Antiapoptotic and Oncogenic Potentials of Hepatitis C Virus Are Linked to Interferon Resistance by Viral Repression of the PKR Protein Kinase

MICHAEL GALE, JR.,1* BART KWIECISZEWSKI,2 MICHELLE DOSSETT,1 HARUHISA NAKAO,2 AND MICHAEL G. KATZE1,2

Department of Microbiology, School of Medicine,1 and Regional Primate Research Center,2 University of Washington, Seattle, Washington 98195

Received 19 February 1999/Accepted 3 May 1999

Hepatitis C virus (HCV) is prevalent worldwide and has become a major cause of liver dysfunction and hepatocellular carcinoma. The high prevalence of HCV reflects the persistent nature of infection and the large frequency of cases that resist the current interferon (IFN)-based anti-HCV therapeutic regimens. HCV resistance to IFN has been attributed, in part, to the function of the viral nonstructural 5A (NS5A) protein. NS5A from IFN-resistant strains of HCV can repress the PKR protein kinase, a mediator of the IFN-induced antiviral and apoptotic responses of the host cell and a tumor suppressor. Here we examined the relationship between HCV persistence and resistance to IFN therapy. When expressed in mammalian cells, NS5A from IFN-resistant HCV conferred IFN resistance to vesicular stomatitis virus (VSV), which normally is sensitive to the antiviral actions of IFN. NS5A blocked viral double-stranded RNA (dsRNA)-induced PKR activation and phosphorylation of eIF-2α in IFN-treated cells, resulting in high levels of VSV mRNA translation. Mutations within the PKR-binding domain of NS5A restored PKR function and the IFN-induced block to viral mRNA translation. The effects due to NS5A inhibition of PKR were not limited to the rescue of viral mRNA translation but also included a block in PKR-dependent host signaling pathways. Cells expressing NS5A exhibited defective PKR signaling and were refractory to apoptosis induced by exogenous dsRNA. Resistance to apoptosis was attributed to an NS5A-mediated block in eIF-2α phosphorylation. Moreover, cells expressing NS5A exhibited a transformed phenotype and formed solid tumors in vivo. Disruption of apoptosis and tumorogenesis required the PKR-binding function of NS5A, demonstrating that these properties may be linked to the IFN-resistant phenotype of HCV.

Eukaryotic viruses establish persistent infection by avoiding the innate defenses of the host cell, escaping acquired immunity, and blocking host-mediated programmed cell death (20, 22, 64). Hepatitis C virus (HCV), a hepacivirus and member of the Flaviviridae (16, 38), mediates persistent infection within a majority of infected individuals. Viral persistence is a major factor contributing to the accumulating prevalence of HCV, which now exceeds 2% of the world population (2). Persistent HCV infection often leads to chronic hepatitis and liver cirrhosis and is strongly associated with the development of hepatocellular carcinoma and lymphoproliferative disorders (65, 66, 86). The molecular mechanisms of HCV persistence and pathogenesis are poorly understood, although these processes clearly involve avoidance of the host immune response through the evolution of viral quasispecies (12, 20, 22, 52) and alteration of host signaling pathways by interaction with specific viral proteins (60, 62).

Of central importance to these problems is the high level of viral resistance to alpha interferon (IFN-α) therapeutic regimens for the treatment of HCV infection. It is now clear that IFN therapies are effective in only approximately 30% of treated patients, though response rates differ between HCV genotypes (36, 37, 43). The recent introduction of IFN with ribavirin combination therapeutic regimens has moderately improved the response rate to anti-HCV therapy (55). However, overcoming IFN resistance remains a major challenge for effective IFN-based therapy and future management of the HCV pandemic. Problematically, resistance to IFN and development of persistent infection are major features of the most widespread HCV genotypes, 1A and 1B (53). Thus, pathogenesis due to HCV may be more severe in individuals infected with HCV genotype 1. Indeed, in independent studies, genotype 1 infection was the single factor consistently associated with IFN resistance, development of persistent infection, and severe liver pathology (3, 11, 23, 25, 77). These features support the hypothesis that HCV persistence and pathogenesis may be linked to the IFN-resistant phenotype.

We have recently demonstrated that the nonstructural 5A (NS5A) protein from IFN-resistant strains of HCV genotypes 1A and 1B can repress the actions of the IFN-induced protein kinase PKR, an immediate-early effector of the cellular antiviral response induced by IFN (29, 31, 32). PKR mediates the antiviral actions of IFN, in part by phosphorylating the alpha subunit of eukaryotic initiation factor 2 (eIF-2α), resulting in acute inhibition of mRNA translation and a concomitant block in viral replication (56, 57; reviewed in references 17, 30, and 76). In addition, PKR facilitates IFN-induced transcriptional programs by participating in the activation of nuclear factor kappa B (NF-kB) and IFN-regulatory factor 1 (IRF-1) (46). Along with its antiviral properties, PKR has been defined as a tumor suppressor (58), and it is an important regulator of cellular pathways that control gene expression and specific apoptotic programs within dividing cells (17). Our results suggest that HCV represses PKR function through the actions of...
the viral NS5A protein, which binds and inhibits PKR in vivo (29, 32). Importantly, mutations within a discrete region of the PKR-binding domain of NS5A (previously termed the IFN-sensitivity-determining region [ISDR] (Fig. 1)), which are identical in IFN-sensitive strains of HCV (14, 23, 24, 47), rendered NS5A unable to bind PKR and inhibit PKR catalytic activity (29, 32). In the present report, we demonstrate that expression of NS5A in mammalian cells provides viral resistance to IFN by removing the IFN-induced, PKR-imposed block on mRNA translation during virus infection. NS5A repression of PKR similarly blocked PKR-dependent eIF-2α phosphorylation and the initiation of host apoptotic programs induced by double-stranded RNA (dsRNA). Our results suggest that disruption of PKR-dependent translational control and apoptotic programs may confer oncogenic potential to HCV.

MATERIALS AND METHODS

Construction of NS5A expression plasmids. The NS5A constructs used in these studies are depicted in Fig. 1. NS5A 1A and NS5A 1B were independently cloned from HCV RNA isolated from separate patients infected with HCV genotypes 1A and 1B, respectively (32). In each case, the patient had failed to respond to IFN therapy, and the resulting viral isolates were labeled as IFN resistant. NS5A 1B-5 is isogenic to NS5A 1A except that it lacks aa 2209 to 2248, which correspond to the entire ISDR. We have previously determined that this region is required for interaction with PKR and inhibition of protein kinase activity (29, 32). The PKR-binding property of each construct is indicated.

Fig. 1. Structural representations of the HCV polyprotein and functional characteristics of the NS5A expression constructs used in this study. (A) The HCV polyprotein and NS5A cleavage product (filled region). (B) Structural representations of full-length NS5A representing HCV 1A and HCV 1B isolates (upper) and HCV 1A-NSDR and HCV 1B-NSDR, respectively (32), into the corresponding sites of pcDNA1Neo (Invitrogen). (DMEM) containing 10% fetal bovine serum (FBS), L-glutamine (200 

Cell culture and transfection. The Tet-Off gene expression system and HeLa S3 Tet-Off cells (Clontech) were used to establish the HeLa 1B and HeLa 1B-5 cell lines harboring pTRE-NS5A 1B and pTRE-NS5A 1B-5, respectively. In this system, expression of NS5A is induced by removal of Tet from the culture medium. HeLa S3 Tet-Off cells (Clontech) were transfected with pTRE-NS5A 1B or pTRE-NS5A 1B-5 and selected in Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 1-glutamine (200 μg/ml), G418 (100 μg/ml), hygromycin B (100 μg/ml), and Tet (2 μg/ml). Clonal lines of HeLa 1B and HeLa 1B-5 were propagated in selective DMEM containing 100 

Vector control NIH 3T3 cell lines (Neo) and those expressing NS5A 1A or the ΔISDR NS5A mutant were derived by transfecting cells via the DEAE-dextran–chloroquine method (84) with 3 to 10 μg of pcDNA1Neo, pNeoNS5A 1A, or pNeoΔISDR, respectively. Stable transfected cell lines were selected (28). Cells were maintained in selective DMEM containing 10% FBS and 600 

Drug-resistant clones were isolated, expanded, and tested for stable transgene expression. By this method, we isolated several clones expressing high or low levels of wild-type or mutant NS5A. Except for the tumorigenicity studies (below), Neo control clone 5-2, ΔISDR clone 3C2, and Neo 1A clone 3C6 were used in all analyses.

Cell growth analysis and tumorigenicity assays. Growth characteristics of stable NIH 3T3 cell lines constitutively expressing NS5A or P58IPK (control) were determined as described elsewhere (6). Determination of culture saturation density, cells were seeded at 105 cells/55-mm-diameter culture dish in selective DMEM containing 10% FBS and 400 μg of G418 per ml. Cells were counted every 24 h, and saturation density was determined by measuring the number of total cells in culture 4 days after reaching confluence. To assess cloning efficiency, 5 × 103, 5 × 104, or 105 cells were suspended in 0.35% agar–DMEM solution with 20% FBS and overlaid in duplicate onto six-well culture dishes containing 0.7% agar–DMEM with 10% FBS. Cloning efficiency was determined 14 days later and is presented in Table 1 as a percentage of the number of colonies observed/total number of cells plated. Colonies were defined as an isolated cluster of four or more cells. Determination of oncogenic potential was made by injecting 4- to 6-week-old athymic nude mice (mu/mu; Charles River) subcutaneously near the left hind limb with 2 × 105 cells in phosphate-buffered saline (PBS). Mice were housed in microisolator cages in a pathogen-free facility and observed for up to 50 days for the formation of solid tumors. For analysis of tumor phenotypes, tumors were excised from mu/mu mice under sterile conditions, washed in PBS, and minced into 1- to 5-mm fragments. Tumor fragments were homogenized by first incubating in 1% collagenase–0.1% Dispase (Gibco BRL) in PBS and then blending in a Dounce vessel homogenizer. Homogenized tumors were incubated at 37°C for 2 h, centrifuged at 600 × g for 10 min, and resuspended in fresh DMEM with 10% FCS. Tumor cell suspensions were seeded into multiwell plates for the generation of tumor-derived clonal cell lines. Cell growth characteristics are shown in Table 1 and are representative of four experiments from each of four independent clones within the groups examined.

Downloaded from http://jvi.asm.org/ on November 9, 2017 by guest
detected by probing the membranes with a primary monoclonal antibody (Mab) specific to NS5A (a generous gift from T. Imagawa, Osaka University), human PKR (48) (generously provided by A. Hovanessian, Pasteur Institute), murine PKR (Santa Cruz Biotechnology), or mammalian eIF-2α (generously provided by Scot Kimball, Pennsylvania State University). Proteins were visualized by enhanced chemiluminescence and autoradiography.

For protein biosynthetic labeling, cultured cell monolayers were rinsed three times with ice-cold PBS and then incubated for 5 h in methionine- and cysteine-deficient medium containing 50 μCi of [35S]methionine-cysteine (Dupont) per ml. Labeled cells were rinsed three times with ice-cold PBS and subjected to extract preparation as described above. Radiolabel incorporation was quantitated from autoradiograms by using a Bio-Rad GS700 imaging densitometer and computer software supplied by the manufacturer.

RESULTS

NS5A from IFN-resistant HCV provides viral resistance to IFN. Previous work demonstrated that NS5A could directly inhibit the translational regulatory properties of PKR in vivo (29, 32). These results suggested that HCV might mediate resistance to IFN, at least in part, through NS5A repression of PKR. Thus, it was essential to determine if NS5A could overcome the IFN-induced block on viral mRNA translation when expressed during viral infection. Since HCV does not replicate efficiently in cell culture, we developed a system based on VSV infection of stable cell lines expressing NS5A from IFN-resistant and IFN-sensitive HCV (Fig. 1). VSV replication involves dsRNA intermediates, which are potent activators of PKR. We chose the VSV model because unlike many eukaryotic viruses, VSV does not encode a mechanism to inhibit the antiviral properties of PKR that are activated during infection (79). Hence, VSV is sensitive to the antiviral actions of IFN mediated through PKR phosphorylation of eIF-2α (49, 79).

We prepared clonal HeLa S3 cell lines (HeLa 1B) that express NS5A, isolated from IFN-resistant HCV-1B (32) from...
of VSV mRNA translation in IFN-treated cells was dependent on the PKR-regulatory function of NS5A, we similarly prepared a HeLa cell line expressing the NS5A 1B-5 mutant from a Tet-regulated promoter. The NS5A 1B-5 construct is isogenic to NS5A 1B but harbors four amino acid substitutions within the ISDR, corresponding to IFN-sensitive HCV (23, 24, 29; reviewed in reference 34). The ISDR mutations in NS5A 1B-5 map to within the PKR-binding domain and render the protein nonfunctional and unable to bind or regulate PKR (29) (Fig. 1). We examined the ability of NS5A 1B and NS5A 1B-5 to rescue VSV mRNA translation upon parallel infection of the respective IFN-treated cell lines (Fig. 3). Removal of Tet from the culture medium induced expression of NS5A 1B and NS5A 1B-5 to approximately equal levels (Fig. 3A). Interestingly, each protein migrated as a 55/58-kDa dimer when separated by high-resolution SDS-PAGE, consistent with isoforms representing physiological levels of NS5A phosphorylation (reviewed in reference 60). Each cell line was infected with VSV in the absence of Tet and increasing amounts of IFN. Analysis of protein synthetic rates demonstrated an acute sensitivity of VSV mRNA translation to IFN in HeLa 1B-5 cells. As shown in Fig. 3B (lower panel), synthesis of the viral matrix protein was completely abolished in HeLa 1B-5 cells treated with an IFN concentration of 400 U/ml. In contrast, viral protein synthesis was sustained in HeLa 1B cells throughout the range of IFN concentrations (Fig. 3B, upper panel), supporting our previous observations (Fig. 2). Determination of the viral matrix protein translation ratio demonstrated that expression of NS5A 1B-5 was not sufficient to rescue viral protein synthesis from the antiviral actions of IFN (Fig. 3C). Expression of NS5A 1B conferred nearly a 12-fold increase in the level of viral protein synthesis compared to cells expressing the nonfunctional NS5A 1B-5 mutant. Importantly, these results suggest that ISDR mutations (corresponding to IFN-sensitive

a Tet-regulated promoter (Fig. 1). Removal of Tet from the culture medium induced the stable expression of NS5A 1B in HeLa 1B cell lines (Fig. 2A), and the level of NS5A 1B was discretely regulated by titrating Tet back into the culture medium (not shown). We used HeLa 1B cells to determine if expression of NS5A could prevent the IFN-induced block on viral mRNA translation imposed by PKR during VSV infection. HeLa 1B cells were infected with VSV in the presence or absence of Tet and increasing amounts of IFN. As revealed by 

\[^{35}S\]methionine-cysteine pulse-labeling and quantitation of VSV matrix protein synthesis, IFN treatment significantly reduced viral mRNA translation in Tet\(^+\) HeLa 1B cultures not expressing NS5A 1B, and a complete block in viral mRNA translation was achieved at 300 U of IFN per ml (NS5A 1B [Fig. 2B, upper panel]). These results are consistent with the PKR-mediated antiviral actions of IFN (30, 40). In contrast, induction of NS5A 1B expression (NS5A 1B\(^+\)) in Tet\(^-\) cultures supported viral mRNA translation in the presence of increasing concentrations of IFN (Fig. 2B, lower panel). As shown in Fig. 2C, determination of the ratio of VSV matrix protein translation in NS5A 1B\(^+\) to that in NS5A 1B\(^-\) cultures revealed an apparent rescue of VSV mRNA translation in NS5A 1B cultures, beginning at 300 U of IFN per ml. Though the rescue of VSV mRNA translation extended over the range of IFN concentrations, the strongest rescue effect was clearly seen at IFN concentrations of 400 and 500 U/ml (Fig. 2C). These studies provide evidence that NS5A from IFN-resistant HCV can mediate viral resistance to IFN. Importantly, our results suggest that NS5A may provide IFN resistance by preventing the PKR-imposed block on viral mRNA translation.

**IFN sensitivity is conferred by ISDR mutations within the PKR-binding domain of NS5A.** To determine if NS5A rescue of VSV mRNA translation in IFN-treated cells was dependent on the PKR-regulatory function of NS5A, we similarly prepared a HeLa cell line expressing the NS5A 1B-5 mutant from a Tet-regulated promoter. The NS5A 1B-5 construct is isogenic to NS5A 1B but harbors four amino acid substitutions within the ISDR, corresponding to IFN-sensitive HCV (23, 24, 29; reviewed in reference 34). The ISDR mutations in NS5A 1B-5 map to within the PKR-binding domain and render the protein nonfunctional and unable to bind or regulate PKR (29) (Fig. 1). We examined the ability of NS5A 1B and NS5A 1B-5 to rescue VSV mRNA translation upon parallel infection of the respective IFN-treated cell lines (Fig. 3). Removal of Tet from the culture medium induced expression of NS5A 1B and NS5A 1B-5 to approximately equal levels (Fig. 3A). Interestingly, each protein migrated as a 55/58-kDa dimer when separated by high-resolution SDS-PAGE, consistent with isoforms representing physiological levels of NS5A phosphorylation (reviewed in reference 60). Each cell line was infected with VSV in the absence of Tet and increasing amounts of IFN. Analysis of protein synthetic rates demonstrated an acute sensitivity of VSV mRNA translation to IFN in HeLa 1B-5 cells. As shown in Fig. 3B (lower panel), synthesis of the viral matrix protein was completely abolished in HeLa 1B-5 cells treated with an IFN concentration of 400 U/ml. In contrast, viral protein synthesis was sustained in HeLa 1B cells throughout the range of IFN concentrations (Fig. 3B, upper panel), supporting our previous observations (Fig. 2). Determination of the viral matrix protein translation ratio demonstrated that expression of NS5A 1B-5 was not sufficient to rescue viral protein synthesis from the antiviral actions of IFN (Fig. 3C). Expression of NS5A 1B conferred nearly a 12-fold increase in the level of viral protein synthesis compared to cells expressing the nonfunctional NS5A 1B-5 mutant. Importantly, these results suggest that ISDR mutations (corresponding to IFN-sensitive
FIG. 4. The PKR-regulatory function of NS5A is required for rescue of viral mRNA translation and ablation of virus-induced eIF-2α phosphorylation in IFN-treated cells. (A) Constitutive expression of NS5A 1A and the ΔISDR construct in NIH 3T3 cell lines. Immunoblots of extracts prepared from Neo control (lane 1), NS5A 1A (wild type [wt]; lane 2), and ΔISDR (lane 3) cells were probed with a MAb specific to NS5A. Arrowheads denote the positions of NS5A 1A and the ΔISDR proteins. (B) Removal of the IFN-induced block on viral mRNA translation requires the PKR-regulatory function of NS5A. NIH 3T3 cell lines were mock infected or infected with VSV in the presence (+) or absence (−) of 100 U of IFN per ml, as shown above each lane. Proteins were pulse-labeled with [35S]methionine-cysteine, separated by SDS-PAGE, and visualized by autoradiography. Shown are representative analyses of Neo control (lanes 1 to 4), NS5A 1A (lanes 5 to 8), and ΔISDR (lanes 9 to 12) cell lines. Positions of molecular mass standards are indicated in kilodaltons. Arrows at right show the positions of the five VSV proteins. (C) NS5A prevents virus-induced eIF-2α phosphorylation in IFN-treated, VSV-infected cells. Soluble extracts were prepared from mock-infected (lanes 1, 3, and 5) or VSV-infected (lanes 2, 4, and 6) IFN-treated cells and subjected to single-dimension isoelectric focusing. Proteins were transferred to nitrocellulose and subjected to immunoblot analysis with a MAb specific to eIF-2α. Arrowheads point to the positions of basally phosphorylated eIF-2α (lower band) and eIF-2α phosphorylated on serine 51, the site phosphorylated by PKR (upper band) (75). Serine 51-phosphorylated eIF-2α as a percentage of the total eIF-2α present in each sample was quantitated by scanning densitometry and is shown below each lane as % P.
dsRNA signaling pathways remained intact in these cells. In contrast, cells expressing NS5A were resistant to dsRNA-induced apoptosis and retained this resistant phenotype even when exposed to high levels of pIC (Fig. 5). Thus, resistance to dsRNA-induced apoptosis required the intact PKR-binding domain of NS5A. Taken together, these results demonstrate that NS5A from IFN-resistant HCV can block PKR-dependent apoptotic signaling induced by dsRNA.

Phosphorylation of eIF-2α serine 51 by PKR is essential for dsRNA-induced apoptosis (81). To determine if the block in apoptosis imposed by NS5A could be attributed to inhibition of eIF-2α phosphorylation, we examined the levels and extent of phosphorylated eIF-2α after exposure of cells to dsRNA. As seen in Fig. 6, pIC induced a greater than fivefold increase in the level of serine 51-phosphorylated eIF-2α in Neo control cells, consistent with the dsRNA-dependent actions of PKR. In contrast, cells expressing NS5A 1A were refractory to dsRNA-dependent signaling, and the induction of serine 51 phosphorylation was completely abolished (Fig. 6, lanes 6 and 7). Importantly, the loss of PKR-binding activity of the ΔISDR mutant restored serine 51 phosphorylation induced by pIC, and these cells exhibited an approximate sixfold increase of phosphorylated eIF-2α. Thus, constitutive expression of NS5A 1A from IFN-resistant HCV blocks serine 51 phosphorylation of eIF-2α by dsRNA. Together, these results suggest that NS5A may block PKR-dependent signaling events during HCV infection, including the activation of PKR by viral dsRNA. Importantly, inhibition of PKR provides a molecular link by which HCV can both resist the antiviral actions of IFN and avoid host apoptotic programs induced by dsRNA.

PKR inhibition confers oncogenic potential to NS5A from IFN-resistant HCV. The translational control and antiproliferative properties of PKR have defined this protein kinase as a tumor suppressor (17). We therefore hypothesized that by blocking PKR function, NS5A might alter the growth properties of HCV-infected cells. To begin to examine this hypothesis, we characterized the growth properties of cells expressing NS5A 1A or the nonfunctional ΔISDR NS5A mutant. As shown in Table 1, comparison with Neo control and P58-20 cells (which overexpresses the cellular PKR inhibitory protein...
P58IPK [5]) indicated that both NS5A 1A and DIISR cells exhibited a growth-stimulatory phenotype with a characteristic reduction in doubling time and an increase in culture saturation density. Moreover, constitutive expression of NS5A 1A or the DIISR mutant supported colony formation of cells cultured on soft-agar medium (Table 1 and Fig. 7A). In contrast, cells expressing NS5A 1A, but not those expressing the DIISR nonfunctional NS5A mutant, generated solid tumors after injection into athymic mice. As noted previously, those mice receiving control P58-20 cells exhibited aggressive tumor growth (6). NS5A expression was confirmed in tumor-derived cells prepared from those tumors recovered from cells expressing NS5A 1A (Fig. 7B). This study demonstrates that constitutive expression of NS5A 1A from IFN-resistant HCV can induce malignant transformation of immortalized cells. The anchorage-independent growth observed in the DIISR cell lines suggests that NS5A can potentiate the immortalized phenotype of NIH 3T3 cells and stimulate cell growth through PKR-independent pathways. However, perturbation of these pathways themselves is not sufficient to induce oncogenic transformation. Importantly, our studies demonstrate that NS5A oncogenicity, defined by the ability to form tumors in vivo, required the PKR-regulatory function of NS5A.

DISCUSSION

Inhibition of PKR function and rescue of viral mRNA translation in IFN-treated cells: a molecular mechanism of HCV IFN resistance. Our results demonstrate that the NS5A protein from HCV can provide viral resistance to IFN by removing the IFN-induced, PKR-imposed block on mRNA translation during an actual viral infection. The following evidence allows

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>Saturation density (10^5 cells/cm²)</th>
<th>Cloning efficiency (%)</th>
<th>Mice with tumors/5 tested</th>
<th>Latency (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neo 5-10</td>
<td>27.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>P58-20</td>
<td>17.3 ± 1.5</td>
<td>3.7 ± 0.2</td>
<td>15</td>
<td>5</td>
<td>12–20</td>
</tr>
<tr>
<td>NS5A 1A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clone 5C6</td>
<td>17.7 ± 0.6</td>
<td>4.0 ± 0.2</td>
<td>14</td>
<td>5</td>
<td>17–25</td>
</tr>
<tr>
<td>Clone 4A1</td>
<td>22.4 ± 2.1</td>
<td>3.3 ± 0.1</td>
<td>4</td>
<td>4</td>
<td>22–44</td>
</tr>
<tr>
<td>DIISR clone 3C2d</td>
<td>17.5 ± 1.3</td>
<td>3.8 ± 0.3</td>
<td>3.9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*Neo 5-10 and P58-20 are negative and positive control cell lines, respectively, and data for each represent results from five independent clonal cell lines. NS5A 1A clone 5C6 cells express NS5A to levels approximately fivefold higher than those expressed by NS5A 1A clone 4A1 cells. Results are representative of four independent cell lines from each group of high- and low-expressing NS5A clones and four DIISR clones. Values shown are averages from four experiments.

B

FIG. 7. The PKR-regulatory function of NS5A is required for solid tumor growth in vivo but not for cell growth on soft-agar medium. (A) Soft-agar colony formation of Neo control (clone 5-2; image 1), NS5A 1A (clones 4A1 and 5C6; images 2 and 4, respectively), and DIISR cell lines (clone 3C2; image 3). For reference, panel 1 shows a single Neo control cell. Magnification is ×100. (B) NS5A is expressed in solid tumors generated from NS5A 1A cells. Extracts prepared from Neo control (lane 1) and NS5A 1A cells (lane 2), or solid tumor-derived cells recovered from mice inoculated with the NS5A 1A 5C6 clone (lane 3), were subjected to immunoblot analysis with anti-NS5A MAb. Positions of molecular mass standards are shown in kilodaltons.
Disruption of host dsRNA signaling may be critical for HCV, shown that NS5A from IFN-resistant HCV can disrupt by which viruses maintain persistent infection. Here we have persistence. During IFN therapy by quasispecies mutations that abolish our studies indicate that the level of HCV mRNA translation, production of IFN-sensitive quasispecies (63), consistent with worthy that recent analyses of HCV dynamics during IFN ability of NS5A to bind and inhibit PKR (29, 32). It is note-

Moreover, previous analyses of NS5A, representing a limited quasispecies diversity and viral fitness. Compared to those viral quasispecies that failed to respond to IFN therapy, IFN-sensi-

T-lymphocyte response.

Maintaining the translational competence of the host cell is critical for HCV, which replicates at an extremely high rate that can average in excess of 10^{12} virion particles/day/ml of blood examined (63). Coupled with the high rate of virion production, the pressure exerted by administration of thera-

peutic doses of IFN is a major factor in the generation of HCV quasispecies diversity and viral fitness. Compared to those viral quasispecies that failed to respond to IFN therapy, IFN-sensi-

tive quasispecies of HCV 1B have been shown to harbor mutations within the ISDR, an important region of the PKR-

binding domain of NS5A (23, 29). Though the majority of these studies have been conducted within Japanese patient populations (where this correlation remains strong [34]), it should be noted that these observations may be controversial, as the correlation between ISDR mutations and IFN sensitivity has not been reliably reproduced in patient populations out-

side Japan (35, 41, 67, 90). However, our results suggest that ISDR mutations may confer IFN sensitivity to viral replication by rendering NS5A unable to repress PKR (Fig. 3 and 4). Moreover, previous analyses of NS5A, representing a limited subset of IFN-sensitive HCV quasispecies, revealed that ISDR and PKR-binding domain mutations or deletions abolish the ability of NS5A to bind and inhibit PKR (29, 32). It is noteworthy that recent analyses of HCV dynamics during IFN therapy indicated that IFN functions to block de novo virion production of IFN-sensitive quasispecies (63), consistent with the antiviral actions of PKR. As demonstrated here with VSV, our studies indicate that the level of HCV mRNA translation, and hence viral persistence, would be severely compromised during IFN therapy by quasispecies mutations that abolish NS5A function.

Disruption of PKR-dependent apoptosis is associated with the IFN-resistant phenotype of HCV: implications for viral persistence. Evasion of host apoptosis is an important element by which viruses maintain persistent infection. Here we have shown that NS5A from IFN-resistant HCV can disrupt dsRNA-induced host apoptotic signaling by inhibiting PKR. Disruption of host dsRNA signaling may be critical for HCV, which has the potential to activate PKR through interactions with stem-loop dsRNA structures located within the 5’ and 3’ untranslated regions of the HCV genome (10, 50). NS5A in-

hibition of PKR and the resulting block in eIF-2a phosphorylation may therefore allow HCV to avoid host apoptosis induced by viral dsRNA.

Activation of PKR and suppression of cellular mRNA translation are necessary for initiation of apoptotic programs induced by dsRNA and such proinflammatory mediators as bac-

terial endotoxin and tumor necrosis factor alpha (9, 18, 81, 87). In combination with the transcriptional regulation of apoptotic effector genes, such as Fas/Apo1, FADD, and BAX, eIF-2a phosphorylation is thought to promote apoptosis by limiting mRNA expression and the synthesis of protective, anti-apo-

ptotic gene products (4, 18, 81). Recent evidence indicates that PKR can signal apoptosis through FADD-dependent mecha-

nisms (4), suggesting that NS5A may additionally allow HCV to avoid dsRNA-independent mechanisms of apoptosis by blocking death receptor signaling cascades. It is important to note that the HCV core protein has been shown to potentiate death receptor signaling and apoptosis in response to tumor necrosis factor alpha (88, 91). Inhibition of PKR function by NS5A may counteract the apoptotic potential of both HCV dsRNA and the viral core protein, thereby blocking the initi-

ation of PKR-dependent host antiviral programs during HCV infection. The ability to block dsRNA apoptotic signaling re-

quired an intact PKR-binding domain on NS5A (Fig. 5 and 6), confirming that NS5A blocks dsRNA-induced apoptosis at the level of PKR activity. Taken together, our data provide evi-

dence that HCV evasion of dsRNA-induced host apoptosis may be limited to those viral quasispecies that can inhibit PKR. We propose that NS5A inhibition of PKR links IFN resistance with the ability of HCV to evade host apoptosis and thereby establish persistent infection. This idea is supported by the observations that viral persistence is not a common feature in infections with HCV genotypes 2 to 6 (53), which collectively exhibit a higher response rate to IFN therapy (3, 59). Accord-

ingly, IFN resistance may now define a major determinant in the progression from acute to persistent HCV infection (51, 59).

Disruption of PKR function by NS5A links IFN resistance with the oncogenic potential of HCV. HCV RNA is present in a high frequency of liver tumors found in patients with chronic HCV infection (33, 73), and recent studies have identified HCV sequences in non-Hodgkin’s lymphoma B cells of HCV carriers (61). These studies implicate HCV in the etiology of virus-related malignancy (19). However, the molecular mech-

anisms underlying the oncogenic potential of HCV remain unclear. Others have shown that the NS3 and core proteins of HCV have oncogenic potential when overexpressed in NIH 3T3 cells (13, 70, 74). Work by Ray et al. (69, 71) suggests that the oncogenic potential of the HCV core protein may reside within its ability to repress transcription from the p53 and p21NFkappaB/Cip1/Ind1 promoters. However, evidence that the viral core protein can potentiate Fas-induced apoptosis (72) and enhance cell death signaling though interactions with members of the tumor necrosis factor receptor family suggests that this viral protein may also have antiproliferative properties (54, 88, 91). Here we provide evidence supportive of a role for NS5A in HCV-related cellular proliferative disorders. We have demon-

strated that expression of NS5A from IFN-resistant HCV, and constitutive inhibition of PKR, can induce a transformed phenotype in murine NIH 3T3 cell lines (Table 1). This is consistent with previous work from our laboratory and others demonstrating that disruption of eIF-2a phosphorylation through expression of an S51A eIF-2a mutant, dominant-negative PKR, or cellular PKR inhibitors could induce malignant
transformation of NIH 3T3 cells (6–8, 44). Taken together, these studies indicate that PKR exerts its antiproliferative effects, at least in part, by phosphorylating serine 51 of eIF-2α and limiting mRNA translation.

Recent work suggests that PKR activity is strictly regulated during the cell division cycle (27, 89). Moreover, Aktaş et al. (1) have demonstrated that PKR-mediated eIF-2α phosphorylation is required for the control of cyclin D1 translation and G1 cell cycle arrest that occur in response to intracellular calcium depletion and activation of PKR (80). In accordance with these observations, Balachandran and colleagues (4) demonstrated that inducible expression of PKR results in altered cell cycle kinetics, accumulation of cells in G1, and potentiation of apoptosis induced by dsRNA. In contrast, loss of PKR function or abrogation of eIF-2α phosphorylation induced oncogenic transformation (5, 6, 8, 21) and rendered cells refractory to apoptosis induced by PKR, including dsRNA (4, 18, 81). Thus, the PKR pathway may regulate cell growth, cell cycle kinetics, accumulation of cells in G1, and potentiation of apoptosis induced by dsRNA. Importantly, however, we emphasize that the oncogenic potential of TAR RNA binding protein TRBP and its regulatory interaction with RNA-dependent protein kinase PKR. EMBO J. 16:611–624.


