

Regulation of mRNA Translation and Cellular Signaling by Hepatitis C Virus Nonstructural Protein NS5A

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The NS5A nonstructural protein of hepatitis C virus (HCV) has been shown to inhibit the cellular interferon (IFN)-induced protein kinase R (PKR). PKR mediates the host IFN-induced antiviral response at least in part by inhibiting mRNA translation initiation through phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2 α). We thus examined the effect of NS5A inhibition of PKR on mRNA translation within the context of virus infection by using a recombinant vaccinia virus (VV)-based assay. The VV E3L protein is a potent inhibitor of PKR. Accordingly, infection of IFN-pretreated HeLa S3 cells with an E3L-deficient VV (VV Δ E3L) resulted in increased phosphorylation levels of both PKR and eIF2 α . IFN-pretreated cells infected with VV in which the E3L locus was replaced with the NS5A gene (VVNS5A) displayed diminished phosphorylation of PKR and eIF2 α in a transient manner. We also observed an increase in activation of p38 mitogen-activated protein kinase in IFN-pretreated cells infected with VV Δ E3L, consistent with reports that p38 lies downstream of the PKR pathway. Furthermore, these cells exhibited increased phosphorylation of the cap-binding initiation factor 4E (eIF4E), which is downstream of the p38 pathway. Importantly, these effects were reduced in cells infected with VVNS5A. NS5A was also found to inhibit activation of the p38-eIF4E pathway in epidermal growth factor-treated cells stably expressing NS5A. NS5A-induced inhibition of eIF2 α and eIF4E phosphorylation may exert counteracting effects on mRNA translation. Indeed, IFN-pretreated cells infected with VVNS5A exhibited a partial and transient restoration of cellular and viral mRNA translation compared with IFN-pretreated cells infected with VV Δ E3L. Taken together, these results support the role of NS5A as a PKR inhibitor and suggest a potential mechanism by which HCV might maintain global mRNA translation rate during early virus infection while favoring cap-independent translation of HCV mRNA during late infection.

Chronic hepatitis C virus (HCV) infection has become a worldwide health problem, affecting an estimated 170 million people worldwide, including about 4 million Americans (1). Chronically infected patients often develop progressive liver disease, cirrhosis, hepatic failure, and hepatocellular carcinoma (36). There is no vaccine available against HCV, and current therapies, including the combination of alpha interferon (IFN- α) and ribavirin, are effective at viral eradication in only a small percentage of patients (26, 33, 34). However, the development of more effective anti-HCV therapeutic agents has been hampered by the lack of an efficient cell culture system and an adequate animal model for HCV infection and replication.

HCV is a hepacivirus belonging to the *Flaviviridae* family. The positive-sense single-stranded enveloped RNA genome is translated in an internal ribosomal entry site-dependent manner to generate a single polyprotein, which is proteolytically processed into at least nine proteins (40). Among these pro-

teins, the NS5A nonstructural protein is receiving increasing attention as a potential target for anti-HCV therapy. The initial interest in NS5A stemmed from the observation that mutations within a discrete region of NS5A from certain HCV genotypes, termed the IFN sensitivity-determining region, correlated with increased sensitivity to IFN treatment (14, 15). Although the existence of the IFN sensitivity-determining region has been rather controversial (25), subsequent studies have shown that NS5A interacted with and inhibited protein kinase R (PKR), which is a key mediator of the host IFN antiviral response (18, 19, 20). PKR exerts its effects by phosphorylating the GTP-binding eukaryotic initiation factor 2 (eIF2) (11, 12). The eIF2 facilitates binding of the initiator Met-tRNA^{Met} to the 40S ribosomal subunit during translation initiation. Phosphorylation of the α subunit of eIF2 (eIF2 α) 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. Thus, NS5A-mediated inhibition of PKR may counteract the IFN-induced translational arrest, alluding to a possible mechanism by which HCV induces or sustains resistance to IFN treatment.

More recently, we have found that NS5A binds to a Src-homology 3 (SH3) domain of Grb2 (51). Grb2 is an adapter protein that mediates intracellular signaling by nucleating the

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formation of signal transduction complexes. The SH3 domains of Grb2 bind the nucleotide exchange factor Sos to trigger a series of signaling cascades in response to growth factor stimulation (10, 13). Stimulation with epidermal growth factor (EGF) induces the SH2 domain of Grb2 to bind to the EGF receptor, thereby recruiting Grb2 and Sos into a complex with the receptor and stimulating Sos to activate Ras. Activated Ras triggers the mitogen-activated protein kinase (MAPK) cascades, including the extracellular signal-regulated kinase (ERK) pathway (23, 57). ERKs phosphorylate a number of substrates, including the transcription factors E1k-1 and c-Jun, as well as the protein kinases Mnk1 and 3pK. Intriguingly, the phosphorylation of eIF4E by Mnk1 suggests a potential mechanism of translational control mediated through growth factor signaling (48, 49). Furthermore, the ERK pathway may play a role in IFN signaling and/or viral replication efficiency (28, 32), alluding to another possible mechanism of HCV resistance to IFN. In addition to NS5A, the E2 envelope protein of HCV has also been implicated as an inhibitor of PKR (54). The exact mechanism by which E2 inhibits PKR is not known, but inhibition may be mediated through E2 sequence homology with the autophosphorylation sites of PKR. Thus, HCV likely employs multiple strategies, including a two-pronged attack on PKR that incorporates the inhibitory functions of both NS5A and E2 proteins, to evade the IFN-induced antiviral response.

The use of cell lines inducibly expressing NS5A to determine the relevance of NS5A-mediated inhibition of PKR in HCV resistance to IFN have led to conflicting results (16, 19, 20, 39). In this report, we investigated the role of NS5A in PKR inhibition in the setting of a virus infection by using an established recombinant vaccinia virus (VV)-based system. We found that expression of NS5A in this system perturbed PKR-mediated signaling cascades, including the p38 pathway, possibly resulting in opposing effects on mRNA translation. Our results provide for the first time evidence supporting the PKR inhibitory role of NS5A in virus-infected cells and highlight a potential mechanism by which HCV subverts cellular signaling pathways to favor cap-independent translation of its mRNA.

MATERIALS AND METHODS

Cells and virus. HeLa S3 cells (ATCC CCL-2.2) were maintained in Dulbecco's minimal essential medium (Gibco/BRL) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml), and 2 mM L-glutamine and were grown at 37°C in 5% CO₂. VV (Copenhagen strain VC-2) was propagated, and working stocks were generated as described by Tartaglia et al. (53). The generation of recombinant VV devoid of E3L (VVΔE3L) and recombinant VV expressing NS5A in place of E3L (VVNS5A) was previously described (3, 51). VV infection of HeLa S3 cells was performed according to the protocol described by Kibler et al. (30). Maintenance, NS5A induction, and EGF treatment of stable Tet-Off HeLa cells (Clontech) inducibly expressing NS5A were performed as previously described (51).

In vivo radioisotope labeling of VV-infected HeLa S3 cells. At 2, 4, and 6 h postinfection, HeLa S3 cells untreated or pretreated with human type I IFN (400 IU/ml) for 24 h (Access) and infected with VV, VVΔE3L, or VVNS5A at multiplicity of infection of 10 were pulse-labeled with [³⁵S]Met (50 μCi/ml) (NEN) for 30 min (3). Cell lysates were prepared and protein concentration was determined as previously described (51). Protein lysates (20 μg/sample) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14% gel) and visualized by autoradiography. Protein synthesis levels were also quantified by subjecting cell lysates (2 μg/sample) to trichloroacetic acid (TCA) precipitation followed by quantification with a Beckman Coulter LS6500 scintillation counter.

Radioimmunoprecipitation assays. Radioimmunoprecipitation assays were performed as previously described (47). Briefly, 5 μl of antiserum against VV

proteins (provided by Bertram Jacobs) was added to 10 μg of [³⁵S]Met-labeled protein lysates prepared from VV-infected cells and incubated on ice with occasional agitation for 1 h. Protein A-agarose beads (20 μl) (Boehringer Mannheim) were added to the antiserum and lysate mixture and were incubated on ice for 2 h with occasional agitation. After incubation, the beads were washed three times with ice-cold phosphate-buffered saline buffer and resuspended in 20 μl of 2× SDS protein loading buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 2% SDS, 2% β-mercaptoethanol, and 0.02% bromophenol blue). Bound proteins were removed by boiling the beads for 5 min. Proteins were resolved by SDS-PAGE (14% gel) and visualized by autoradiography.

Analysis of PKR autophosphorylation. Cell lysates were prepared and protein concentration was determined as previously described (51). Lysates from VV-infected cells were subjected to SDS-PAGE (7.5% gel) followed by electroblotting to nitrocellulose filters (Schleicher & Schuell). The membranes were probed with a rabbit polyclonal antiserum specific for PKR (2), and detection was performed by using enhanced chemiluminescence (Amersham Pharmacia Biotech).

Determination of eIF2α, p38, and eIF4E phosphorylation. For analysis of eIF2α, p38, and eIF4E phosphorylation, equal amounts of cell lysates (20 μg) were subjected to SDS-PAGE and electroblotting. Filter immunoblots were probed with antibodies specific to phosphorylated forms of eIF2α (Ser51; Research Genetics), p38 (Thr180/Tyr182; New England Biolabs), or eIF4E (Ser209; New England Biolabs). The blots were stripped and reprobed with a monoclonal antibody that recognizes eIF2α (a gift from the E. C. Henshaw laboratory) or with antibodies directed against p38 (Santa Cruz Biotech) or eIF4E (Transduction Laboratories) to estimate the amount of expressed proteins. The relative levels of protein phosphorylation were determined by quantifying the immunoblots with ImageQuant (version 5.1). The signals from the phospho-specific immunoblots were normalized against their individual control signals, and the ratio of phospho-specific signal to control signal was determined.

RESULTS

Transient expression of NS5A from a recombinant VV system. VV is relatively resistant to the antiviral effects of IFN-α because the virus encodes a number of proteins that can interfere with components of the IFN signaling pathways (5, 37, 42, 44). One of these proteins is E3L, a double-stranded RNA (dsRNA)-binding protein that potently inhibits PKR, apparently by both sequestering dsRNA activators of PKR (8) and directly binding to the protein kinase (46). Accordingly, a recombinant VV lacking the *E3L* gene is exquisitely sensitive to IFN treatment (3, 4, 9). To use this system to characterize the PKR inhibitory function of NS5A, we inserted the NS5A coding sequence (obtained from an HCV genotype 1b isolate from a patient who did not respond to IFN therapy; see reference 18) into VVΔE3L such that its expression was under the control of the *E3L* promoter (51). This enabled us to examine the effects of NS5A expression within the context of virus infection and IFN treatment.

In all experiments, HeLa S3 cells were pretreated with human type I IFN (400 IU/ml) for 24 h and then washed with fresh medium prior to infection with different recombinant VV. Expression of NS5A was confirmed by immunoblot analysis of lysates derived from IFN-pretreated HeLa S3 cells infected with wild-type VV, VVΔE3L, or VVNS5A by using an antibody specific to NS5A. Using this analysis, NS5A was detected as early as 2 h postinfection (Fig. 1A), consistent with the fact that the early *E3L* promoter regulates NS5A expression. The immunoblot analysis also revealed multiple forms of NS5A, presumably due to differential phosphorylation (41, 52) or other forms of posttranslational modifications. A similar pattern of NS5A protein expression was observed in the absence of IFN treatment (data not shown).

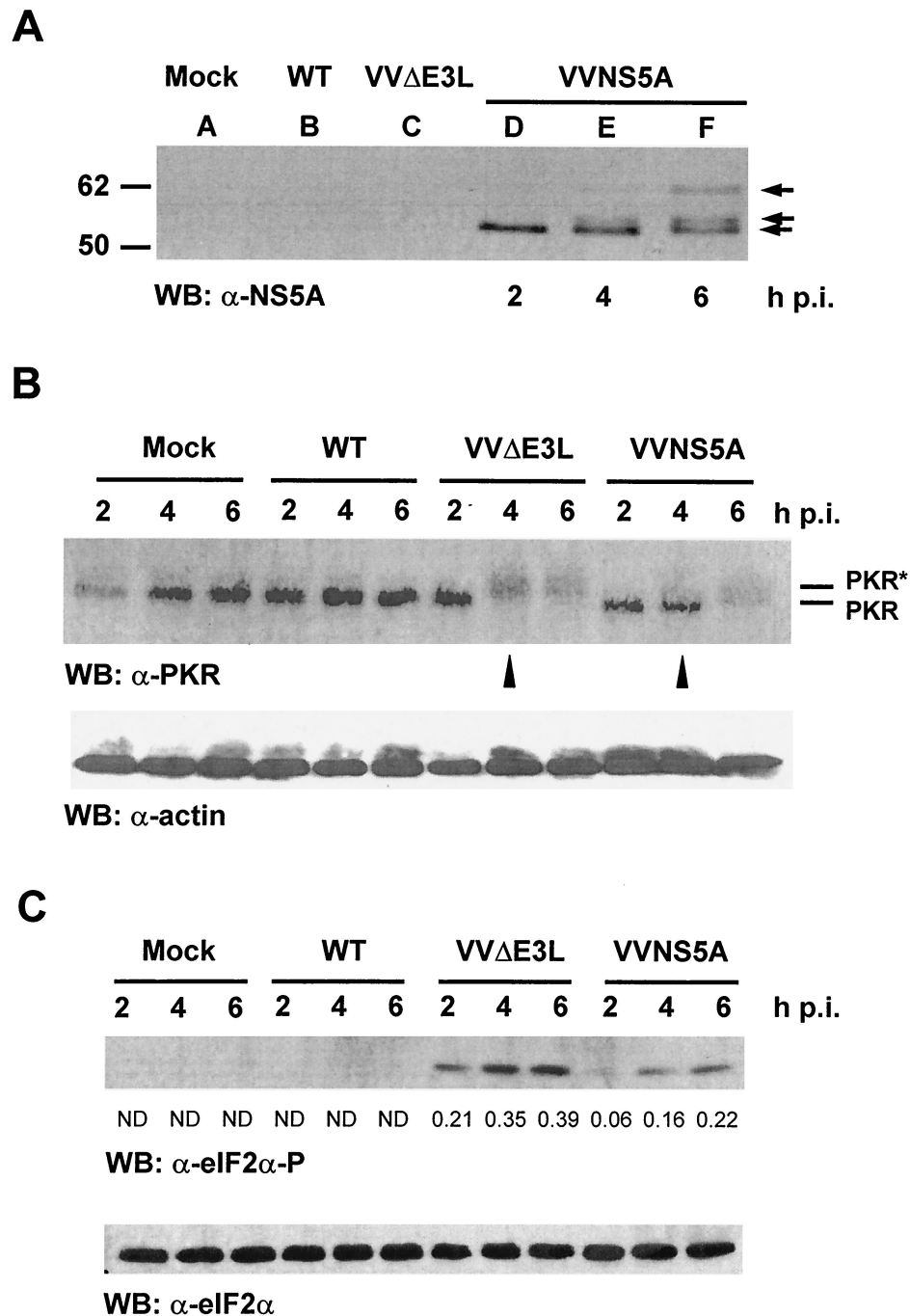


FIG. 1. NS5A reduces virus-induced PKR autophosphorylation and eIF2 α phosphorylation. (A) Expression of NS5A in VVNS5A-infected cells. Lysates (20 μ g of protein) from HeLa S3 cells pretreated with IFN- α / β and mock-infected (lane A) or infected with wild-type (WT) VV (lane B), VV Δ E3L (lane C), or VVNS5A (lanes D through F) at 2, 4, or 6 h postinfection (p.i.) were resolved by SDS-PAGE (14% gel). For mock infections and infections with wild-type VV or VV Δ E3L, only samples from the 6-h time point are shown. NS5A was detected by Western blotting (WB) using an anti-NS5A antibody (ID Labs). Arrows indicate the various migrating forms of NS5A. Sizes are indicated in kilodaltons. (B) Transient inhibition of virus-induced PKR autophosphorylation by NS5A. Lysates (20 μ g of protein) used for panel A were resolved by SDS-PAGE (7.5% gel). PKR (top panel) and actin (bottom panel) proteins were detected with an anti-PKR antibody and an anti-actin (ICN) antibody, respectively. Hyperphosphorylated PKR protein is denoted by an asterisk. (C) Reduced eIF2 α phosphorylation in the presence of NS5A. Lysates were immunoblotted with an antibody specific to the Ser51-phosphorylated form of eIF2 α (top panel) or an anti-eIF2 α antibody to detect protein levels (bottom panel). The relative levels of eIF2 α phosphorylation were determined by quantitative densitometry and normalized against the individual total protein amounts. The ratio of the phospho-specific signal to the total protein signal is indicated below the lanes for lysates from VV Δ E3L- and VVNS5A-infected cells. ND, not detectable.

Inhibition of virus-induced phosphorylation of PKR by NS5A. We first measured the activity of PKR in IFN-pretreated HeLa S3 cells infected with the various recombinant VV. Previous studies established that autophosphorylation of PKR correlates with its activation and that higher autophosphorylation leads to lower electrophoretic mobility (17, 27, 45, 46). We thus examined differential phosphorylation of PKR based on differential migration in SDS-PAGE (Fig. 1B). Mock-infected cells served as a negative control in which PKR was unphosphorylated and inactive. In lysates from IFN-pretreated wild-type VV-infected cells, PKR migrated at the same rate as that in lysates from mock-infected cells, indicating that in wild-type VV-infected cells PKR was unphosphorylated and inactive. In lysates from IFN-treated cells infected with VV Δ E3L, PKR migrated at slower rates 4 h after virus infection, indicating the presence of phosphorylated and active PKR. In contrast, PKR migrated at faster rates in lysates from VVNS5A-infected cells, indicating that NS5A could inhibit the phosphorylation and activation of PKR (Fig. 1B, arrows). This effect was transient, as the phosphorylated forms of PKR reappeared at 6 h postinfection. The difference in PKR abundance is noteworthy since it was specific and highly reproducible. This dissimilarity may be due to the fact that PKR downregulates its own expression at the translational level by a mechanism that depends on its phosphorylation, such that PKR abundance is inversely proportional to its kinase activity (45, 46). Alternatively, the phosphorylated PKR forms may be degraded by an unknown mechanism, as is the case during poliovirus infection (6, 7). This assay was also performed on lysates from VV-infected cells not treated with IFN, and similar results were observed (data not shown).

Virus-induced phosphorylation of PKR substrate, eIF2 α , is also inhibited by NS5A. PKR exerts its antiviral effects in part by phosphorylating eIF2 α on Ser51. To test whether the inhibition of PKR by NS5A affected the phosphorylation status of eIF2 α , we measured the relative amount of eIF2 α phosphorylation by using an antibody specific to the Ser51 phosphorylated form of the protein. In agreement with the results above, mock-infected or wild-type VV-infected cells produced no detectable level of eIF2 α phosphorylation at Ser51 (Fig. 1C). As expected, in VV Δ E3L-infected cells, there was a significant increase in phosphorylation of eIF2 α by PKR. Infection with VVNS5A, however, clearly decreased the proportion of phosphorylated eIF2 α compared with that of VV Δ E3L-infected cells, while the protein level of eIF2 α remained unaffected. On average, NS5A caused a two- to threefold decrease in eIF2 α phosphorylation. Previous studies have established that as little as a 15% change in the phosphorylation levels of eIF2 α can result in a dramatic impact on protein synthesis initiation (11). The different kinetics and levels of PKR activation and eIF2 α phosphorylation at different time points postinfection are likely due to the presence of other eIF2 α kinases that can also phosphorylate the initiation factor (12). Taken together, these results support the role of NS5A in counteracting PKR activity and subsequent phosphorylation of eIF2 α , suggesting a potential mechanism by which NS5A evades the antiviral effects of IFN.

Subversion of the p38 MAPK signaling pathway by NS5A during viral infection. Recently, it has been suggested that PKR might act upstream of the p38 MAPK pathway (24, 29,

59). We thus examined whether the p38 pathway is affected by NS5A in the VV system. As shown in Fig. 2A, in mock- and wild-type VV-infected cells pretreated with IFN, there was no detectable p38 activation, as indicated by the absence of phosphorylation at Thr180/Tyr182. VV Δ E3L-infected cells exhibited a significant increase in p38 activation despite the presence of comparable protein levels of p38 in all samples, consistent with the scenario where PKR is activated in the absence of E3L. Importantly, the activation of p38 was significantly decreased (approximately twofold on average) in VVNS5A-infected cells, presumably at least in part due to NS5A inhibition of PKR.

An important substrate of p38 is MAPK-interacting protein kinase 1 (Mnk1), which in turn phosphorylates the translation initiation factor eIF4E. Phosphorylation of eIF4E on residue Ser209 by Mnk1 can enhance the cap-binding activity of the translation initiation factor (48, 49). We thus examined the effect of NS5A on eIF4E phosphorylation in recombinant VV-infected HeLa S3 cells pretreated with IFN. In mock- and wild-type VV-infected HeLa S3 cells a low basal level of eIF4E phosphorylation was detectable (Fig. 2B). A slight increase in eIF4E phosphorylation was observed in wild-type VV-infected cells, possibly due to the activation of MAPK pathways by the virus-encoded EGF homologue. Consistent with the p38 activation profile described above, a much higher level of eIF4E phosphorylation was observed in VV Δ E3L-infected cells. As predicted, the presence of NS5A reversed this effect by approximately two- to fivefold, indicating that NS5A can subvert the PKR-p38 pathway. The inhibition of p38 and eIF4E phosphorylation by NS5A is specific, since we did not observe an inhibition of the unrelated AKT cell survival pathway in VVNS5A-infected cells (data not shown).

NS5A inhibits EGF-induced phosphorylation of p38 and eIF4E. To confirm our data and examine the effect of NS5A on the p38-eIF4E pathway outside the context of virus infection, we compared the levels of EGF-induced activation of p38 and eIF4E in an inducible stable NS5A-expressing Tet-Off HeLa cell line (51). In accordance with the results that NS5A could affect the p38 MAPK-eIF4E pathway during viral infection, and in agreement with a previous study that NS5A can interact with the Grb2 adaptor molecule and perturb the downstream MAPK pathway (51), we found that cells stably expressing NS5A were indeed more refractory to EGF-induced activation of p38 than control cells lacking NS5A (Fig. 3A). Likewise, NS5A expression also inhibited EGF-induced phosphorylation of eIF4E (Fig. 3B). Importantly, the protein levels of both p38 and eIF4E were expressed to comparable levels in the cells. Inducible expression of NS5A in these cells was confirmed by Western blot analysis (data not shown). Taken together with the data obtained from the VV infection studies, these results suggest NS5A, whether expressed from a tetracycline-regulated promoter or as a virus-encoded protein, has the capability to inhibit the p38 signaling pathway and downregulate eIF4E phosphorylation. Furthermore, these results support the significance of our findings, demonstrating the ability of a single viral protein to regulate different translation initiation factors in different settings by modulating different cellular pathways.

NS5A inhibits IFN-induced translational arrest during virus infection. A prediction from the inhibition of eIF2 α phos-

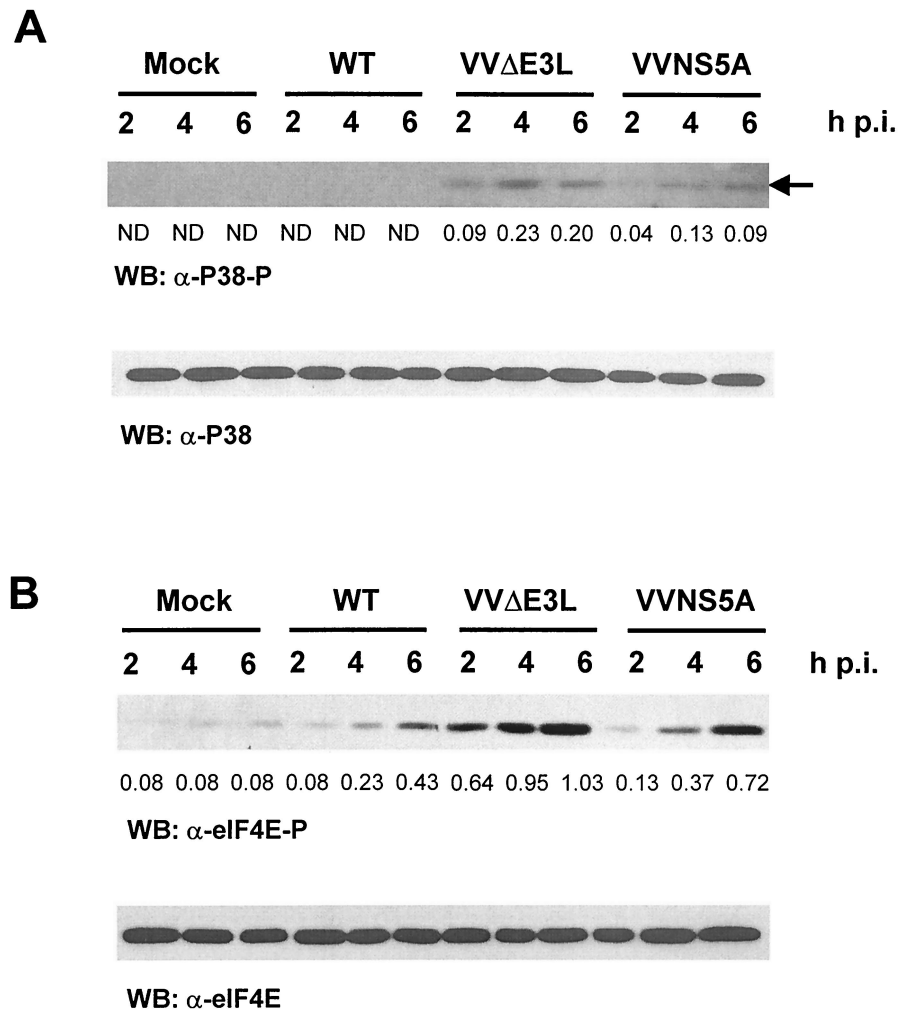


FIG. 2. Inhibition of p38 activation and eIF4E phosphorylation by NS5A expression in VV-infected cells. (A) Virus-induced p38 activation in HeLa S3 cells infected with different recombinant VV at various time points postinfection (p.i.). Lysates (20 μ g of protein) were fractionated by SDS-PAGE. The activation state of p38 was then determined by Western blotting (WB) using antibody specific for the dually phosphorylated activated form of p38 (Thr180/Tyr182) (top panel) or the total p38 protein kinase (bottom panel). The position of activated p38 is indicated by an arrow. The relative levels of p38 phosphorylation were determined by quantitative densitometry and normalized against the individual total protein amounts, and the phospho-specific signal/total protein signal ratios are indicated below the lanes for lysates from VV Δ E3L- and VVNS5A-infected cells. ND, not detectable; WT, wild type. (B) Inhibition of Ser209 phosphorylation of eIF4E by NS5A expression in VV-infected cells. Phosphorylation levels of eIF4E at Ser209 were measured by Western blotting of the lysates used for Fig. 1 by using a phospho-specific antibody (top panel), while protein levels of eIF4E were determined by using an anti-eIF4E antibody (bottom panel). The relative levels of eIF4E phosphorylation were determined by quantitative densitometry and normalized against the individual total protein amounts. The ratio of the phospho-specific signal to total protein signal is indicated below the lanes.

phorylation through PKR downregulation by NS5A (Fig. 1) is maintenance of cellular protein synthesis during virus infection. However, the decrease in eIF4E phosphorylation (Fig. 2 and 3), possibly due to NS5A-mediated inhibition of the PKR-p38 pathway, would also argue for a reduction of cap-dependent translation of cellular mRNAs. We therefore examined the consequences of such opposing effects on mRNA translation in VVNS5A-infected cells. HeLa S3 cells pretreated with IFN were infected with wild-type or recombinant VV and pulse-labeled with [35 S]Met at 2, 4, or 6 h postinfection. As previously reported (3, 4, 43), IFN pretreatment had little effect on global protein synthesis in wild-type VV-infected cells (Fig. 4A), as confirmed by quantification of protein translation

levels in VV-infected cells by TCA precipitation followed by scintillation counting (data not shown). In contrast, cells infected with VV Δ E3L displayed a significant decrease in global protein synthesis, an effect largely attributed to the activation of PKR and phosphorylation of eIF2 α (3, 4, 57). On average, VV Δ E3L infection caused an approximately 10-fold decrease in protein translation levels (data not shown) compared with those of mock- and wild-type VV-infected cells. As predicted, NS5A partially and transiently restored global protein synthesis in VVNS5A-infected cells, consistent with the notion that the restoration is a net result of dephosphorylation of eIF2 α and eIF4E. On average, NS5A expression resulted in an approximately sixfold increase in protein translation levels, with

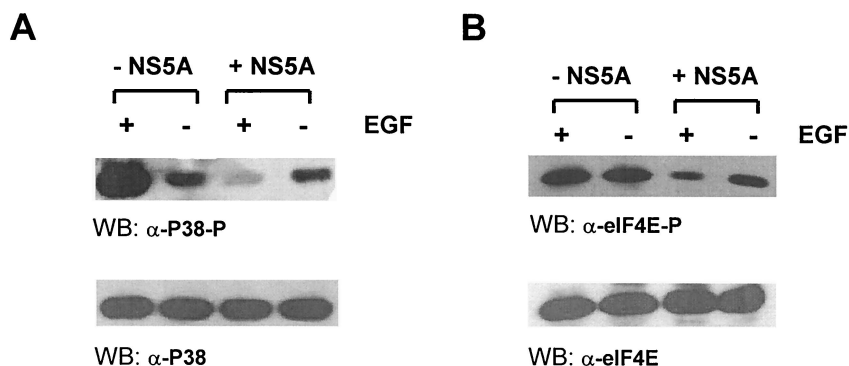


FIG. 3. Inhibition of EGF-induced phosphorylation of p38 and eIF4E in an inducible stable NS5A-expressing HeLa cell line system. Tet-Off HeLa cells cultured in the presence (-NS5A) or in the absence of tetracycline (+NS5A) were mock- or EGF-treated (20 ng/ml for 2 min), washed, and incubated for 4 h prior to lysis. The activation state of p38 (A) and Ser209 phosphorylation of eIF4E (B) were then determined by immunoblotting using antibody directed against the phosphorylated forms of the proteins as described in the legend to Fig. 2. Total protein levels were determined as described in the legend to Fig. 2. WB, Western blotting.

the most significant effect observed at 4 h postinfection (an approximately 18-fold increase).

We next investigated whether NS5A could rescue viral protein synthesis from the IFN-induced translational arrest phenotype observed in VV Δ E3L-infected cells. Cell lysates were prepared from wild-type and recombinant VV-infected HeLa S3 cells that were pulse-labeled with [³⁵S]Met at 4 h postinfection. Viral proteins were then immunoprecipitated from these lysates using a rabbit polyclonal antiserum raised against VV proteins. The immunoprecipitated proteins were resolved by SDS-PAGE and visualized by autoradiography. While efficient viral protein synthesis could be detected in HeLa S3 cells infected with wild-type VV, there was a significant decrease in viral protein synthesis in VV Δ E3L-infected cells (Fig. 4B). As

predicted, viral protein synthesis was partially restored by NS5A expression in VVNS5A-infected cells. These assays were also performed with VV-infected cells not treated with IFN, and a similar, although less significant, effect of NS5A expression on global and viral protein translation was seen (data not shown). Thus, these observations also suggest that the effect of reduced eIF2 α phosphorylation may offset the effect of reduced eIF4E phosphorylation during NS5A expression. It therefore appears that NS5A can functionally replace E3L as an inhibitor of PKR and restore global protein synthesis during virus infection.

The ability of NS5A to interact with Grb2 is not required for restoration of protein synthesis during virus infection. We previously showed that NS5A also binds Grb2, an adapter

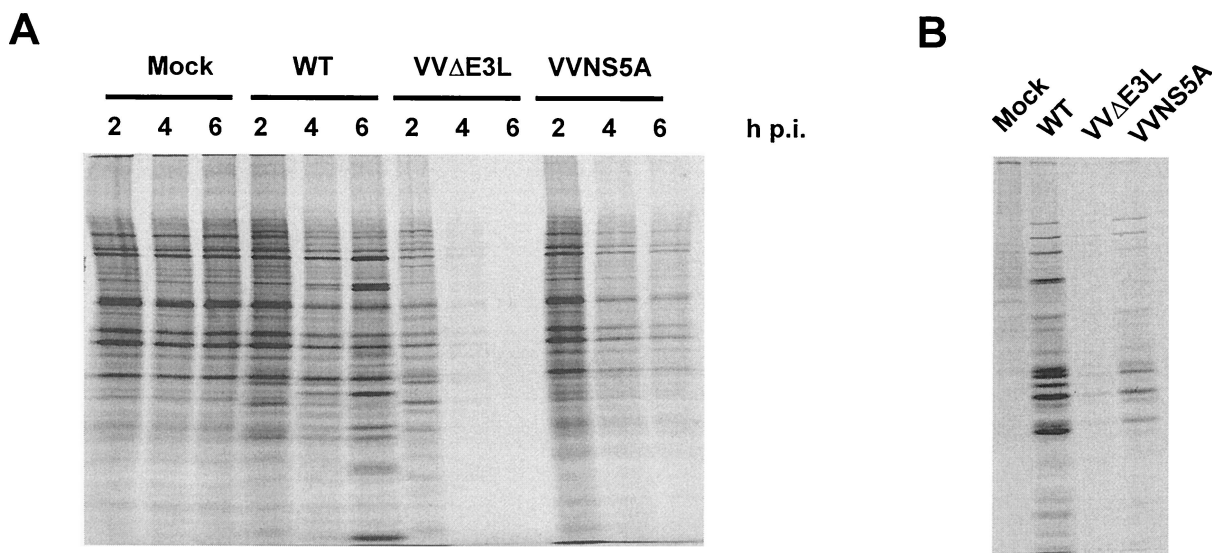


FIG. 4. NS5A partially reverses the translational arrest phenotype observed in VV Δ E3L-infected cells. (A) Profiles of global protein synthesis in recombinant VV-infected cells. HeLa S3 cells were pretreated with IFN- α / β and mock-infected or infected with wild-type (WT) VV, VV Δ E3L, or VVNS5A. Cells were pulse-labeled with [³⁵S]Met and harvested at 2, 4, and 6 h postinfection (p.i.) Lysates (20 μ g of protein) were resolved by SDS-PAGE and detected by autoradiography. The protein translation levels were also quantified by subjecting lysates to TCA precipitation followed by scintillation counting. (B) Profiles of viral protein synthesis in recombinant VV-infected cells. The lysates used for panel A were immunoprecipitated with a rabbit polyclonal antiserum against total VV proteins, and precipitated proteins were subjected to SDS-PAGE and autoradiography.

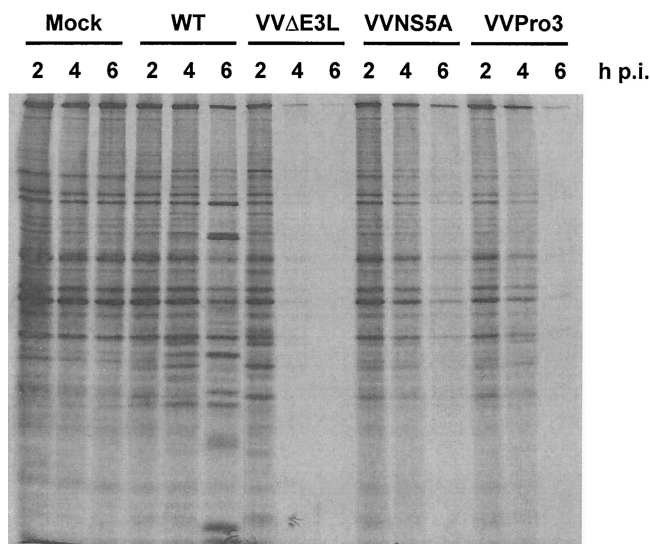


FIG. 5. NS5A-mediated reversal of the translation arrest phenotype observed in VV Δ E3L-infected cells is independent of its ability to bind Grb2. Analyses of global protein synthesis in HeLa S3 cells pretreated with IFN- α/β and infected with a recombinant VV expressing a Grb2 binding-defective form of NS5A (VVPro3) were performed as described in the legend to Fig. 4. WT, wild type; p.i., postinfection.

protein that mediates signaling by nucleating the formation of signal transduction complexes in response to growth factor stimulation. An effect of NS5A interaction with Grb2 is an inhibition of downstream MAPK activation by EGF (51). A proline-rich motif present in the C-terminal region of NS5A is required for Grb2 binding and ERK1/2 inhibition (51; H. Nakao et al., unpublished data). Because Grb2 also mediates signaling to the p38 pathway, we cautioned that the observed protein synthesis restoration by NS5A might also involve downregulation of p38 via the interaction of NS5A with Grb2. To test this, we examined the ability of a recombinant VV that expresses a NS5A variant containing point mutations within the proline-rich motif (VVPro3) to restore global protein synthesis during virus infection. As shown in Fig. 5, expression of the Grb2 binding-deficient NS5A protein restored mRNA translation to an extent similar to that of wild-type NS5A, as confirmed by quantification of protein translation levels as described above (data not shown). Thus, the rescue is not dependent on Grb2-mediated pathways in this system but is likely due to NS5A-mediated inhibition of PKR.

DISCUSSION

In the present report, we used a recombinant VV-based assay to study the PKR inhibitory effects of NS5A within the context of virus infection for the first time. In the absence of a robust HCV cell culture infection system, it is necessary to use surrogate systems such as the vaccinia system described herein. Consistent with the notion that E3L is a potent inhibitor of PKR, infection of IFN-treated cells with VV Δ E3L resulted in increased phosphorylation of PKR and eIF2 α and a marked arrest in protein synthesis initiation (Fig. 1 and 4). Importantly, expression of NS5A in VVNS5A-infected cells decreased the phosphorylation of PKR and eIF2 α and alleviated the trans-

lational block in a partial and transient manner. Stimulation of protein synthesis by NS5A was likely directly caused by inhibition of the PKR pathway rather than the Grb2 pathway (51; H. Nakao et al., unpublished) since the PxxP Grb2 binding-deficient NS5A mutant stimulated protein synthesis to the same degree as the wild type. We are currently constructing a VV containing a PKR binding-deficient NS5A mutant to prove this point unequivocally. Despite the need for this and other mutants to pinpoint precise mechanisms, we should stress that we have utilized four separate VV in the present study to provide the necessary controls and to prove that the effects are specific to the HCV NS5A. In all experiments, we compared the results to both wild-type and isogenic VV lacking the E3L gene. We also included an additional virus containing NS5A with the described proline mutations. It is also relevant to note that our present results build upon previous reports from our laboratory and others, which have detailed the PKR inhibition by wild-type and mutant NS5A in a "nonviral" setting (18, 19, 20). These data are also consistent with reports from several independent laboratories that have shown that NS5A, expressed in *trans*, can confer IFN resistance to otherwise IFN-sensitive viruses (39, 50).

Perhaps most importantly, our results highlight a previously unknown effect of NS5A: the inhibition of p38 activation during VV infection or EGF stimulation (Fig. 2 and 3). This is supportive of previous reports that p38 lies downstream of the PKR signaling pathway (24, 29, 59) and our previous finding that NS5A can perturb the MAPK pathway by targeting Grb2 (51; H. Nakao et al., unpublished). Relevant to translational regulation, p38 phosphorylates Mnk1 protein kinase, which in turn phosphorylates the translation initiation factor eIF4E (48, 49). Indeed we can now demonstrate that reductions in p38 activity caused by HCV NS5A also decreased 4E phosphorylation levels, perhaps having a negative impact on cap-dependent mRNA translation. Curiously, however, p38 is best known for its role in the stress-activated transcriptional regulatory pathways (35). At this point one might only speculate that HCV encodes a mechanism to downregulate this pathway to enhance and/or sustain infection and negate the ability of the infected cell to properly respond to stress-mediated signaling.

One unanswered question relates to why only a transient and partial restoration of cellular and viral protein synthesis by NS5A occurs. There is always the possibility that the lack of total rescue of protein synthesis late after infection was simply due to the massive activation of PKR and resultant eIF2 α phosphorylation. This suggests that NS5A simply cannot fully compensate for the loss of E3L. The latter could be due to the inherently different properties of E3L and NS5A. In fact, the VV system mainly has been used to study only dsRNA-binding inhibitors, including the reovirus S4 protein (3), the porcine group C rotavirus NSP3 protein (31), the *Escherichia coli* RNase III protein (47), and the cellular TAR RNA-binding protein (38). In this regard, it is interesting that NS5A, which has not been shown to possess dsRNA-binding activity, is capable of downregulating PKR in the VV system. E3L, by virtue of its ability to bind dsRNA, can probably inhibit other PKR-independent IFN-stimulated dsRNA-dependent pathways (4). Indeed, a recent study showed that VV E3L could function as an inhibitor of the IFN-induced 2-5A synthetase enzyme (43).

Thus, it may not be surprising that NS5A may not fully substitute for E3L.

We rather favor an alternative explanation for the incomplete restoration of mRNA translation by NS5A. We suggest that NS5A may exert counteracting effects on mRNA translation during viral infection. While NS5A-mediated inhibition of PKR could function to ensure the availability of functional eIF2 α to maintain global mRNA translation, it could also lead to dramatic decreases in eIF4E phosphorylation and function, thereby incapacitating cap-dependent translation of cellular mRNAs. Since translation of HCV mRNA occurs via an internal ribosomal entry site-dependent, cap-independent mechanism (55, 58), it is tempting to speculate that NS5A-mediated disruption of the PKR-p38-Mnk1-eIF4E pathway during HCV infection may ultimately favor the translation of viral mRNAs over host mRNAs and therefore contribute to the replication and pathogenesis of HCV. This scenario is reminiscent of the strategies utilized by poliovirus that encodes dual mechanisms to inhibit PKR activity (6, 7, 21, 22) and disrupts cap-dependent translation (56). Indeed, eukaryotic viruses in general display an impressive array of strategies to ensure the efficient and selective translation of viral mRNAs (for a comprehensive and recent review see reference 22). Unfortunately, complete examination of translational regulatory strategies employed by HCV awaits the development of an in vitro infection system.

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