoprotein of HCV has also recently been shown to interact with and inhibit PKR (Taylor *et al.,* 1999). This hypothesis originally stemmed from the observation that the E2 protein contains a 12-amino-acid sequence that shares high homology with several putative autophosphorylation sites within the N-terminal regulatory domain of PKR, as well as its natural substrate, eIF2α. This region termed PePHD (for PKR-eIF2α phosphorylation homology domain) was subsequently shown to be required for E2 inhibition of PKR in yeast. Although the exact mechanism by which E2 inhibits PKR is not clear, a recent follow-up study suggests E2 is unlikely to act as pseudosubstrate to compete with the PKR autophosphorylation sites (Taylor *et al.,* 2001). Interestingly, E2 sequences from HCV gentoypes 1a and 1b, which share higher homology to PKR than genotypes 2a, 2b, or 3a, are generally more resistant to IFN therapy than genotypes 2 and 3, suggesting that E2 inhibition of PKR may also contribute to HCV resistance to IFN treatment. However, an increasing number of clinical studies demonstrate that the PePHD is a highly conserved region with no significant mutations accumulated during IFN therapy (Berg *et al.,* 2000; Chayama *et al.,* 2000; Cochrane *et al.,* 2000; Gerotto *et al.,* 2000; Polyak *et al.,* 2000; Sarrazin *et al.,* 2000). Furthermore, the ability of E2 to confer IFN resistance to IFN-sensitive viruses has not been demonstrated. Thus, the role of E2 in mediating HCV resistance to IFN action remains to be established.

Relevance of NS5A Trans-activating Properties

There is also evidence that the carboxyl-terminal domain of NS5A, including the ISDR, contains nonspecific trans-activating activity in both yeast and mammalian cells when fused to the DNA-binding domain of GAL4, suggesting that NS5A might function as a viral transactivator (Tanimoto *et al.,* 1997; Kato *et al.,* 1997). Interestingly, the introduction of mutations derived from HCV isolates from IFN-responsive patients into the ISDR can enhance the trans-activating activity of NS5A in the artificial GAL4 system (Fukuma *et al.,* 1998). Despite the presence of a putative nuclear localization signal (NLS) in its C-terminal region (Ide *et al.,* 1996) (Fig. 1), NS5A is predominantly localized in the cytoplasm and/or nuclear periplasmic membrane. Furthermore, it has been reported that NS5A may be cleaved by a caspase-like protease(s) in mammalian cells (Song *et al.,* 2000), possibly in an apoptotic-dependent manner (Satoh *et al.,* 2000). The cleaved NS5A forms, which lack potential NLS-masking sequence in the N-terminal region, are localized in the nucleus and display transcriptional activity when co-expressed with the α-catalytic subunit of protein kinase A (PKA), which has previously been shown to phosphorylate NS5A *in vitro* (Ide *et al.,* 1997). Several recent studies found that NS5A is capable of interacting with cellular transcriptional factors, including a novel cellular transcription factor SRCAP (Ghosh *et al.,* 2000) and P53 (Majumder *et al.,* 2001), as well as a nuclear import machinery component named karyopherin β3 protein (Chung *et al.,* 2000). However, the biological consequences of these interactions are not known.

A recent study found elevated levels of the pro-inflammatory chemokine, interleukin-8 (IL-8), in IFN nonresponsive patients as well as in NS5A-expressing HeLa cells (Polyak *et al.,* 2001). Interestingly, expression of N-terminal truncated forms of NS5A stimulates the IL-8 promoter to levels higher than the full-length NS5A protein in HeLa cells, as determined by transcriptional activation of a luciferase gene driven by IL-8 specific promoter. This stimulation also correlates with increased nuclear localization of the truncated NS5A proteins. These results suggest NS5A may be capable of inducing the transcription of IL-8, which was previously shown to inhibit the antiviral actions of IFN *in vitro* (Khabar *et al.,* 1997), providing an additional mechanism for IFN resistance in chronic hepatitis C. However, the clinical correlation between NS5A trans-activating activity, IL-8 levels, and IFN sensitivity has not been clearly established. Furthermore, the functional significance of the truncated form of NS5A is not known.

INTERCEPTION OF THE IFN SIGNALING BY HCV

PKR inhibition by NS5A or E2 is likely not the only mechanism responsible for HCV evasion of host IFN response. Viruses also disrupt the ability of cells to respond to IFN, either through the use of decoy receptors, inhibitors of signaling components or repressors of gene transcription (reviewed in Cebulla *et al.,* 1999; Alcami and Koszinowski, 2000). The blockade of IFN signaling pathways is an efficient strategy because it would have a pleiotropic effect on the antiviral activities of IFN and thus would be economical for viruses with limited gene-coding capacity.

Viral Strategies against IFN Signaling: More Than Meets the Eye

The binding of IFNs to species-specific cell surface receptors elicits multiple signal transduction pathways, which lead to the induction of gene expression (Darnell 1997, 1998; Stark *et al.,* 1998). The IFN-Stimulated Gene Factor 3 (ISGF3) is responsible for the induction by binding to IFN-stimulated response elements (ISREs) found in numerous IFN-induced genes. Active ISGF3 is composed of three functional subunits. Two of these proteins are named Signal Transducer and Activators of Transcription (STAT) 1 and 2, which reside in the cytoplasm of unstimulated cells. STAT1/2 proteins are tyrosine phosphorylated by JAK1 and Tyk2 of the Janus kinases (JAK) family. The Tyk2 molecule binds to the intracytoplasmic domain of one of the two cloned IFN-α receptor subunits, IFNAR1, whereas JAK1 is constitutively associated with the second subunit IFNAR2. IFN-α binding likely causes the oligomerization of these receptors, which leads to the autophosphorylation and activation of JAK1 and Tyk2. JAK1 and Tyk2 in turn phosphorylate the IFN receptors on specific tyrosine residues, which function as docking sites for the Src homology (SH) 2 domain of STAT1/2 proteins. Recruited STAT1/2 proteins are subsequently phosphorylated by the JAK kinases on tyrosine residues, heterodimerize and translocate to the nucleus where they bind p48, which is in direct contact with the ISRE, thereby forming the ISGF3 active complex. In addition, the IFN regulatory factor (IRF) family of transcription factors also plays a critical role in regulating IFN and IFN-induced gene expression (reviewed by Taniguchi *et al.,* 1997). Activation of different members of STAT and IRF family and the formation of different homo- and heterodimers are believed to provide specific biological responses to different cytokines or growth factors.

Viruses have evolved different mechanisms to interdict the IFN signaling. Some viruses secrete soluble IFN receptor-like polypeptides that can bind to IFNs to inhibit their biological activities. VV encodes a soluble viral homolog of IFN-α/β receptor (IFN-α/βR), termed B18R, which binds to IFN-α/β (Colamonici *et al.,* 1995). Other viruses perturb the signaling components of the JAK-STAT pathway to subvert IFN action. For example, the viral large T antigen of murine polyoma virus directly binds to JAK1 resulting in the inhibition of its function (Weihua *et al.,* 1998). Human cytomegalovirus (HCMV) apparently uses a different strategy that involves the proteolysis of JAK1 (Miller *et al.,* 1998). Adenoviruses appear to directly interfere with the transcriptional response by using the viral E1A protein (Leonard and Sen 1996). Depending on the cell types, E1A has been shown to downregulate the levels of p48 and STAT1 (Leonard and Sen, 1997), or disrupt the interaction of STAT proteins with transcriptional co-activator CBP/p300 in the nucleus (Bhattacharya *et al.,* 1996).

Human herpesvirus 8 uses a yet another mechanism to disrupt the IFN responsive transcription apparatus. This virus codes for a viral homolog of the IRF family proteins, which presumably forms inactive heterodimers with other members of the family (Zimring *et al.,* 1998). Human papilloma virus (HPV), which is also resistant to IFN-α treatment, encodes two proteins to interfere with IFN signaling: E6 binds to and inhibits Tyk2 activity (Li *et al.,* 1999), while E7 binds p48 and blocks its nuclear translocation (Barnard and McMillan 1999). The terminal protein of HBV has been shown to interrupt both Type I and II IFN signaling by disrupting ISGF3 formation but the exact mechanism is not known (Foster *et al.,* 1991). Sendai virus (SeV) may use several strategies, including a partial inhibition of Tyk2 activation (Komatsu *et al.,* 2000) and suppression of STAT1 levels (Garcin *et al.,* 2000). The latter effects appear to require the viral C proteins to interact with unknown cellular components.

Is HCV an Enemy of the STAT?

Could HCV also encode a mechanism to disarm the JAK-STAT pathway? Heim and colleagues (1999) addressed this question by generating stably transfected human US-OS cells expressing full-length HCV genome. They found a complete inhibition of IFN-α-induced activation of ISGF3 in the HCV-expressing cells, whereas the control cells exhibited the expected activation of ISGF3, as judged by ISGF3 binding to the ISRE by gel shift assays. STAT1/3 tyrosine phosphorylation was not impaired in these cells as revealed by immunoblot analysis using phospho-specific antibodies to the activated forms of STAT1/3. Furthermore, ISGF3 showed normal nuclear transportation and tyrosine phosphorylation in the HCV-expressing cells. Although reduced expression of STAT1 and p48 was detected in the HCV-expressing cells, it is not clear whether the decrease in protein expression was the cause or result of the observed inhibition of IFN signaling. It should also be pointed out that serine phosphorylation of STAT1/3 has been reported to modulate the transcriptional activity of STAT1/3 (see discussion below); future studies should examine whether this step is affected by HCV. Thus, the exact mechanisms by which HCV proteins inhibit IFN signaling remain to be determined. Nevertheless, these results are interesting in light of previous findings that STAT1 knockout mice display liver necrosis, syncytial cell formation and an inflammatory response in their liver, which is reminiscent of viral hepatitis (Durbin *et al.,* 1996). The possibility that HCV may interfere with the JAK-STAT pathway should be followed up in future studies, including the determination of the HCV protein(s) responsible for such action. In regard to the role of NS5A, HeLa cells that express NS5A do not appear to interfere with STAT1 tyrosine phosphorylation or ISGF3 DNA binding activity (Polyak *et al.,* 2001).

Recently, we found that HCV may utilize the viral NS5A

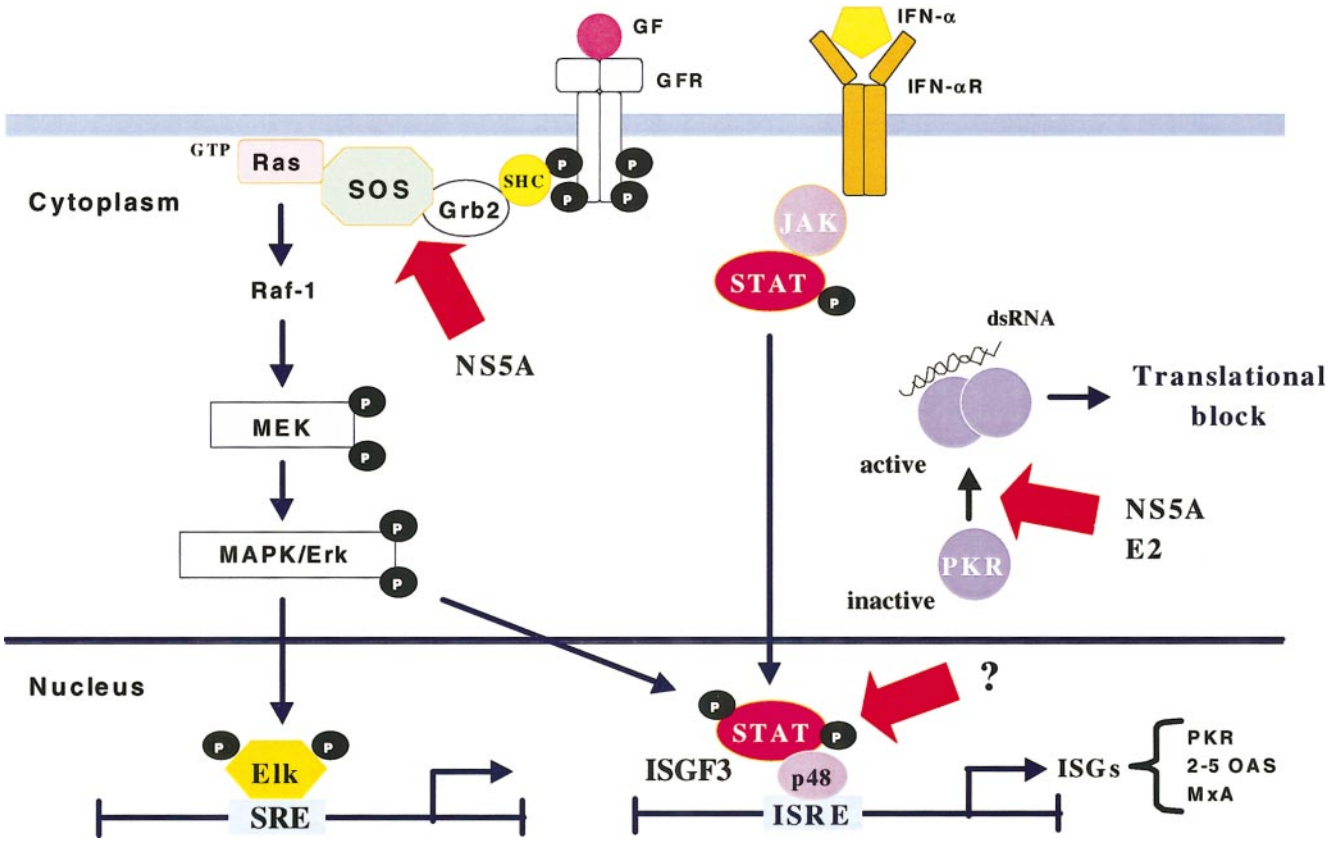

FIG. 2. Potential HCV strategies against IFN response. Cross-talk between the JAK-STAT and ERK signaling pathways is shown. Grb2 mediates the Ras-Raf-ERK MAP kinase pathway by nucleating the formation of signal transduction complexes at the plasma membrane. ERK-mediated phosphorylation of STAT1 at serine 727 may be required for maximal transcriptional activity of the ISGF3 complex. JAK represents JAK1 and TYK2; STAT includes STAT1 and STAT2; GF, growth factor; GFR, growth factor receptor; IFN-αR, IFN-α receptor; dsRNA, double-stranded RNA; SRE, serum response element; ISRE, IFN-stimulated response element; ISGF3, IFN-stimulated gene factor complex 3; ISGs, IFN-stimulated genes. ISGs with known antiviral functions include but are not limited to PKR, MxA, and 2′-5′ oligoadenylate synthetase (2-5 OAS) (Vilcek and Sen, 1996). Block arrows indicate postulated target sites for HCV antagonism of IFN response, including NS5A- and E2-mediated inhibition of PKR, NS5A interaction with Grb2, and downregulation of ISGF3. See text for further details.

protein to perturb host intracellular signaling pathways through its ability to interact with the adaptor protein Grb2 (Tan *et al.,* 1999). Using *in vitro* and *in vivo* binding assays, we found that the NS5A protein of hepatitis C virus (HCV) physically interacted with the cellular growth factor receptor-bound protein 2, Grb2. The NS5A-Grb2 interaction occurred in a SH3/ligand-dependent manner; the ligand was mapped to a highly conserved proline-rich sequence within the C-terminal end of NS5A (Fig. 1). Grb2 is a SH3 domain-containing adaptor protein that mediates growth factor-induced signaling by coupling the membrane receptor tyrosine kinases (RTKs) to the Ras-MAPK (mitogen-activated protein kinase) pathway (Fig. 2). Expression of NS5A can inhibit epidermal growth factor (EGF)-induced phosphorylation of the MAP kinases, extracellular-regulated kinase 1 and 2 (ERK1/2), in HeLa cells. Furthermore, interaction with Grb2 is necessary for NS5A to inhibit activation of ERK1/2 by EGF in HeLa cells (He *et al.,* in preparation). Together, these studies suggest that NS5A is a viral inhibitor of mitogenic signaling, at least in part because of its ability to interact with Grb2. It should be noted, however, that signaling responses vary depending on the cell type and the nature, strength and duration of the signal. It is therefore important to corroborate these results, as well as the effects of other growth factors and cytokines, particularly those implicated in liver proliferation and apoptosis, such hepatic growth factor, TNF-α and IL-6, in liver cells. It is also not clear how NS5A achieves specificity in recognizing Grb2 upon mitogenic stimulation. Because NS5A and Grb2 interaction is inducible by EGF treatment (Tan *et al.,* 1999), NS5A is probably transiently associated with Grb2 in the cytoplasm. Interestingly, Grb2 binds to a proline-rich sequence of NS5A immediate to the putative nuclear localization signal (Fig. 1), which could block NS5A translocation to the nucleus during mitogenic stimulation.

Because ERK1/2 and other mitogen-activated protein kinases (MAPKs) have been implicated in the JAK-STAT pathway, NS5A inhibition of Grb2 may also affect the expression of IFN-stimulated genes (reviewed by Leaman *et al.,* 1996, Winston and Hunter, 1996). A recent

study showed that full transcriptional activation of ISGF3 requires the p38 MAPK pathway (Goh *et al.,* 1999; Williams, 1999). p38 was activated by Type I IFNs and its function was essential for IFNα-induced ISRE-dependent gene transcription but not for the DNA-binding activity of the ISGF3 complex. Interestingly, PKR has been implicated upstream of the p38 signaling pathway (Goh *et al.,* 2000; Iordanov *et al.,* 2000; Williams, 1999), suggesting yet another mechanism by which NS5A inhibition of PKR could result in suppression of IFN action. Several recent papers also suggest a "cross-talk" between the JAK-STAT pathway and the ERK pathway (Fig. 2). It has been shown that ERK-mediated serine phosphorylation of STAT1 and STAT3 is required for the optimal activation of these STAT proteins (David *et al.,* 1995, 1996; Wen and Darnell, 1995; Zhang *et al.,* 1995), although this may be cell type-specific (Chung *et al.,* 1997; Jain *et al.,* 1998). Thus, NS5A perturbation of both the ERK and the p38 MAPK pathways, through its ability to bind Grb2 and/or PKR, may decrease the serine phosphorylation of STAT1/3, resulting in the disruption of IFN-induced gene expression, thereby providing an alternative mechanism for HCV resistance to IFN. In this regard, it is interesting to note that SeV and simian virus 5, both of which disrupt IFN signaling, have been shown to reduce the serine phosphorylation of STAT1α (Young *et al.,* 2000).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Many hypotheses regarding HCV pathogenesis and the host IFN response to HCV infection are derived mainly from clinical observations and from expression analyses of selected genes in nonhuman cells or nonhepatocyte cells. Thus the vast amounts of data generated must be validated in normal human hepatocyte cells, relevant animal models, as well as in the context of a natural HCV infection. Exciting prospects are anticipated with the recent success in the development of infectious HCV molecular clones in small animal models (Gale and Beard, 2001), and the advent of large-scale gene expression profiling technologies (Manger and Relman, 2000). In an interesting study using suppression-subtractive hybridization, expression of several IFN-α/β-induced genes in the liver of chronic hepatitis C patients was found to be significantly enhanced, indicating that HCV may be an inducer of IFN-α/β-inducible genes (Patzwahl *et al.,* 2001). These genes, which include IP-10, MxA, IFI-56K, and p44, have been previously implicated in the cellular antiviral response. Whether HCV also encodes mechanisms to counteract these gene products remains to be determined. However, since common sequences have been removed from the subtracted library, this analysis is not an accurate reflection of the infected liver gene expression profile. The use of differential display analysis or DNA microarray technology should provide a more comprehensive survey of cellular gene expression in the presence or absence of HCV infection. With respect to IFN response, these methods have been used to identify IFN-induced genes whose expression is altered during infection by HCMV (Zhu *et al.,* 1997), HPV (Chang and Laimins, 2000), and HSV (Mossman *et al.,* 2001).

NS5A is likely to play an important role in virus–host interaction and the regulation of viral replication, and the literature in general supports the role of NS5A in the suppression of IFN-induced antiviral activity. As with other viruses, HCV likely employs several mechanisms to counteract the IFN response (Fig. 2). NS5A is phosphorylated on multiple serine residues and exists in two species: a basal phosphorylated form of 56 kDa and a hyperphosphorylated form of 58 kDa (Kaneko *et al.,* 1994). Although several cellular protein kinases have been shown to phosphorylate NS5A *in vitro* (Idle *et al.,* 1997; Kim *et al.,* 1999), the physiological relevance of these results remain to be shown. In this regard, we have recently found that serine 2194 is a highly conserved phosphorylation site of NS5A (Katze *et al.,* 2000). PKR does not appear to phosphorylate NS5A (Reed and Rice, 1999) and mutation of serine 2194 does not effect the ability of NS5A to bind PKR *in vitro* (Katze *et al.,* 2000). Interestingly, adapted HCV replicons that have acquired higher replication efficiency contain mutations in several putative hyperphosphorylation sites of NS5A (Fig. 1), suggesting that hyperphosphorylation of NS5A is not essential for viral replication (Blight *et al.,* 2000). The hyperphosphorylated NS5A is apparently generated slower and less stable compared to the basal phosphorylated NS5A and other nonstructural proteins, suggesting that the two NS5A variants may serve distinct functions during different stages of HCV replication (Pietschmann *et al.,* 2001). Clearly, the identification of the cellular protein kinase(s) that is responsible for differential phosphorylation of NS5A will provide further insights into NS5A function and regulation.

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