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Suppression of the ERK1/2 signaling pathway from HCV NS5A protein expressed by herpes simplex recombinant viruses

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Summary. Two herpes simplex type 1 (HSV-1) recombinant viruses carrying the hepatitis C virus (HCV) NS5A open reading frame under the control of the cytomegalovirus immediate early (IE) or a herpes simplex chimeric promoter ($\alpha 4\gamma 1$UL19) were constructed and characterized. Expression studies showed that both HSV-NS5A recombinant viruses were able to express high levels of the NS5A protein in infected cells. Most importantly, using this system, we demonstrated that the NS5A protein interacts with the growth receptor-bound protein 2 (Grb2) and inhibits the phosphorylation of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) in HeLa, NIH3T3 or liver infected cells. Thus, our studies confirm the ability of the NS5A protein to perturb the extracellular signal-regulated kinase (ERK) pathway in HeLa cells by the use of an alternative system for NS5A expression and extend this observation to additional cell lines. We conclude that HSV-based viral vectors may provide a useful system for studying the expression and selected functional properties of the HCV NS5A protein.

Abbreviations: herpes simplex virus type 1 (HSV-1), hepatitis C virus (HCV), mitogen activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), RNA-activated protein kinases (PKR), green fluorescent protein (GFP), growth factor receptor bound protein 2 (Grb2).

Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, often leading to liver failure, cirrhosis or hepatocellular carcinoma [1]. HCV is a classified member within the *Flaviridae* family [31]. The virus has an approximately 9.6 kb positive-stranded RNA genome that encodes a precursor polyprotein, which yields the individual structural (C, E1, E2, p7) and non-structural (NS2-5) viral proteins after host and viral protease-mediated cleavage [17]. NS5A is a multifunctional

phosphoprotein with postulated important roles in HCV pathobiology [26]. However, its detailed function has yet to be defined as its role in HCV-host interaction and viral replication is still being elucidated. The protein is phosphorylated mainly on serine residues and the degree of phosphorylation determines its molecular weight (56–58 kDa) [19, 26]. The full-length NS5A protein is mainly positioned on the cytoplasmic side of the endoplamic reticulum and it is believed to be part of the viral replicase complex [5]. On the other hand, it was recently shown that NS5A may be proteolytically processed by a caspase-like protease to produce smaller molecular weight forms [33]. N-terminal deleted forms of the NS5A protein translocate to the nucleus and appear to display distinct biological functions [7, 14, 37].

Several studies suggest that the NS5A protein interacts with various cellular proteins including members of different cellular signaling pathways, the transcription activation machinery and cell cycle-regulatory kinases and many of these interactions are directly related with the anti-apoptotic and oncogenic properties of the NS5A protein [18, 25]. Notwithstanding, most previous studies link the NS5A protein with HCV resistance to the antiviral effects of α-interferon (IFN-α) [18, 25, 38]. Furthermore it was shown that NS5A from genotypes 1a or 1b binds to the IFN-induced double-stranded RNA-activated protein kinase (PKR), a key component of the IFN-mediated antiviral response, and represses its activity [9, 10]. However, the exact host molecular targets involved in the interferon-α resistance remain controversial as most recent studies provide strong evidence that NS5A uses additional, PKR-independent, mechanisms to counterattack the IFN-mediated antiviral response [16, 18, 27]. In this context, Tan et al. have recently reported that NS5A acts as a viral inhibitor of the mitogenic signaling pathway at least in part by the direct interaction to Grb2 [39]. Grb2 is a SH_3–domain containing adaptor protein that mediates intracellular signaling by the formation of a complex with the guanine nucleotide exchange factors Son of sevenless (Sos) 1 and 2 upon growth factor-mediated stimulation of receptor tyrosine kinases. During this process Sos triggers the Ras-MAPK pathway [4]. Expression of NS5A was found to suppress the phosphorylation of the ERKs. Interestingly, it was suggested that the perturbation of the MAPK pathway by NS5A may exert an effect on the interferon signaling pathway because of the cross-talk between the MAPK and JAK/STAT pathway [18, 38, 39]. However, these experiments were performed only with HeLa cells and the level of suppression was substantially lower in stably transfected cells rather that in HeLa cells infected by the NS5A-expressing recombinant vaccinia virus [39].

Because of the lack of a cell culture system for HCV, the functional studies of the HCV proteins are restricted by the use of heterologous expression systems and the recently described replicons [3]. In this context recombinant viruses expressing HCV proteins may have the advantage to serve as surrogate virus for HCV infection. Although considerable progress has been made by the use of such approaches, none of those systems represents the physiological cellular environment for HCV infection. As such, novel functional properties should be confirmed by the use of additional independent approaches.

Herpes simplex virus type 1 (HSV-1) is an enveloped DNA virus, with many favorable properties for expression of heterologous genes. These include the ability to efficiently infect a variety of mammalian cells, the large capacity for exogenous transgene insertion combined with the high expression levels of the transgene, and the easiness of manipulating the system [32]. Over the past decades HSV-1 has been extensively characterized and a series of genetic modifications have generated a number of different types of innovated viral vectors suitable for gene therapy and/or vaccine development [32]. Thus, in this study, we explored the possibility of using HSV-1 as an alternative viral expression system to study selected functions of the NS5A protein. As viral functions related to the signaling responses are likely to vary depending on the cell type and the methods of protein expression, we sought to further confirm and analyze the interplay between NS5A and MAPK/ERK signaling pathway using this system.

To this end we have constructed recombinant HSV-1 viruses expressing the NS5A from HCV-1a and examined the possible interaction of the NS5A protein with ERK signaling pathways in different cell lines. For this purpose we have produced a polyclonal antibody that specifically reacts with the different forms of the NS5A protein. We show here that in HeLa, NIH3T3, WRL68 or liver cells infected with HSV-NS5A recombinant viruses, the NS5A protein coimmunoprecipitates with the Grb2 adaptor protein and disrupts the activation of ERK1/2.

Materials and methods

Cells and viruses

Vero, 143TK$^-$ and rabbit skin cells (RSC) were kindly provided by B. Roizman (University of Chicago), WRL68 were kindly provided by A. Budkowska (Institut Pasteur, Paris) and NIH3T3 were from ATCC. Cells were maintained in DMEM supplemented with 5 % (Vero, RSC) or 10 % (143TK$^-$, WRL68, NIH3T3) fetal calf serum, penicillin, streptomycin and 2 mM L-glutamine at 37 °C with 5 % CO_2. For the ERK phosphorylation experiments all cells used were synchronized by starvation for 24 h in 0,1 % serum containing medium. HSV-1(F) is a limited passage wild type viral isolate and the HSV-1(F)Δ305 recombinant contains a deletion within the coding sequences of the thymidine kinase (*tk*) gene [29]. Both viruses were kindly provided by B. Roizman (University of Chicago, Chicago). Recombinant viruses RF640, RF680 were constructed in this study as previously described [12]. Briefly, intact HSV-1(F) DNA was cotransfected with pHPI640 or pHPI680 plasmid DNA (see construction of plasmids) into RSC. The progeny of the transfection were plated on 143TK$^-$ cells in the presence of 100 μg/ml bromodeoxyuridine to select for recombinant *tk*$^-$ viral progeny [30]. Individual plaques were picked, plaque purified, and tested for the presence of insert by Southern blot analyses. For the RF680 recombinant virus, selection was facilitated by the expression of GFP fluorescent protein.

Construction of plasmids

The HCV NS5A coding sequence (447 aa) was obtained by PCR using a plasmid containing cloned cDNA sequences from HCV-H(1a) (kindly provided by G. Inchauspe) and primers: sense 5′A<u>GATATC</u>**ATG**AGCTCCGGTTCCTG 3′ and antisense 5′ <u>CTCGAG AAGC</u>**<u>TTA</u>**GC AGCACACGA 3′ (*Eco*RV and *Xho*I restriction sites are underlined, initiator ATG and stop

codons are in bold). PCR conditions were as follows: 35 cycles of 94 °C for 1 min for denaturation, 65 °C for 30 sec for annealing, 72 °C for 2 min for extension, followed by 72 °C for 10 min. The PCR product were digested with *Eco*RV-*Xho*I, blunt-ended with Klenow and cloned into the *Sma*I site of pUC18 to yield plasmid pHPI611. Subsequently, the 1350 bp *Hin*dIII-*Hin*dIII fragment containing the NS5A coding sequence was obtained from plasmid pHPI611 and inserted into the TK shuttle vectors pHPI636 or pHPI669 under the control of an HSV-1 chimeric αγ (kindly provided by B. Roizman) or immediate-early CMV (IECMV) promoters respectively. Both vectors are designed to allow homologous recombination with the HSV-1 genome at the TK locus and are derived from plasmid pRB3823. Plasmid pRB3823 contains the upstream *Bam*HI-*Pvu*II (nt 48634–48106) and downstream *Bam*HI-*Bgl*I (nt 45055–47855) fragments from HSV-1 TK sequences cloned into the *Hin*dIII and *Bam*HI sites respectively, of pUC19. The αγ promoter is a novel chimeric HSV promoter that consists of the upstream regulatory promoter elements of the α4 gene (nt 146201–146761) fused at position −12 relative to the α4 cap site with the downstream regulatory sequences of the γ1UL19 promoter sequences (nt 40581–40782). UL19 encodes for the VP5, the major capsid protein of the virus. The α4 upstream promoter sequences were derived by ligation of a ∼500 bp *Nar*I-*Bgl*I DNA fragment (−520 to −88 relative to the α4 cap site) obtained from pRB168 [28, 30] to a synthetic oligonucleotide designed to bridge from −88 to −12 in the α4 promoter region. In addition a *Not*I site was induced at −40 position and a *Bam*HI at −12 site to facilitate the cloning of the γ1UL19 downstream promoter sequences. The ligation product was cloned into pGEM-3z vector cut with *Acc*I and *Bam*HI to yield plasmid 4295. The γ1UL19 downstream promoter sequence were obtained as a 200 bp *Bam*HI-*Bst*YI fragment containing the sequences from −11 to +188 of the UL19 promoter. This fragment was originally cloned into the *Bam*HI site of pGEM-3z to convert *Bst*YI into *Bam*HI recognition site, cleaved with *Bam*HI and recloned into the induced *Bam*HI site (described above) of pRB4295 to yield plasmid pRB4297 [King, R. W., Barker, D. E., and Roizman, B., unpublished data]. The α4γ1UL19 promoter was obtained as *Sac*I-*Pst*I fragment from pRB4297 and cloned into the *Sal*I site of pRB3823. Furthermore, a linker introducing a *Hin*dIII cloning site and stop codons within the three open reading frames was inserted at the *Xba*I site downstream of the promoter sequences.

Plasmid pHPI680 was made in two steps. Firstly, the 2428 bp *Pac*I-*Pac*I fragment carrying the GFP coding sequence under the control of the 3′RSV LTR and the CMV promoter in the opposite direction was derived from pIMJ28 (kindly provided by Xenova, UK) and cloned into the *Xba*I site of pRB3823 to yield pHPI669. Secondly, the *Hin*dIII-*Hin*dIII fragment from pHPI611 was inserted into the *Hin*dIII site of pHPI669. To prepare NS5A protein in *E. coli*, the *Bam*HI fragment obtained from plasmid pHPI611 was inserted into the *Bam*HI site of pGEX 2T to yield plasmid pHPI642. The correct frame was confirmed by sequencing.

Preparation of NS5A antibody

Rabbit polyclonal anti-NS5A serum was used to follow the expression of HCV NS5A protein in infected cells. The GST-NS5A fusion protein, obtained after 3 h induction of an XL-1 *E. coli* strain transformed with plasmid pHPI642 in the presence of 0.5 mM IPTG, was purified with Glutathione-Sepharose4B beads according to the manufacturer's protocol (Pharmacia). The purified protein was used to immunize rabbits as previously described [11].

Coimmunoprecipitation and immunoblot analysis

Cell monolayers were infected with HSV-1(F), HSV-1(F)Δ305, or the recombinants RF640 and RF680 at a multiplicity of infection (m.o.i) of 5. At different times post-infection, the cells were collected, and resuspended in ice-cold triple detergent buffer (50 mM Tris pH8,

150 mM NaCl, 0.1 % SDS, 100 μg/ml PMSF, 1 % NP40, 0.5 % sodium deoxycholate), or ice-cold lysis buffer containing 1 % Triton X-100, 50 mM KCl, 10 mM Tris (pH 7.5), 1 mM DTT, 2 mM $MgCl_2$, 1 mM PMSF depending on their intended use, electrophoretically separated on 10 % or 12 % SDS-polyacrylamide gels, electrotransferred onto nitrocellulose membranes, tested with appropriate antibodies and finally detected by enhanced chemiluminescence (ECL Pierce).

For immunoprecipitation, cells were resuspended in triple detergent or lysis buffer and incubated with the NS5A polyclonal antibody on a rotary shaker at 4 °C overnight. Resulting complexes were precipitated for 2 h at 4 °C with 50 μl of 10 % protein A-Sepharose beads (Sigma). Immunoprecipitations for the Grb2 interaction were performed by 5-fold dilution of the cell lysates (0.5 mg) in dilution buffer without Triton X-100 and overnight incubation

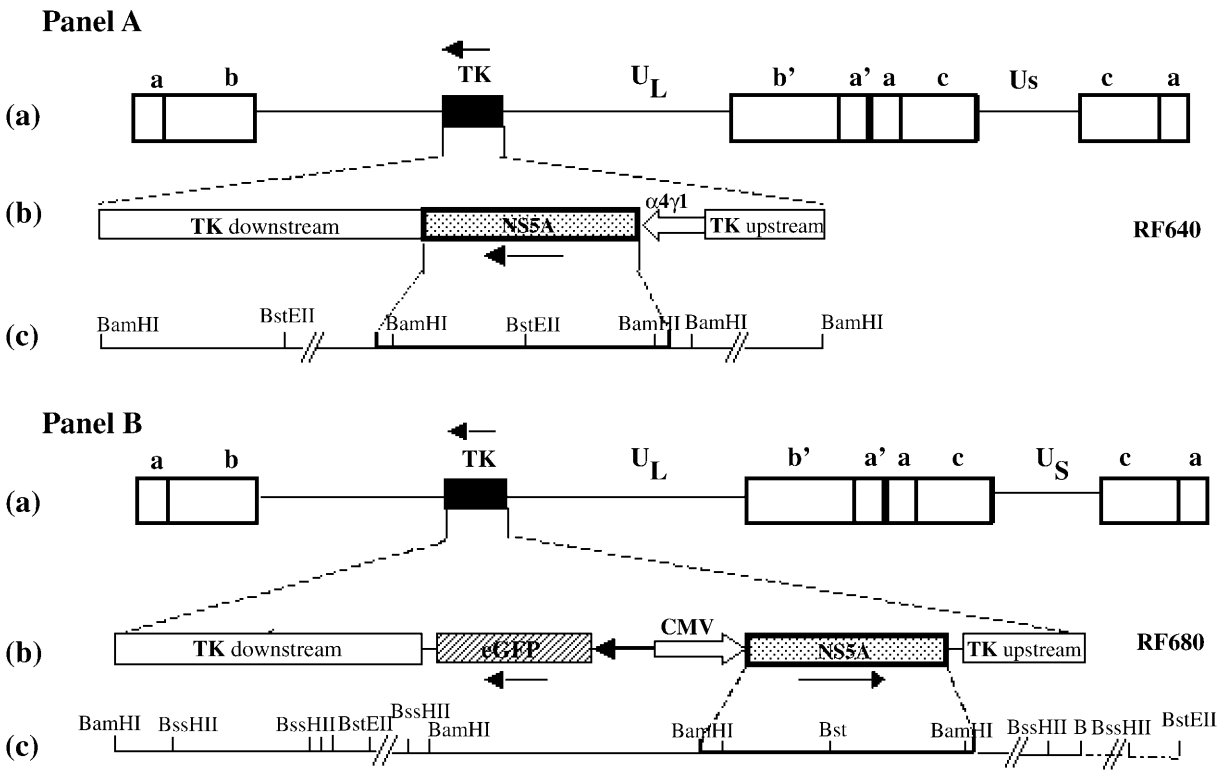

Fig. 1. The DNA sequence arrangement and structure of the HSV-1(F) genome and the recombinant viruses RF640 and RF680. **A:** (**a**) Schematic representation of the HSV-1(F) genome in the prototype arrangement, showing the unique sequences (lines) flanked by the inverted repeats ab, b′a′, a′c′, and ca (rectangles) and the location in the genome of the TK gene. (**b**) The NS5A coding region is inserted between the TK coding sequences under the control of the α4γ1UL19 chimeric promoter. (**c**) Restriction endonuclease map of the TK chimeric region including the *Hin*dIII-*Hin*dIII fragment from pHPI640 containing the coding sequences of the NS5A gene. **B:** (**a**) HSV-1(F) genome as in panel A. (**b**) A *Pac*I-*Pac*I fragment containing the CMV promoter, the RSV 3′LTR and the eGFP coding region is inserted between the tk upstream and downstream flanking sequences. The NS5A coding region is cloned under the control of the CMV promoter of pHPI669. (**c**) Restriction endonuclease map of the TK chimeric region including the *Hin*dIII-*Hin*dIII fragment from pHPI640 containing the coding sequences of the NS5A and GFP genes

with 8 μl of the sc-255 Grb2 antibody (Santa Cruz Biotechnology, Inc.) followed by the addition of 50 μl of protein A-Sepharose. After 2 h incubation, immune complexes were pelleted (14000 × g for 1 min) and washed three times with ice-cold PBS. Electrophoresis and immunoblot analysis were done as above described. Labelling experiments were done by pulse-labeled Vero cells for 20 min with 50 μCi ^{35}S-methionine (Amersham) at 10, 15 and 22 h post-infection. The cells were harvested immediately after the pulse and the labeled polypeptides were analysed by immunoprecipitation.

Determination of the ERK1/2 activation

For analysis of the ERK1/2 phosphorylation equal amounts of protein from each sample (80 μg) were subjected to SDS/PAGE electrophoresis and immunoblotting. Nitrocellulose filters were probed with the E10 monoclonal antibody (New England, Biolabs), specific for the dually phosphorylated (Thr202/Tyr204) form of ERK1/2. In order to quantitatively compare the immunoblot, equal total protein amounts were loaded on the gels. Furthermore the same membranes were stripped and reprobed with the sc-94 ERK1 antibody (Santa Cruz Biotechnology, Inc.), which reacts with ERK1(p44) and, to a lesser extent, with ERK2(p42).

Results and discussion

Construction of HSV-NS5A recombinant viruses

Because the HCV NS5A protein is known to modulate expression of viral promoters, two replication-competent HSV-NS5A recombinant viruses were made. Both viruses were designed to contain the HCV NS5A gene into the TK locus of the HSV-1(F) genome so that its expression would be under the control of an HSV chimeric α4γ1UL19 promoter for the RF640 or the heterologous immediate early (IE) CMV promoter for the RF680 recombinant viruses. TK mutant progeny viruses were plaque purified and viral DNAs were analyzed for the presence of the NS5A expression-casette by Southern blotting. Viral DNA prepared from Vero cells infected with HSV-1(F) or the recombinant viruses were digested with *Bam*HI or *Bam*HI-*Bst*EII for RF640 and *Bss*HI or *Bam*HI-*Bst*EII for RF680 recombinant viruses. Restriction-digested fragments were electrophoretically separated, transferred and probed with pRB103 plasmid containing the TK coding sequences (*Bam*HI-Q fragment) [30] and pHPI611 plasmid containing the *Hin*dIII NS5A fragment (Fig. 2).

Hybridization patterns of the *Bam*HI digests of DNA from RF640 when probed with ^{32}P-labeled TK probe revealed as expected the presence of a 2.7 kb fragment derived from the TK (UL23) downstream and the carboxyterminus of NS5A and 1 kb from the γ1 leader to the upstream TK (lane 3). Parental virus with an intact *Bam*HI Q fragment yielded a single 3.5 kb band upon digestion with *Bam*HI (lane 1). The same fragments hybridized with ^{32}P-labeled NS5A probe yielded a single 1.3 kb fragment which contains the NS5A sequence (lane 7). The hybridization pattern of the *Bam*HI-*Bst*EII digest of RF640 hybridized with ^{32}P-labeled TK probe yielded a 1.7 kb fragment from the carboxyterminus of NS5A to TK downstream, as well as two fragments of 1 kb from TK downstream and 0.9 kb from the TK upstream to the α4γ1 (lane 4) while the parental virus yielded

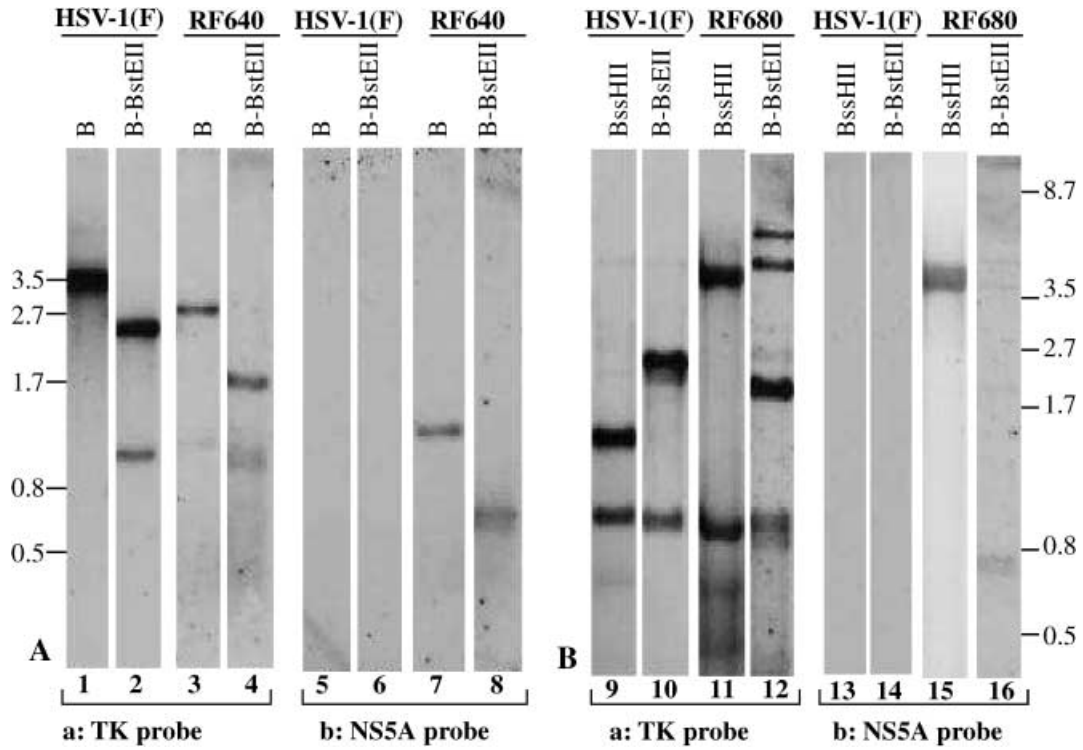

Fig. 2. A: Autoradiograms of electrophoretically separated *Bam*HI (*B*) and *Bam*HI-*Bst*EII restriction enzyme digests of recombinant RF640 and HSV-1(F) viral DNAs, hybridized to: (**a**) the ^{32}P-labeled TK probe (pRB103) [lanes 1–4] and (**b**) the ^{32}P-labeled NS5A probe (pHPI611) [lanes 5–8]. **B:** Autoradiograms of electrophoretically separated *Bss*HII and *Bam*HI-*Bst*EII restriction enzyme digests of recombinant RF680 and HSV-1(F) viral DNAs, hybridized to: (**a**) the ^{32}P-labeled TK probe (pRB103) [lanes 9–12] and (**b**) the ^{32}P-labeled NS5A probe (pHPI611) [lanes 13–16]

a 2.5 kb and 1 kb fragments from the TK sequences (lane 2). The same digest with NS5A probe for the RF640 yielded two fragments of 0.6 and 0.7 kb derived from NS5A sequences, because of a *Bst*EII site approximately in the middle of the NS5A region (lane 8).

We used the same procedure for viral DNAs of the RF680 recombinant virus for the presence of the NS5A expression cassette. The hybridization pattern of RF680 after digestion with *Bam*HI-*Bst*EII and hybridisation with TK yielded 1 kb fragment from TK downstream, 1.75 kb from TK downstream to GFP and a 0.537 kb fragment from the TK upstream to the carboxyterminus of NS5A (lane 12). When the same fragments were hybridized with NS5A two bands of 0.7 kb and 0.6 kb derived from NS5A sequence were detected (lane 16). The parental virus yielded two fragments of 2.5 kb and 1 kb when hybridized with TK (lane 10). Furthermore, the *Bss*HII digest yielded a 5.5 kb band derived from the TK upstream to the end of GFP gene, a 0.758 kb fragment from the TK upstream and 1.1 kb and 0.452 kb from the TK downstream sequences, when probed with TK

sequences (lane 11). The same fragments hybridized with NS5A probe resulted in a 5.5 kb band (lane 15). The parental HSV-1(F) virus yielded the expected bands (lane 9).

Expression of HCV NS5A protein by HSV-NS5A recombinant viruses in tissue culture

To analyze the ability of the RF640 and RF680 recombinant viruses to support the expression of the NS5A protein, replicate cultures of Vero cells were either mock infected or infected with 5 PFU of HSV-1(F), RF640 or RF680 per cell. Lysates of cells harvested at 3, 6, 9, 12, 16 and 22 hours after infection were analyzed by Western blotting using a novel anti-NS5A rabbit polyclonal serum produced for this study as described in Materials and methods. As shown in Fig. 3A, the NS5A, anti serum reacted strongly with a protein of an apparent mass of 56–58 kDa, as expected for HCV NS5A protein, only in the RF640 or RF680 infected cells, whereas no specific bands were detected in lysates of mock- or HSV-1(F)-infected cells. Notably, the immunoblotting analysis also revealed multiple bands migrating faster than the authentic NS5A protein in the RF640 or RF680 infected cell lysates (Fig. 3A, parts b, c). Although the nature of these proteins is unknown, it is of interest to note that Satoh et al. [33] recently reported a similar pattern for the NS5A protein in mammalian cells and provided evidence for proteolytic processing of the protein by caspase(s)-like proteases. The NS5A protein expression was detected as early as 6 h post infection and accumulated at high levels at 22 h post-infection by both recombinant viruses. This indicates that the NS5A was efficiently expressed in the context of the HSV-1 genome by both the CMV IE or the HSV-1 $\alpha 4 \gamma 1$UL19 promoters (Fig. 3).

To further assess the efficiency of the NS5A expression a kinetic study was conducted. Vero cells infected with either the RF640 or the RF680 were pulse-labeled with ^{35}S-methionine for 20 minutes at various times post infection and lysates were immunoprecipitated with the anti-NS5A antibody. As shown in Fig. 3B, both viruses induced similar levels of NS5A protein throughout infection, indicating a similar behavior for the two viral promoters (Fig. 3B part b). To confirm the identity of the NS5A protein band, the same nitrocellulose was also probed with anti-NS5A antibody (Fig. 3B part c). It should be noted that the strong protein band, which migrated with slower mobility than the NS5A band (Fig. 3B), is the HSV-1 glycoprotein gE (gE), known to be immunoprecipitated by any rabbit antiserum due to its Fc binding activity [24]. This is demonstrated in Fig. 3B, which shows ^{35}S-immunoprecipitated polypeptides from HSV-1(F) and RF640 infected Vero cells at 12 h post infection. For the studies described in the rest of this manuscript only the RF640 recombinant virus was used.

NS5A interacts with Grb2 adaptor protein in RF640 infected cells and perturbs the ERK1/2 phosphorylation

It has been previously reported that the NS5A protein perturbs the MAPK/ERK signaling pathway when expressed in HeLa by a NS5A vaccinia recombinant virus

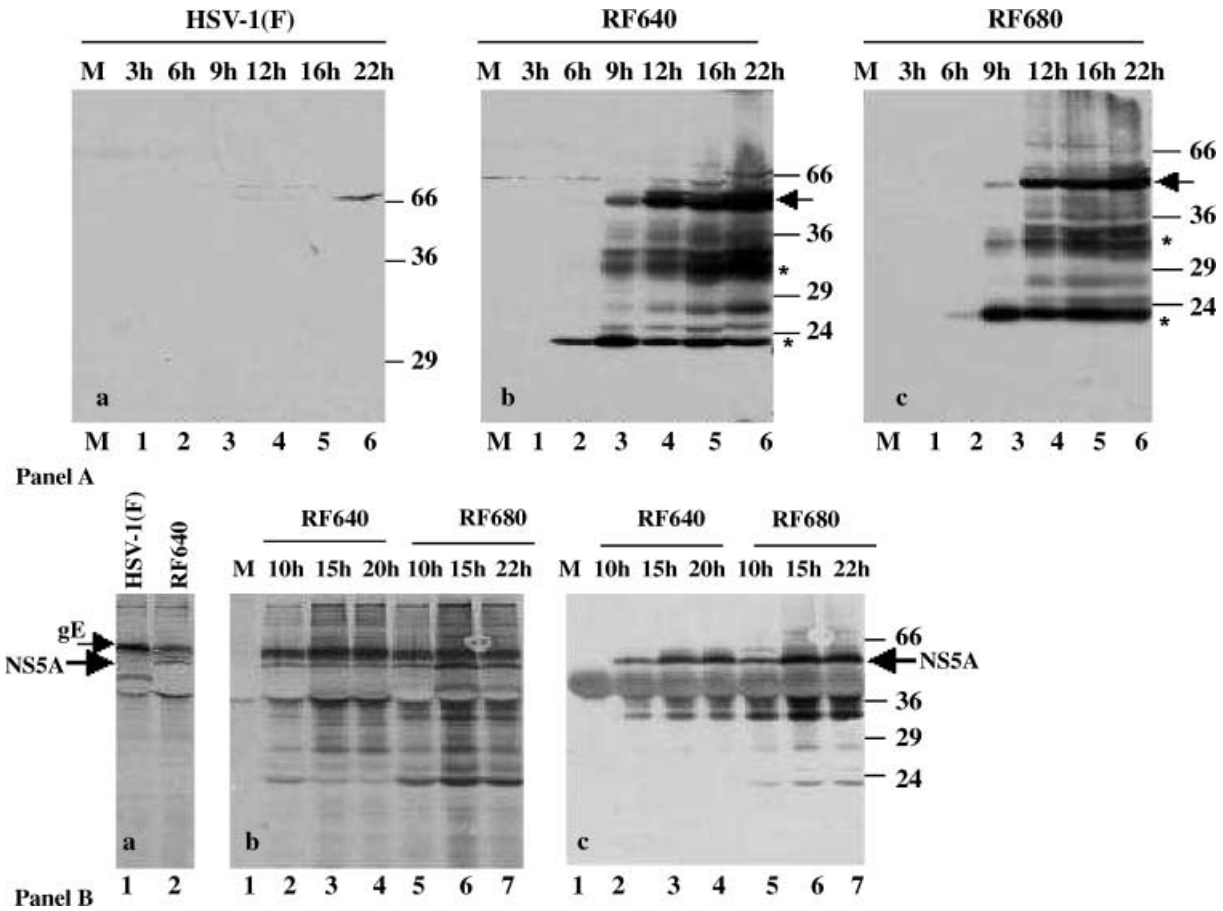

Fig. 3. Analysis of NS5A protein expression in Vero cells. **A:** Cells were separately infected with HSV-1(F) (**a**), RF640 (**b**), RF680 (**c**) viruses and harvested at 3, 6, 9, 16, 22 h (lanes *1, 2, 3, 4, 5, 6* respectively) post-infection, electrophoresed and immunoblotted with NS5A antiserum. *M*: mock infected cells. Arrow indicates the 56–58 kDa NS5A, (*) indicates the major small faster migrating forms of the NS5A protein. **B:** (**a**) Immunoprecipitation of total cell lysates from Vero cells infected with HSV-1(F) and RF640 viruses labeled with ^{35}S-methionine for 2 h at 12 h post-infection. (**b**) Mock (*M*), RF640 or RF680 infected cells were pulse labeled with ^{35}S-methionine as indicated in Materials and methods. Cells were harvested immediately after the pulse and analyzed by immunoprecipitation using the NS5A antiserum. The thin arrow indicates the presence of the HSV-1 encoded gE protein in the immunoprecipitates. (**c**) Western blot of the same NC membrane as in (b) to see the total amount of NS5A expressed at the selected time points

and to a lesser extent in stably transfected cells. In the next step we sought to explore the possibility of using the HSV-NS5A recombinant viruses as an alternative system to generalize and further analyze this phenomenon. To this end cells were infected with RF640 or control viruses and the ability of the NS5A to interact with the Grb2 protein was investigated by co-immunoprecipitation experiments. Furthermore, since the physiological responses associated with certain MAPK pathways may differ depending on the cell type [21, 34], in addition to HeLa,

NIH 3T3 and liver cells were used. Cells were infected with RF640 or HSV-1(F) parental virus and at 12 or 15 h after infection cell lysates were immunoprecipitated with anti-Grb2 antibody. Immunoblotting of the Grb2 immunoprecipitated material with anti-NS5A antibody revealed the NS5A protein in cells infected with the RF640 but not with HSV-1(F) or HSV-1(F)Δ305 (data not shown) control viruses, suggesting the formation of a NS5A-Grb2 complex (Fig. 4a). These data are in agreement with the previous studies [39] and indicate that the NS5A protein has the ability to interact with the Grb2 adaptor protein in the environment of HeLa, NIH 3T3, and WRL68 liver cells infected by an HSV-NS5A recombinant virus. Notably, the level of NS5A protein that was co-immunoprecipitated with Grb2 varied in the different cell lines used in this study most likely reflecting different levels of NS5A expression in different cellular environments. (compare Figs. 4 and 5). Panel b for each cell type is an immunoblot for NS5A of total cell lysates demonstrating the differences in the amount of NS5A expression in each cell line.

Next the effect of the NS5A protein on the ERK1/2 (p44/42) activation was tested by western blotting with an antibody specific for the phosphorylated forms of ERK1/2. Infections were performed in the presence of 1 % FCS in cells that had been previously starved for 24 h in 0.1 % FCS. In the RF640 infected cells a significant decrease of the ERK1/2 tyrosine phosphorylated forms was observed late post-infection as compared to ERK1/2 phosphorylation in mock or HSV-1(F) infected cells (Fig. 5). Immunoblot analysis of the same membrane with the total ERK1/2 specific antiserum revealed that equivalent levels of MAPK protein were present in the immunoprecipitates. The results were similar regardless of the cell line used. Interestingly, in WRL68 and particularly in HeLa cells the HSV-1(F) infection resulted in an increase of the ERK1/2 phosphorylation at 10 and 18 h post infection respectively, whereas no significant changes at the level of the phosphorylated ERK1/2 were observed in HSV-1(F) NIH3T3 and HepG2 (data not shown) infected cells.

Taken together, these results confirm the interaction of the NS5A protein with the MAPK/ERK1/2 signaling pathway in HeLa cells and indicate that the suppression of the ERK1/2 phosphorylation is independent of the cell line or the experimental procedure used. ERK activation is closely associated with cell proliferation and inhibition of apoptosis [4]. Activated ERK is known to translocate into the nucleus [21] and modulate the expression and function of key genes in the cell cycle (induce entry to S phase) or apoptosis. On the other hand, NS5A is known to have an antiapoptotic function and oncogenic properties [2, 8, 13, 15, 40]. Thus, the inhibition of the ERK1/2 activation by NS5A may have a biological function other than in cell proliferation or apoptosis. Interestingly, recent studies support a cross talk between the JAK-STAT pathway and the ERK pathway [22]. It was, therefore, suggested that the NS5A-mediated inhibition of the ERK might be involved in the HCV resistance to IFN by the downregulation of IFN-induced gene expression [18, 25, 38]. Notably, the observed inhibition of the ERK phosphorylation/activation occurs in the absence of EGF or mitogenic stimulation factor. Furthermore, in contrast with other viruses such as vaccinia virus, it is unclear whether HSV-1 has a protein able to trigger mitogenic Ras/MEK/MAPK

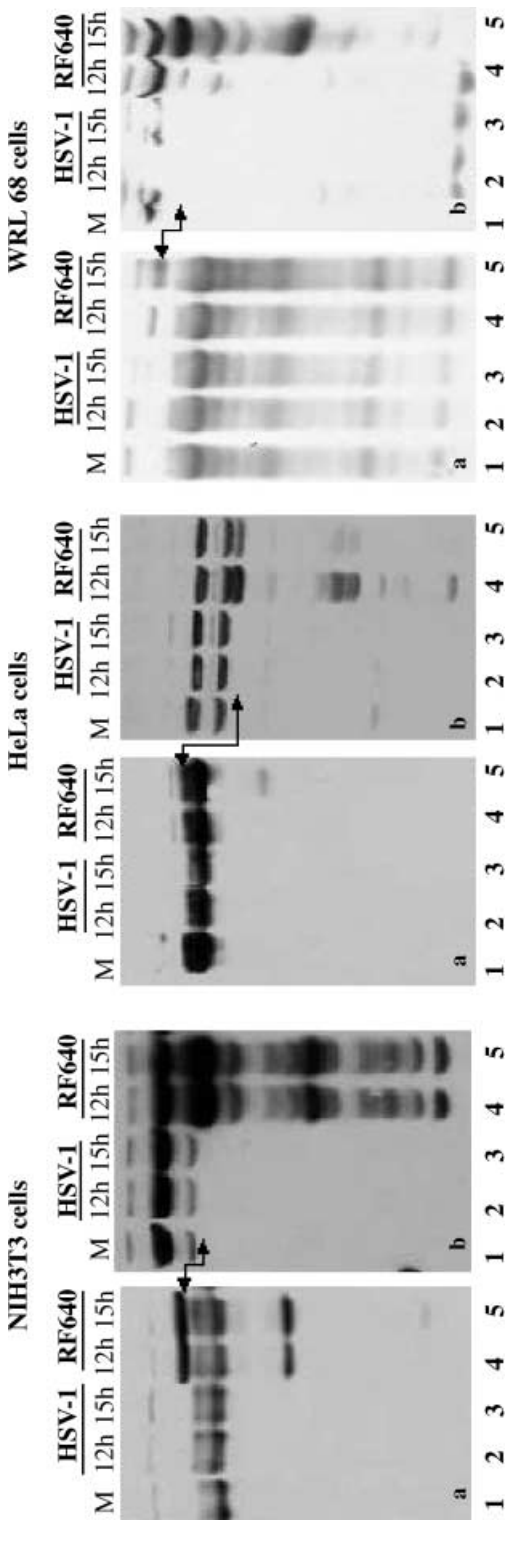

Fig. 4. Grb2-NS5A interaction in various cell lines infected with NS5A-expressing recombinant herpes simplex viruses. (**a**) Immunoprecipitation of total cell lysates from NIH3T3, HeLa cells and WRL68 cells infected with HSV-1(F) and RF640 recombinant viruses. Cells were harvested at 12 (lanes 2, 4) or 15h post-infection (lanes 3, 5) and analyzed by immunoprecipitation using the Grb2 polyclonal antiserum (Santa Cruz, Biotechnology). The immunoprecipitates were electrophoretically separated on a 12 % polyacrylamide gel, transferred on nitrocellulose and immunoblotted with the NS5A antiserum. The arrow indicates the presence of NS5A after the immunoprecipitation with Grb2. (**b**) Total cell lysates immunoblotted for the presence of NS5A protein

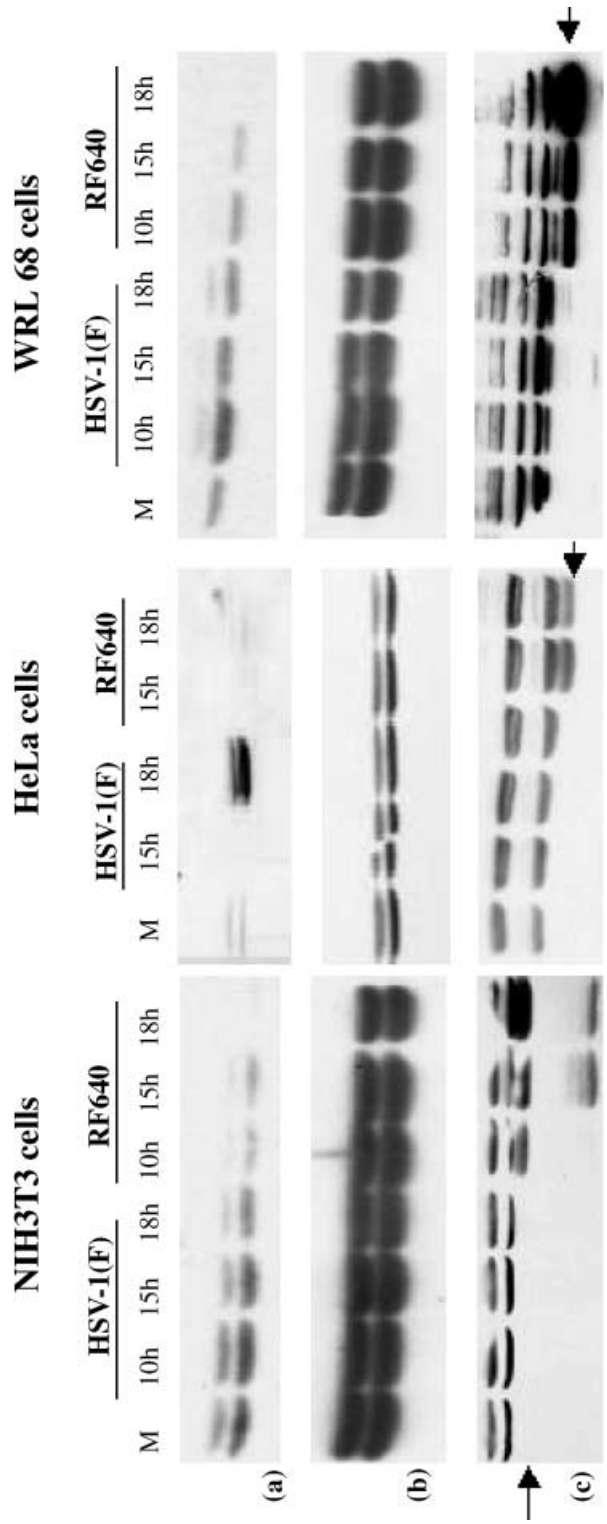

Fig. 5. (**a**) ERK1/2 phosphorylation in various cell lines infected with RF640 virus expressing NS5A or control HSV-1(F) virus at different times post infection. Total cell lysates were electrophoresed and analyzed by immunoblot for the presence of: (**a**) the phosphorylated forms of ERK p44/42 (Thr 202/Thr 204) using the E10 monoclonal antibody (New England Biolabs) (**b**) total p44/42 ERK proteins using the sc-94 antibody (New England Biolabs) (**c**) NS5A expression using the specific NS5A antiserum

pathway [20, 36]. In fact, a recent report supports the lack of ERK activation during the first 10 h post-infection [23]. However, in our studies under certain conditions, an increase in the phosphorylation status of ERK1/2 (p44/42) was detected at late hours post infection, suggesting a possible exploitation of the MAPKs pathway by the HSV-1 virus in order to promote late viral events.

Finally, it is interesting to note that HCV NS5A and core proteins appear to have, opposite roles in the mitogenic signaling cascade, as core was recently shown to induce ERK1/2 activation [6, 35]. In addition, NS5A and core may act antagonistically in cells undergoing apoptosis or may antagonize each other or cooperate in supporting cell proliferation. Thus, an intriguing question is to understand how the NS5A- and core-mediated interaction with the signaling pathway is regulated within the HCV infected cell.

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