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NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling

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ABSTRACT **Although hepatitis C virus (HCV) infection is an emerging global epidemic causing severe liver disorders, the molecular mechanisms of HCV pathogenesis remain elusive. The NS5A nonstructural protein of HCV contains several proline-rich sequences consistent with Src homology (SH) 3-binding sites found in cellular signaling molecules. Here, we demonstrate that NS5A specifically bound to growth factor receptor-bound protein 2 (Grb2) adaptor protein. Immunoblot analysis of anti-Grb2 immune complexes derived from HeLa S3 cells infected with a recombinant vaccinia virus (VV) expressing NS5A revealed an interaction between NS5A and Grb2 *in vivo*. An inactivating point mutation in the N-terminal SH3 domain, but not in the C-terminal SH3 domain, of Grb2 displayed significant diminished binding to NS5A. However, the same mutation in both SH3 regions completely abrogated Grb2 binding to NS5A, implying that the two SH3 domains bind in cooperative fashion to NS5A. Further, mutational analysis of NS5A assigned the SH3-binding region to a proline-rich motif that is highly conserved among HCV genotypes. Importantly, phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) was inhibited in HeLa S3 cells infected with NS5A-expressing recombinant VV but not recombinant VV control. Additionally, HeLa cells stably expressing NS5A were refractory to ERK1/2 phosphorylation induced by exogenous epidermal growth factor. Moreover, the coupling of NS5A to Grb2 in these cells was induced by epidermal growth factor stimulation. Therefore, NS5A may function to perturb Grb2-mediated signaling pathways by selectively targeting the adaptor. These findings highlight a viral interceptor of cellular signaling with potential implications for HCV pathogenesis.**

Hepatitis C virus (HCV) is the major etiologic agent of non-A non-B hepatitis worldwide (1). An estimated 2% of the world population is clinically affected by HCV; more than half of these cases progress into chronic infection, often developing liver cirrhosis (2). Currently the leading cause of adult liver transplantation in the United States, HCV infection has also been epidemiologically linked to the development of hepatocellular carcinoma. However, research on HCV replication and pathogenesis, as well as the development of therapeutic strategies, has been severely hindered because of the lack of a reliable cell culture system and an adequate animal model for HCV infection and propagation (3–5). Nevertheless, HCV-encoded proteins have been identified by *in vitro* and *ex vivo* systems expressing cloned viral cDNA (6–9). Recent research efforts have thus focused on the properties and functions of individual HCV gene products in the interest of unraveling the mechanisms of viral pathogenicity.

The largest ORF in the HCV genome produces a polyprotein precursor that is cleaved by host and viral proteases, resulting in at least nine mature viral structural and nonstructural proteins (10). The latter include a 56- to 58-kDa phosphoprotein NS5A. NS5A recently became a subject of intense investigation when a cluster of amino acid mutations within a discrete region of the protein (amino acids 237–276) from the HCV genotype 1b (HCV-1b) was correlated with increased resistance to IFN (11–14). This site, termed the "interferon sensitivity determining region" (ISDR), is thought to mediate the resistance of HCV-1b to IFN-α therapy, the only currently available treatment for HCV infection. Although the exact molecular basis by which NS5A might mediate IFN resistance is not clear, we have recently demonstrated that NS5A is a potent inhibitor of the IFN-induced double-stranded RNA-activated protein kinase (PKR), a key mediator of the host IFN-induced antiviral and antiproliferative response (15). Subsequent studies show that mutations within the ISDR that were observed in clinically IFN-sensitive HCV-1b strains disrupt the ability of NS5A to interact with and repress PKR activity, supporting the notion that NS5A mediates HCV resistance to IFN through the down-regulation of PKR (16). Interestingly, there is also evidence that the C-terminal domain of NS5A, including the ISDR, contains transcriptional activity, suggesting that NS5A might function as a viral transactivator (17–18). Apparently, clinically relevant mutations within the ISDR also influence the transcriptional function of NS5A (19), but the relation between transcriptional activity and IFN sensitivity and/or viral replication is unclear.

A potential clue that NS5A may also perturb other cellular signaling functions is the presence of several proline-rich (PXXP) sequences, which are highly conserved among HCV species (Fig. 1). Such PXXP motifs bind to the Src homology (SH) 3 domain found in a diverse group of signal-transducing molecules (20). Another well characterized signaling modular domain that mediates selective protein–protein interaction is SH2 domain, which binds to phosphotyrosine-containing se-

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Abbreviations: PKR, RNA-activated protein kinase; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; Grb2, growth factor receptor-bound protein 2; GST, gluthathione-*S*-transferase; HCV, hepatitis C virus; SH, Src homology; Sos; Son of sevenless; VV, vaccinia virus; ISDR, interferon sensitivity determining region; MPA, mycophenolic acid; SD, synthetic defined; AD, activation domain; BD, binding domain; −His, lacking His.

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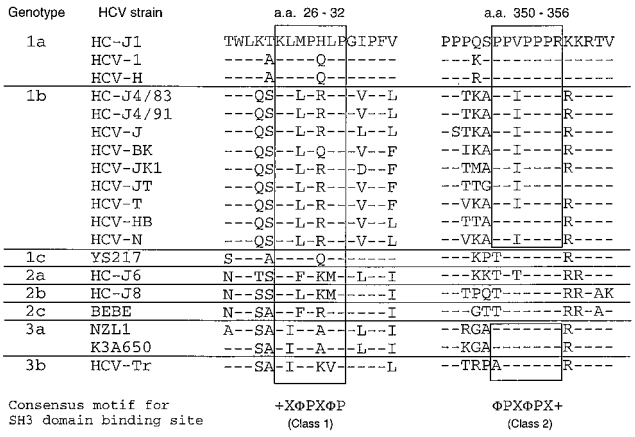

FIG. 1. Sequence of the proline-rich SH3-binding motifs in NS5A. Amino acid (a.a.) sequence alignment of NS5A from various HCV species revealed conservation (residues enclosed in boxes) of both class I and II proline-rich motifs (26). *P* denotes the highly conserved proline residue. + tends to be basic residue; Φ, hydrophobic residue; X, any residue.

quences. SH2/SH3-related signaling proteins are generally categorized into two classes: proteins that contain distinct enzymatic activities (e.g., cytoplasmic protein tyrosine kinases Abl, Csk, Src, and Syk) and nonenzymatic adaptor proteins consisting mostly of SH2 and SH3 domains (e.g., Crk2, Grb2, Nck, and Shc). These SH2/SH3 signaling enzymes and adaptors form distinct multiprotein complexes that allow extracellular signals to transmit to downstream effectors that regulate various cellular responses.

In the present study, we demonstrate that HCV NS5A specifically and directly associated with growth factor receptor-bound protein 2 (Grb2) in a SH3 ligand/domain-dependent manner. Further, HeLa cells stably expressing NS5A or HeLa S3 cells infected with a recombinant vaccinia virus (VV) expressing NS5A were refractory to epidermal growth factor (EGF) or a virally induced phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2), respectively. Our results suggest that NS5A may bind to Grb2 to interfere with normal signal transduction pathways of the infected cell, alluding to a potential mechanism for HCV pathogenesis.

MATERIALS AND METHODS

Plasmid Construction. All molecular cloning techniques were performed as described (21). DNA restriction endonucleases, T4 DNA ligase (New England Biolabs), and Pfu polymerase (Stratagene) were used as recommended by the suppliers. Construction of pGEX2T-NS5A and pAD-NS5A was described (15). To create pAD-NS5A 1–81 and 1–236, fragments encoding NS5A amino acids 1–81 and 1–236 were released from the corresponding pCR2.1 constructs (16) by digestion with *Nde*I-*Pst*I and *Nde*I-*Sal*I, respectively, and ligated into the appropriate sites of pGAD425 (15). pAD-NS5A 148–304 was generated by inserting an *Eco*RI-*Pst*I fragment from pBD-NS5A 148–304 (16) into the corresponding sites of pGAD425. Fragments encoding NS5A amino acids 237–447 and 277–447 were amplified by PCR by using pBD-NS5A and the appropriate restriction site-linked primers and cloned into the *Eco*RI-*Pst*I sites of pGAD425 to yield pAD-NS5A 237–447 and 277–447. pAD-NS5A PXXP3 was constructed by replacing a 1,042-bp *Apa*I-*Aat*II fragment with a PCR-generated fragment containing the appropriate point mutations (see Fig. 4).

Construction, Expression, and Purification of Recombinant NS5A. A DNA fragment encoding full-length NS5A (HCV strain 1b) was generated by PCR by using oligonucleotides containing suitable restriction sites. The resultant DNA fragment was entirely sequenced and introduced into the *Bam*HI/*Xba*I cloning sites of the plasmid pFASTBAC1 (GIBCO/BRL). Isolation of recombinant baculovirus DNA (Bacmid) containing the NS5A gene under the transcriptional control of the polyhedrin promoter and transfection of Sf9 cells (Invitrogen) were performed as described by the manufacturer (BAC-TO-BAC baculovirus expression system, GIBCO/BRL). For protein expression, Sf9 cells were infected with the recombinant baculovirus at a density of 2×10^6 cells/ml and a ratio of about five virus particles per cell. After 4 days of infection at 27°C, cells were collected and the protein was purified by conventional chromatographic methods. Details of the purification procedure will be described elsewhere.

Construction of NS5A-Expressing Recombinant VV. *In vivo* recombination and selection of recombinants were performed as previously described (22). NS5A was cloned into the VV insertion plasmid, pMPE3LΔGPT, by using *Hin*dIII digestion. This plasmid has an *ecogpt* gene, which enables selection of transfected cells by treatment with mycophenolic acid (MPA) and flanking arms of E3L on either side of the multiple cloning sites. *In vivo* recombination was performed in RK-13 cells by infecting VVΔE3L (VV in which the E3L genes have been replaced with β-galactosidase gene of *Escherichia coli*) at a multiplicity of infection of 10 plaque-forming units/cells. The cells were then infected with pMPE3LΔGPT NS5A by using Lipofectace (GIBCO) according to the manufacturer's instructions. After 36 h, cells were harvested by centrifugation, and virus released from these cells by three rounds of freezing and thawing was used to infect confluent RK-13 cells that have been pretreated with MPA. Plaques formed by MPA-resistant viruses were picked and replaqued twice in the presence of MPA. This virus was used to infect cells in the absence of MPA to allow resolution of the inserted plasmid. Recombinant virus having the β-galactosidase gene in the E3L locus replaced by NS5A was identified by 5-bromo-4-chloro-3-indolyl β-D-galactoside staining and confirmed by PCR and sequencing.

Cell Lines, Virus Infection, and Lysate Preparation. Tet-Off Gene Expression System and HeLa Tet-Off cells (CLONTECH) were used to establish tetracycline-regulated NS5A stable expressing stable cell lines. Details of the generation of the cell lines will be described elsewhere. Cells were maintained as described by the manufacturer. For EGF stimulation, medium containing tetracycline (Tet) was removed at 12 h postseeding NS5A-expressing cells and replaced with fresh medium lacking Tet. After 12 h, the cells were cultured in 0.1% FBS-containing medium lacking Tet for another 12 h to induce starvation before EGF treatment at 20 ng/ml for 2 min. Untreated and EGF-treated cells were lysed 2 or 6 h after EGF stimulation in 300 μl of low-salt lysis buffer [20 mM Tris (pH 7.5)/50 mM KCl 50 mM NaCl/0.5% Triton X-100/1 mM EDTA/20% glycerol/1 mM PMSF/1 mM DTT/10 μg/ml aprotinin/1 μM okadaic acid/1 mM Na_3VO_4].

HeLa S3 cells (ATCC CCL-2.2) were maintained in DMEM supplemented with 10% FBS/100 units/ml penicillin/streptomycin/2 mM L-glutamine at 37°C in 5% CO_2. Cell monolayers (70% confluent) were infected with recombinant VV (Vp1080) or recombinant VV expressing NS5A (VpNS5A) as previously described (22). At 2, 4, and 6 h postinfection, the cells were rinsed twice with ice-cold Hanks' solution and lysed with ice-cold disruption buffer [1% Triton X-100/50 mM KCl/10 mM Tris (pH 7.5)/1 mM DTT/2 mM $MgCl_2$/0.2 mM PMSF/100 μg/ml Aprotinin]. Lysates were clarified by centrifugation at 14,000 × *g* for 2 min at 4°C. Protein concentrations of lysates were determined by using the Bio-Rad detergent-compatible protein assay.

Precipitation and Immunoblotting. Glutathione-*S*-transferase (GST)-Grb2 and mutant constructs were previously described (23). GST fusion proteins were purified and coprecipitation assay was performed as described (24). Whole-cell lysates (0.5 mg) or purified recombinant NS5A (5 ng) were

incubated with GST fusion protein (100 ng or 500 ng) bound to glutathione-agarose beads for 4 h at 4°C while gently rotating. The glutathione-agarose resin was pelleted at 14,000 × *g* for 2 min and washed three times with ice-cold PBS. Precipitated proteins were subjected to 12% SDS/PAGE, followed by electroblotting to nitrocellulose filters (Schleicher & Schuell). Filters were probed with an NS5A-specific antibody, and detection was performed by using enhanced chemiluminescence (ECL, Amersham).

Coimmunoprecipitation and Immunoblot Analysis. Immunoprecipitations were performed by dilution of the cell lysates (0.5 mg) 5-fold in lysis buffer without Triton X-100 and incubation with 2 μg of Grb2 antibody (Transduction Laboratories, Lexington, KY) for 16 h at 4°C, and 50 μl of 50% protein G-agarose (Boehringer Mannheim) was added. After 1 h at 4°C, the immune complexes were pelleted at 14,000 × *g* for 2 min and washed three times with ice-cold PBS. Immunoblot analyses were performed as described above.

Yeast Two-Hybrid System. Yeast strain Hf7c (CLONTECH), which carries the *HIS3* reporter fused to a GAL1 promoter sequence, was used to assay for protein–protein interactions as described (24). For selection of double transformants and determination of transformation efficiency, transformants were plated on solid synthetic defined (SD) medium (Bio101) lacking tryptophan (Trp) and leucine (Leu). To select for His^+ colonies, transformants were grown on SD plates lacking Trp and Leu for 3 days at 30°C and then streaked onto SD plates lacking Trp, Leu, and histidine (His) and allowed to grow for 3 days at 30°C, after which the plates were scored for growth.

Determination of ERK1/2 Phosphorylation. For analysis of ERK1/2 phosphorylation, equal amounts of protein (50 μg) from each sample were subjected to SDS/PAGE and electroblotting. Filters were probed with an antibody specific to the dually phosphorylated (Thr-202/Tyr-204) activated form of ERK1/2 (New England Biolabs). The blots were stripped and reprobed with an ERK1 antibody (Santa Cruz Biotechnology) that recognizes ERK1, and to a lesser extent, ERK2 to determine the amount of expressed proteins.

RESULTS

NS5A Associates with Grb2 Adaptor *in Vitro*. NS5A contains 11% prolines including several PXXP regions with striking similarities to SH3-binding motifs. Sequence homology analysis showed that two of these PXXP motifs of NS5A are highly conserved among several HCV genotypes (Fig. 1). The first domain, which is located at position 26–32 of NS5A, contains the consensus motif for class I SH3-binding site (25). The second PXXP region at position 350–356 of NS5A resembles a class II SH3-binding site. Because similar PXXP motifs, including those from viral proteins, bind to signaling molecules, we investigated whether NS5A could bind to any SH3-related molecule. We used GST recombinant protein, fused to a panel of SH2/SH3 domains from enzymatic (Abl or Src) and adapter proteins (Crk, Grb2, or Nck), as an affinity matrix to precipitate lysates from a HeLa cell line stably expressing NS5A (Fig. 2*A*). The precipitated proteins were analyzed by Western blotting by using an anti-NS5A antibody. GST-Grb2 (Fig. 2*A*, lanes 5 and 6), but not GST or other GST fusions, bound NS5A in a concentration-dependent fashion. Several other nonsignaling SH2/SH3-related proteins were also examined, but none demonstrated an interaction with NS5A (data not shown). The observed doublet band might represent basally and hyperphosphorylated forms of NS5A (26–28) or might be caused by other posttranslational modifications. Western blot analysis by using an anti-GST antibody indicated that all GST fusions were expressed efficiently (Fig.2*A Lower*). To determine whether the interaction is direct, we repeated the precipitation/immunoblotting experiment using highly purified recombinant NS5A proteins expressed from a baculovirus system. The results were consistent with the notion that NS5A specifically and directly interacts with Grb2 (Fig. 2*B*). Furthermore, the NS5A-Grb2 interaction was confirmed by reciprocal experiment by using a GST-NS5A fusion to precipitate endogenous Grb2 from a crude HeLa cell lysate (Fig. 2*C*).

Cooperative Effects of SH3 Domains in Grb2–NS5A Interaction. To gain insight into the molecular basis underlying NS5A–Grb2 interaction, we asked which SH3 domain of Grb2 is responsible for NS5A binding by using the yeast two-hybrid assay (29). Full-length NS5A and Grb2 proteins or deletion mutants were fused to GAL4 activation domain (AD) and GAL4 DNA-binding domain (BD), respectively (Fig. 3*A Upper*). The constructs were coexpressed in a yeast strain containing *HIS3* reporter gene, and a positive interaction between hybrid proteins was scored by the capacity to grow on medium lacking His (−His). Yeast colonies expressing NS5A together with full-length Grb2, but not the N-terminal (SH3-N) or C-terminal (SH3-C) SH3 domain of Grb2, grew on −His medium (Fig. 3*A Lower*). The binding of AD-NS5A fusion protein to BD-Grb2 fusion protein is specific as it did not bind the BD alone or to other unrelated AD chimeras (Fig. 3*A* and results not shown). Conversely, all cotransformants grew on medium containing His (+His), indicating that the lack of growth on −His medium is not because of toxicity caused by

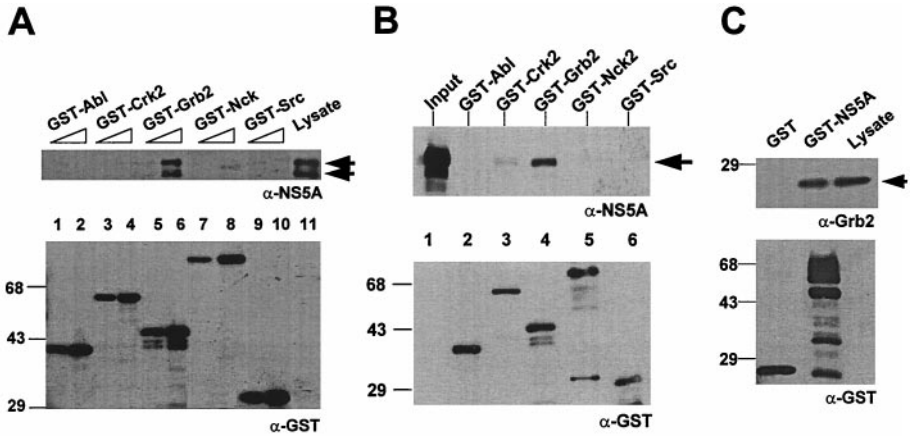

FIG. 2. NS5A specifically and directly interacts with Grb2. Cell lysates from HeLa cells stably expressing NS5A (*A*) or purified recombinant NS5A proteins (*B*) were incubated with GST or the indicated GST fusion protein immobilized on glutathione-agarose beads as described in *Materials and Methods*. Precipitates were washed and resolved by SDS/PAGE, immunoblotted with anti-NS5A (α-NS5A; *Upper*) or anti-GST (α-GST; *Lower*). (*C*) Reciprocal experiments by using GST-NS5A to precipitate endogenous Grb2. The position of NS5A is indicated by the arrow. The molecular size standards are shown in kilodaltons.

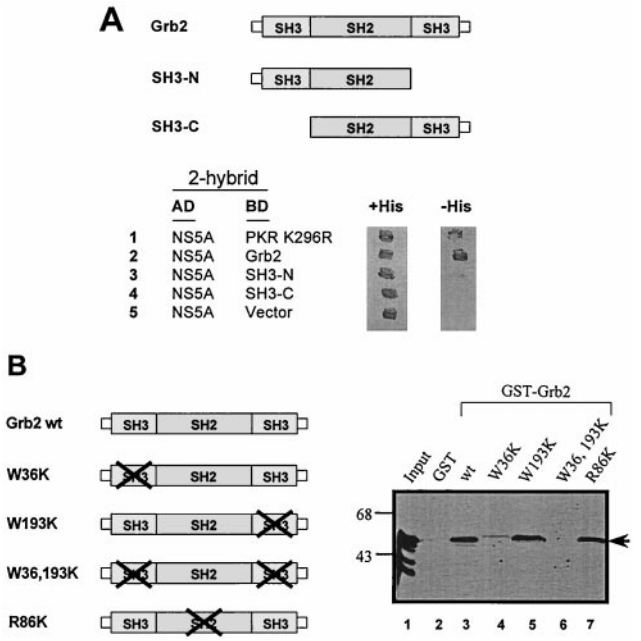

FIG. 3. Localization in Grb2 of the regions of interaction with NS5A. (*A*) Yeast two-hybrid analysis of NS5A and Grb2 interaction. The Hf7c reporter strain was transformed with the indicated plasmids. The interaction between the two hybrid proteins is indicated by the induction of *HIS3* expression (growth on SD medium −His). NS5A-PKR K296R was used as a positive control (15). SH3-N and SH2-C represent the depicted Grb2 deletion mutants (*Upper*). AD denotes fusion with activation domain of GAL4; BD, fusion with DNA-binding domain of GAL4. Double transformants were plated on SD medium lacking Trp and Leu, and then colonies were patched on SD medium lacking Trp/Leu/His. (*B*) *In vitro* binding analysis. Purified recombinant NS5A was incubated with purified GST-Grb2 or GST-Grb2 mutants coupled to glutathione-agarose beads and precipitated as described in Fig. 2. A schematic representation of Grb2 and mutants is shown, but not to scale (*Left*). Mutant domains in which binding activity has been abrogated are indicated by Xs. Precipitated proteins were detected by immunoblotting by using anti-NS5A (*Right*). Amino acid is denoted by standard one-letter code. The position of NS5A is indicated by the arrow.

the overexpression of the relevant hybrid proteins in yeast. Further, all hybrid proteins were expressed to detectable levels in the yeast cells (data not shown). These data imply that the two SH3 domains might bind in a cooperative manner to NS5A.

To more precisely map the regions of Grb2 SH3 domains that mediate NS5A interaction, we used recombinant Grb2 point mutants in the precipitation/immunoblotting assay. NS5A proteins purified from the baculovirus system were incubated with glutathione-agarose beads coated with equivalent amounts of GST, GST-Grb2, or various Grb2 inactive mutants (Fig. 3*B Left*), and the bound proteins were identified by immunoblotting by using an anti-NS5A antibody. A Grb2 mutant containing a point mutation in the N-terminal SH3 domain (W36K), which renders Grb2 unable to bind proline-rich ligands (23), exerted significant diminished binding to NS5A (Fig. 3*B Right*, lane 4). A Grb2 mutant with such mutation in the C-terminal SH3 domain (W193K) retained the ability to effectively bind NS5A (lane 5). The same point mutation in both SH3 regions, however, completely abrogated Grb2 binding to NS5A (lane 6). As a further control, a Grb2 form containing an inactivating point mutation in the SH2 domain (R86K) interacted with NS5A to the level comparable to wild-type Grb2 (lanes 3 and 7). These results demonstrate that the interaction between NS5A and Grb2 is primarily mediated by the N-terminal SH3 domain of Grb2 and that both SH3 domains of Grb2 might cooperate to interact with NS5A.

A Proline-Rich Motif of NS5A Is Required for Grb2 Binding. We next tested which region of the NS5A protein was responsible for interaction with Grb2 using two-hybrid analysis. Because truncated NS5A fragments have been reported to possess transcriptional activity (17–18), we fused all deletion constructs of NS5A to AD (Fig. 4). As summarized in Fig. 4, removing the C-terminal part of NS5A, including the class II PXXP motif, led to complete loss of detectable interaction with Grb2. In contrast, the deletion of the N-terminal fragment of NS5A, which includes the class I PXXP motif, did not reduce Grb2 binding. These observations suggest that the carboxyl class II proline-rich region of NS5A might mediate Grb2 interaction. To further validate that the class II PXXP motif of NS5A was indeed required for Grb2 binding, we mutated three proline residues in the proline motif into alanines (P350A, P351A, and P354A) and tested the ability of the mutant (NS5A PXXP3) to bind Grb2. As expected, the triple-proline mutant was unable to bind Grb2 in the two-hybrid assay. As a control, NS5A PXXP3 retained the capability to bind PKR (15), indicating that the triple point mutant is properly expressed and transported to the nucleus.

Association of NS5A and Grb2 *in Vivo* and Perturbation of Virally Induced ERK1/2 Phosphorylation. To demonstrate NS5A-Grb2 interaction in a cellular context, and perhaps in a more physiologically relevant system (within the context of virus infection), we infected HeLa S3 cells with recombinant VV expressing NS5A before coimmunoprecipitation analysis. Infection of HeLa S3 cells with recombinant VV expressing NS5A, but not recombinant VV control, resulted in an NS5A-Grb2 complex, as demonstrated by the coimmunoprecipitation of NS5A by an anti-Grb2 antibody (Fig. 5*A*; lanes 3 and 4). When a parallel coimmunoprecipitation experiment was performed by using normal mouse serum, neither NS5A (lane 5) nor Grb2 (not shown) was detectable.

Grb2 plays an essential role in coupling the membrane receptor tyrosine kinases to the Ras-ERK pathway (30). Because NS5A interacted with Grb2 in recombinant VV-infected cell, we tested whether NS5A could affect Grb2-mediated signaling to downstream signal transducer molecules during viral infection. Because of the lack of an efficient tissue culture system for HCV infection and replication, we used VV as a surrogate system to test the effect of NS5A on ERK1/2 phosphorylation in a virally

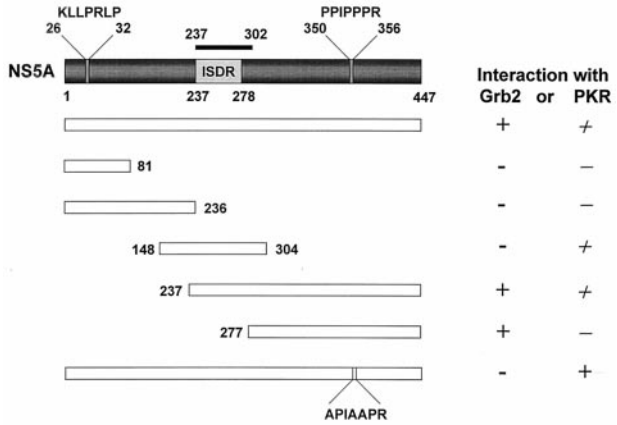

FIG. 4. Mapping of the Grb2-binding site of NS5A by two-hybrid analysis. NS5A deletion and point mutants were constructed in pGAD425, and their interaction with BD-Grb2 was tested as described in Fig. 3*A*. Schematic representation of NS5A mutant proteins is not depicted to scale. Numbers represent terminal aa positions of the NS5A sequence. A summary of Grb2- and PKR-binding activities of NS5A variants is shown on the right. The data indicated here in italics are predicted from previous results (16). Top bar indicates the region required for PKR interaction. ISDR, IFN sensitivity-determining region; −, no growth on SD medium −His; +, growth on the selective medium.

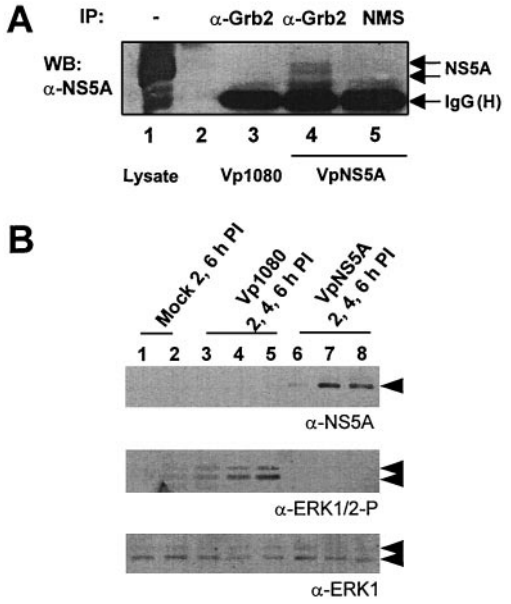

FIG. 5. Physical and functional analysis of NS5A–Grb2 interaction in a virally infected system. (*A*) Anti-NS5A immunoblot comparing total lysate with anti-Grb2 or normal mouse serum (NMS) precipitated protein complexes from HeLa S3 cells infected with recombinant VV expressing NS5A (VpNS5A) or recombinant VV control (Vp1080) at 4 h postinfection. Lane 2 is an empty lane. The position of NS5A is indicated by the arrow. IgG(H) denotes the heavy chain of the mouse Ig. IP, antibody used for immunoprecipitation; WB, antibody for Western blot analysis. (*B*) ERK1/2 phosphorylation in HeLa S3 cells infected by recombinant VV expressing NS5A (VpNS5A) or recombinant VV control (Vp1080) at different time points postinfection. Cell lysates were fractionated with SDS/PAGE and then subjected to immunoblotting by using anti-NS5A [α-NS5A (*A*)], an antibody specific to the dually phosphorylated (Thr-202/Tyr-204) activated form of ERK1/2 [α-ERK1/2-P (*B*)] or anti-ERK1 [α-ERK1 (*C*)]. Position of NS5A and ERK1/2 is indicated by arrow.

infected setting. Furthermore, VV offers the added advantage in that it encodes a viral EGF homolog (31), and viral infection can mimic EGF-induced tyrosine protein phosphorylations (31–32). Consistent with previous results, we found that HeLa S3 cells infected with recombinant VV, but not mock infection (Fig. 5*B Middle*, lanes 1–2), displayed an increase in ERK1/2 activation (lanes 3–5). In contrast, cells infected with a recombinant VV expressing NS5A exhibited a significant inhibition in ERK1/2 phosphorylation (lanes 6–8). Importantly, NS5A was expressed efficiently (Fig. *5B Top*, lanes 6–8) and the protein levels of ERK1/2 accumulated to comparable levels in the cells (*Bottom*).

EGF-Induced NS5A-Grb2 Complex Formation Correlates with Inhibition of ERK1/2 Phosphorylation. To further demonstrate the cellular consequences of the NS5A–Grb2 interaction, we also examined the effect of NS5A expression on EGF-induced ERK1/2 phosphorylation in the stable NS5A-expressing HeLa cell line. In accordance with the assertion that NS5A could affect signal transduction pathways downstream of Grb2, we found that cells stably expressing NS5A were indeed more refractory to EGF-induced ERK1/2 phosphorylation compared with non-NS5A expressing control cells, despite similar protein levels of ERK1/2 (Fig. 6*A*). Inducible expression of NS5A in these cells was confirmed by Western blot analysis (data not shown). It should be pointed out that the inhibitory effect of NS5A on ERK1/2 phosphorylation was not as dramatic as that observed in the VV system (Fig. 5*B*). Experiments are in progress to determine the reasons for these differences. Coimmunoprecipitation experiments were also carried out by using lysates prepared from stable NS5A-expressing HeLa cells to examine the NS5A–Grb2 interaction (without virus involvement) in the presence or absence of exogenous EGF. In addition to confirming NS5A–Grb2 interaction *in vivo*, the results demonstrate that the interaction was inducible by EGF stimulation (Fig. 6*B*, lane 3). Thus, it appears that the interaction between NS5A and Grb2 is not constitutive, a property that is important for transient signaling interactions. Taken together, these results suggest that NS5A is a viral inhibitor of mitogenic signaling pathway, at least in part because of its ability to bind Grb2.

DISCUSSION

Although NS5A contains multiple potential PXXP-defining SH3-binding sites, only two are highly preserved among HCV genotypes, which include both class I (amino acids 26–32) and class II (amino acids 350–356) motifs (Fig. 1). We found that the latter is necessary for NS5A interaction with Grb2, an adaptor protein that consists of one SH2 domain flanked by two SH3 domains. Grb2 forms a complex with the guanine nucleotide exchange factors, Son of sevenless (Sos) 1 and 2 (30). Growth factor-mediated stimulation of receptor tyrosine kinases creates binding sites for the SH2 domain of Grb2, recruiting the Grb2-Sos complex to the plasma membrane, where Sos activates Ras. This activation in turn triggers the Ras-MAPK kinase-ERK pathway. Consistent with the idea that NS5A binds Grb2 and perturbs downstream signaling, we found that EGF- or VV-induced phosphorylation of ERK1/2 was inhibited in HeLa cells stably expressing NS5A or HeLa S3 cells infected with a recombinant VV expressing NS5A, respectively (Figs. 5*B* and 6*A*). The exact mechanism by which NS5A interferes with Grb2 function remains to be elucidated. A closer analysis of NS5A class II motif revealed a complete conservation of the Grb2 N-SH3-binding consensus sequence (PXXPXR), which mediates Sos binding to Grb2. It is therefore possible that NS5A may compete with Sos for binding to Grb2, preventing the relocation of Sos to activated receptor to activate Ras, and hence limiting ERK activation. However, our attempt to coimmunoprecipitate Grb2 and Sos1/2 in the presence or absence of NS5A on EGF stimulation did not yield any significant change in the levels of Grb2-Sos complex (data not shown). It is possible that NS5A may function through a different mode of action, considering that the Grb2 SH3 domains can bind to a number of protein ligands in addition to Sos1/2.

Cells alter their proliferation, differentiation, metabolism, and other cellular processes in response to the different

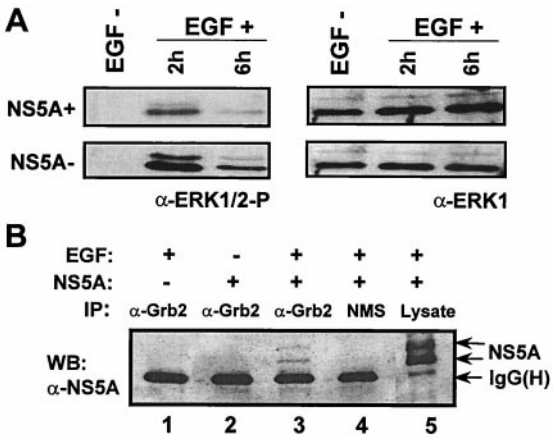

FIG. 6. Physical and functional analysis of NS5A–Grb2 interaction in an inducible stable NS5A expression HeLa cell system. (*A*) EGF-induced ERK1/2 phosphorylation in NS5A- or non-NS5A-expressing HeLa cells at different time points after EGF treatment (20 ng/ml, 2 min). Phosphorylation and protein levels of ERK1/2 were measured as described in Fig. 5*B*. (*B*) Anti-NS5A immunoblot comparing total lysate with anti-Grb2 or NMS-precipitated protein complexes from HeLa cells stably expressing NS5A with (+) or without (−) EGF stimulation.

extracellular signals, including virus infection. The precise and rapid transmission of cellular signals is executed through the concerted actions of a diverse set of signal-transducing molecules, many of which contain distinct modular domains that mediate selective protein–protein interactions (20). It is therefore not surprising that viruses have evolved a multitude of strategies to exploit cellular signaling pathways to both evade host antiviral defense and perturb cellular homeostasis. The viruses that encode genes that function to target signaling molecules include HIV (Nef protein) (33), herpes simplex virus type 2 (ribonucleotide reductase ICP10) (34), and polyomavirus (middle-T antigen) (35). HCV is unique in that it targets an adaptor protein (Grb2) that couples extracellular signals to the ERK pathway, among others. What is the physiological significance of this interaction? HCV often causes persistent infection and silent diseases, which may lead to hepatocellular carcinoma. Thus, HCV may benefit from interception of ERK signaling pathways that transduce growth/activation signals. Such possibility is supported by the observation that induction of cell proliferation/activation pathways may lead to activation of the lytic replication pathway in latently infected cells (36–38) or apoptosis of host cells (38–40). Regarding the latter, it has been suggested that regulation of Grb2 may play a role in apoptosis (41). Furthermore, we recently have shown that NS5A possesses antiapoptotic and oncogenic properties (M. Gale, B. Kwieciszewski, M. Dossett, H.N. & M.G.K., unpublished work).

In relation to IFN therapy for HCV infection, it is interesting that EGF-induced activation of ERK cascade may also play an important role in the induction of IFN gene expression (42). Activated ERK1/2 translocates to the nucleus and activates transcription by phosphorylation of transcription factors, including signal transducers and activators of transcription (43, 44), which plays a critical role in the induction of IFN-induced genes. It is tempting to speculate that NS5A binding to Grb2 might be a mechanism by which HCV induce or sustain IFN resistance, namely by down-regulating IFN gene expression. Intriguingly, HCV genotypes 2a and 2b (Fig. 1), which are highly sensitive to IFN, contain weak class II SH3 consensus-binding sequences. It would be of considerable interest to examine whether the NS5A proteins from these HCV genotypes could bind Grb2 and inhibit IFN signaling.

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