Akt Phosphorylation of Hepatitis C Virus NS5B Regulates Polymerase Activity and Hepatitis C Virus Infection

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Hepatitis C virus (HCV) is a single-stranded RNA virus of positive polarity [ssRNA(+)] that replicates its genome through the activity of one of its proteins, called NS5B. This viral protein is responsible for copying the positive-polarity RNA genome into a negative-polarity RNA strand, which will be the template for new positive-polarity RNA genomes. The NS5B protein is phosphorylated by cellular kinases, including Akt. In this work, we have identified several amino acids of NS5B that are phosphorylated by Akt, with positions S27, T53, T267, and S282 giving the most robust results. Site-directed mutagenesis of these residues to mimic (Glu mutants) or prevent (Ala mutants) their phosphorylation resulted in a reduced NS5B in vitro RNA polymerase activity, except for the T267E mutant, the only non-conserved position of all those that are phosphorylated. In addition, in vitro transcribed RNAs derived from HCV complete infectious clones carrying mutations T53E/A and S282E/A were transfected in Huh-7.5 permissive cells, and supernatant viral titers were measured at 6 and 15 days post-transfection. No virus was rescued from the mutants except for T53A at 15 days post-transfection whose viral titer was statistically lower as compared to the wild type. Therefore, phosphorylation of NS5B by cellular kinases is a mechanism of viral polymerase inactivation. Whether this inactivation is a consequence of interaction with cellular kinases or a way to generate inactive NS5B that may have other functions are questions that need further experimental work.

Keywords: HCV (hepatitis C), NS5B (non-structural protein) polymerase, Akt, virus replication, phosphorylation

INTRODUCTION

Hepatitis C virus (HCV) is a positive single-stranded RNA virus [ssRNA(+)] belonging to Flaviviridae family. HCV genome replication takes place in replication complexes where the non-structural NS5B protein produces positive single-stranded genome copies [RNA(+)] through an intermediary of negative polarity [RNA(−)] (Neufeldt et al., 2018; Tabata et al., 2020). The HCV
protein NS5B is an RNA-dependent RNA polymerase (RDRP) which, like other polymerases in its class, shows a structure that has been compared to the shape of a right hand with three subdomains called fingers, palm, and thumb (Sesmero and Thorpe, 2015). The subdomain of the palm comprises three well-preserved motifs A (D<sub>220</sub>-X(4)-D<sub>225</sub>), B (S<sup>282</sup>-X(8)-N<sup>291</sup>), and C (G<sup>317</sup>D<sup>318</sup>D<sup>319</sup>), which define the catalytic center. The aspartic acid residues D<sup>220</sup> in motif A and D<sup>318</sup> and D<sup>319</sup> in motif C are involved in the coordination of the divalent cations (Mg<sup>2+</sup> and/or Mn<sup>2+</sup>) essential for the formation of the phosphodiester bond. Residues D<sup>225</sup> of motif A, and S<sup>282</sup> and N<sup>280</sup> residues of motif B are involved in selection of ribonucleoside triphosphates over dNTPs and, thus, determine whether RNA rather than DNA is synthesized (Sesmero and Thorpe, 2015).

Viruses are obligate intracellular parasites that usurp the cellular machinery to complete their replicative cycle. This means that viral components need to interact with cellular components to direct cellular activity according to viral interests. HCV is no exception, and an enormous amount of interactions between viral components and cellular proteins have already been described (de Chassey et al., 2008; Dolan et al., 2013; Hagen et al., 2014). HCV proteins NS3, NS5A, and core have shown the most extensive network of interactions with host factors (de Chassey et al., 2008). NS5B also interacts with cellular proteins (Hamamoto et al., 2003; Munakata et al., 2005; Watashi et al., 2005; Kusakawa et al., 2005; Hillung et al., 2012), and some of its interactions require partial denaturation of NS5B and subsequent loss of RDRP activity. For example, the retinoblastoma tumor suppressor is down regulated by interacting with NS5B via amino acids located in the catalytic center (C motif) (Munakata et al., 2005). For this interaction to occur, the NS5B protein must expose amino acids located in the active center. These data indicate that an inactive NS5B may be modulating an important surveillance pathway.

We have previously described the interaction of NS5B with the cellular kinase Akt and the changes in subcellular localization related with this protein:protein interaction (Valero et al., 2016). Furthermore, we documented that Akt phosphorylates NS5B and that inhibitors of Akt affect HCV replication in cell culture (Liu et al., 2012; Qian et al., 2020), has been linked to HCV infection and related metabolic disorders (Eden et al., 2011). The PI3K-Akt pathway, transiently activated during HCV entry (Liu et al., 2012; Qian et al., 2020), has been shown to be involved in the replication of HCV (Qadri et al., 2012; Zhang et al., 2018). Therefore, modulation of Akt and Akt-related proteins could be of great importance for HCV replication.

In this study, we describe the positions of the HCV NS5B protein that are phosphorylated by the cellular kinase Akt. Furthermore, we show that mutants mimicking phosphorylation at these positions lead to proteins with very low RDRP activities. In addition, viruses carrying mutations mimicking Akt phosphorylation of T53 or S282 residues were unable to replicate in cultured cells. Akt:NS5B interaction produces an inactive viral polymerase whose role needs to be elucidated.

### MATERIALS AND METHODS

**Reagents, Expression Plasmids, and Inhibitors**

Plasmid pET_N5SBΔ21 encoding HCV NS5B from strain HC-J4 with a 21 amino acid deletion at the C-terminal end has been described previously (Lopez-Jimenez et al., 2014). Recombinant Akt/PKB was purchased from Biaffin (PK-PKBA-020, Biaffin).

| Name | Sequence (5′→3′) |
|------|-----------------|
| pJ4 S27A s | gaccatgttgtggtgacgcagcaagagttgctgcgtgacg |
| pJ4 S27A as | acgttgccgccgccgcagggcgtgctgacg |
| pJ4 S29A s | acgttgccgccgccgcagggcgtgctgacg |
| pJ4 S29A as | acgttgccgccgccgcagggcgtgctgacg |
| pJ4 T53A s | cttgcgtcctgtcaaaagctacctttttagccctctg |
| pJ4 T53A as | tccttttgagttctccagtctcccagggaggag |
| pJ4 T267A s | ccctttttgagttctcaggccagcggag |
| pJ4 T267A as | ccctttttgagttctcaggccagcggag |
| pJ4 T269A s | ccctttttttagccctctccagggaggag |
| pJ4 T269A as | ccctttttttagccctctccagggaggag |
| pJ4 T267A/T269A s | ccctttttttagccctctccagggaggag |
| pJ4 T267A/T269A as | ccctttttttagccctctccagggaggag |
| pJ4 T53E s | ccctttttttagccctctccagggaggag |
| pJ4 T53E as | ccctttttttagccctctccagggaggag |
| pJ4 T282A s | ccctttttttagccctctccagggaggag |
| pJ4 T282A as | ccctttttttagccctctccagggaggag |
| pJ4 S29E s | ccctttttttagccctctccagggaggag |
| pJ4 S29E as | ccctttttttagccctctccagggaggag |
| pJ4 S27E s | ccctttttttagccctctccagggaggag |
| pJ4 S27E as | ccctttttttagccctctccagggaggag |
| pJ4 T53E s | ccctttttttagccctctccagggaggag |
| pJ4 T53E as | ccctttttttagccctctccagggaggag |
| pJ4 T267E/T269E s | ccctttttttagccctctccagggaggag |
| pJ4 T267E/T269E as | ccctttttttagccctctccagggaggag |
| pJ4 T267E s | ccctttttttagccctctccagggaggag |
| pJ4 T267E as | ccctttttttagccctctccagggaggag |
| pJ4 T269E s | ccctttttttagccctctccagggaggag |
| pJ4 T269E as | ccctttttttagccctctccagggaggag |
| pJ4 S27A/S29A s | ccctttttttagccctctccagggaggag |
| pJ4 S27A/S29A as | ccctttttttagccctctccagggaggag |
| pJ4 S27A s | ccctttttttagccctctccagggaggag |
| pJ4 S27A as | ccctttttttagccctctccagggaggag |
| pJ4 S27A s | ccctttttttagccctctccagggaggag |
| pJ4 S27A as | ccctttttttagccctctccagggaggag |
| pJ4 S27A s | ccctttttttagccctctccagggaggag |
| pJ4 S27A as | ccctttttttagccctctccagggaggag |
| pJ4 T282E s | ccctttttttagccctctccagggaggag |
| pJ4 T282E as | ccctttttttagccctctccagggaggag |
| pJ4 T267E s | ccctttttttagccctctccagggaggag |
| pJ4 T267E as | ccctttttttagccctctccagggaggag |
| pJ4 S282A s | ccctttttttagccctctccagggaggag |
| pJ4 S282A as | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T282A s | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T282A as | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T53A s | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T53A as | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T282E s | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T282E as | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T53E s | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T53E as | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T282A s | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T282A as | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T53A s | ccctttttttagccctctccagggaggag |
| pJ4 NS5B T53A as | ccctttttttagccctctccagggaggag |
Hepatitis C Virus NS5B WT and Mutants Purification

Point mutants in NS5B were generated by site-directed mutagenesis following the manufacturer's instructions (QuikChange Site-Directed Mutagenesis, Agilent Technologies). Synthetic oligonucleotides used for point mutant generation are described in Table 1. NS5B wild type and mutants were overexpressed and purified as described previously (Lopez-Jimenez et al., 2014; Valero et al., 2016).

In vitro Kinase Assay

Kinase assays were performed as previously described (Albentosa-Gonzalez et al., 2021). Briefly, HCV NS5B (3 µg) was incubated in 20 mM Hepes pH 7.4, 10 mM MgCl\(_2\), 10 mM MnCl\(_2\), 1 µCi of \(\gamma^32\)P-ATP, 1 mM DTT, in the presence of 0.5 µg of recombinant Akt/PKB (PK-PKBA-A020, Biaffin GmbH&Co). Following SDS-PAGE electrophoresis, the gel was dried and exposed to phosphorimager screens, and scanned with Typhoon 9600 (Molecular Dynamics) to detect radiolabeled products.

In-Gel Digestion and Reverse Phase-Liquid Chromatography RP-LC-MS/MS Analysis

The identification of phosphorylated NS5B residues was performed as previously described (Albentosa-Gonzalez et al., 2021). Briefly, phosphorylated protein was digested in situ with sequencing grade trypsin (Promega, Madison, WI, United States) and analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro mass spectrometer (Thermo Scientific). The MS/MS spectra from the peptides were analyzed by assigning the fragment ions to the candidate sequence after calculating the series of theoretical fragmentations.

In vitro RNA-Dependent RNA Polymerase Replication Assays

RNA polymerase assays were performed using the symmetric substrate LE-19, which is capable of de novo initiation (DN) and primer-extension (PE), as previously described (Clemente-Casares et al., 2011; Lopez-Jimenez et al., 2014). 200 nM NS5B was pre-incubated for 30 min in a reaction mixture containing 20 mM MOPS, pH 7.3, and 5 mM MnCl\(_2\). Reactions were started by adding 500 µM GTP, 100 µM ATP, and UTP, and 1 µCi of \(\alpha^32\)PCTP (3000 Ci mmol, PerkinElmer Life Sciences). Reactions were stopped by adding EDTA/formamide loading buffer at different time points as indicated. Products were separated using denaturing polyacrylamide (23% PAA, 7 M urea) gel electrophoresis. Gels were exposed to phosphorimager screens and scanned with Typhoon (Molecular Dynamics). Quantification was achieved by running samples on parallel gels and determining band volumes using ImageQuant software (GE Healthcare).

Cell Culture Virus Infection

The origin of the Huh-7.5 cell line, procedures for cell growth in Dulbecco’s modified Eagle’s medium (DMEM), the virus used in the experiments rescued from plasmid Jc1FLAG2(p7-nSgluc2A) (a chimera of J6 and JFH-1 from genotype 2a), and the procedures used to prepare the initial virus stock HCVp0, to titrate viral infectious particles, and to quantify viral RNA have been described previously (Perales et al., 2013; Sheldon et al., 2014). To perform infections for immunofluorescence and RNA quantification assays, \(1 \times 10^5\) Huh-7.5 cells were infected with HCVp0 at a multiplicity of infection (MOI) of 0.5 Tissue Culture Infectious Dose (50%) TCID50/cell. The infected cells were further incubated at 37°C for 6 and 15 days. Absence of contamination was checked by maintaining and titrating mock-infected cells and their supernatants in parallel with the infected cultures. No infectivity in the mock-infected cultures was detected in any of the experiments.

Plasmid Jc1FLAG2(p7-nSgluc2A) was used as a template for constructing T53A, T53E, S282A, and S282E mutants by site-directed mutagenesis using the oligonucleotides described in Table 1. Plasmids carrying the selected mutations were transcribed in vitro and the genomic RNA from WT or mutant virus was used to transfect cells as described above to produce HCVp0 virus stock. The supernatant from these transfections...
was used to titer the virus obtained. Supernatant from infections with mutant virus in which viral titer was obtained was used to purify viral RNA that was retrotranscribed, PCR amplified, and sequenced to test for mutation reversal.

RESULTS

Phosphoproteomic Studies

Previous results from our laboratory have shown that the Akt protein phosphorylates the NS5B polymerase of HCV (Valero et al., 2016). To analyze the effect that this phosphorylation causes in the biology of the virus, we first wanted to identify the residues phosphorylated by this Ser/Thr kinase. To this end, we performed an in vitro Akt phosphorylation assay using recombinant protein NS5B (66 kDa) fused to the fluorescent protein EGFP (NS5B-FP) and γ\[^{32}\]P-ATP as substrates. The fused protein shows a molecular mass of approximately 95 kDa that can easily be distinguished from Akt (68 kDa). The products of the phosphorylation reaction were resolved by SDS-PAGE and two bands were detected, one at about 100 kDa corresponding to NS5B-FP, and the other at about 65 kDa corresponding to the autophosphorylated Akt (Figure 1A). The phosphorylation experiment was repeated with cold ATP for proteomic purposes. The NS5B-FP band was extracted and digested with trypsin, yielding a sequence coverage of 70% in the proteomic analysis (Figure 1B).

Proteomic analysis of the trypsin digestion products allowed the identification of the phosphorylated residues S29, T53, T267, and S282 (Figure 2). Ser29 is located in the subdomain called fingertips, and is involved in the interaction with the thumb (Figure 3A). Residues T53 and T267 are located in the fingers subdomain, and are involved in the helix-helix interactions that stabilize this subdomain (Figure 3A). Finally, the S282 residue is part of the motif B site and interacts with the D225 residue of motif A for the correct positioning of the NTP during the formation of the phosphodiester bond. Two other residues (S27 and S269) could be phosphorylated and were not well identified because of their proximity to the most likely ones (S29 and T267). In any case, it is much less likely that they are phosphorylated and this was the reason they were not included in some experiments.

NS5B Activity Studies

Next, we sought to determine the effect of the phosphorylation of the residues described above on the RDRP activity of the NS5B protein. To this end, mutations that mimic phosphorylated Ser or Thr residues at these positions were introduced by site-directed mutagenesis, yielding proteins carrying the mutations S27E, S29E, T53E, T267E, S269E, and S282E. We also generated mutants carrying Ala in these positions. All these proteins were overexpressed in E. coli and purified to homogeneity by affinity chromatography, as judged by SDS-PAGE (Figure 3B).

RDRP activity assays were carried out using oligonucleotide LE19, which allows de novo initiation and primer extension to be analyzed at the same time. Results with Ala mutants showed activity levels below 50% compared to WT in all cases and for both types of activity. Of all the Ala mutants tested, T267A
showed the highest activity levels (Figure 4). Assays with the Glu mutants showed imperceptible activity levels for all mutants except T267E, which showed RNA polymerase activity similar to or even higher than the WT protein (Figure 5). Therefore, changes that mimic or abrogate phosphorylation at the NS5B protein positions identified to be phosphorylated by Akt yield proteins with much lower levels of RDRP activity than the WT protein for both de novo and primer-dependent synthesis, with the sole exception of the T267E mutant.

Phosphorylation of Ser and Thr residues leads to the introduction of negative net charges in these positions. Ser282, one of the NS5B residues that becomes phosphorylated by Akt, is part of the active center of the polymerase. We therefore wanted to analyze whether the mutant that mimics phosphorylation in this position maintains the ability to bind RNA. For this purpose, we carried out electromobility shift assay (EMSA) experiments comparing WT and S282E proteins. First, we determined the concentration of WT protein that was able to bind and delay a fixed RNA concentration (corresponding to 10,000 cpm) and at a 100 mM NaCl concentration. The results show that at 100 nM protein all RNA is forming RNA:protein complexes (Figure 6A). Then, with those conditions (10,000 cpm RNA and 100 nM protein) as starting point, we performed electromobility shift assays varying the NaCl concentration to compare WT and S282E proteins. Since RNA:protein interactions are predominantly electrostatic, an increase in ionic strength will result in the loss of interaction. When the experiment was carried out at the lowest NaCl concentration (30 mM), all of the RNA with WT protein is forming RNA:protein complexes (Figure 6B). However, with the protein carrying the S282E mutation under these conditions, a free-form RNA band is observed, indicating less interaction between RNA and mutant protein compared to the WT protein (Figure 6B). In addition, whereas the WT protein even shows an intense band corresponding to RNA:protein complexes at the highest NaCl concentration tested (530 mM), the mutant protein shows very faint complex bands at concentrations higher than 330 mM NaCl (Figure 6B). These results indicate that, under the same conditions, the interaction of RNA with the S282E mutant protein is weaker than with the WT protein.

**Effect of Mutations in T53 and S282 Residues on Virus Replication in Cell Culture**

Next, the effect of NS5B phosphorylation by Akt or, alternatively, its inability to completely phosphorylate the polymerase, on HCV’s replicative capacity was analyzed. To do this, we constructed T53A, T53E, S282A, and S282E mutants in the
Jc1FLAG2(p7-nsGluc2A) plasmid, the resulting plasmids were transcribed \textit{in vitro}, and the RNA product of the transcription was used to transfect Huh-7.5 cells. The supernatant from these transfected cells was used to titrate for the presence of HCV following a described procedure (Perales et al., 2013; Sheldon et al., 2014). WT virus could be recovered from the supernatant of all three replicates of transfected cells with the corresponding RNA (Figure 7A). The average viral titer was $4.40 \times 10^3$ TCID50/ml at 6 days post-transfection, and $1.96 \times 10^3$ TCID50/ml at 15 days post-transfection. On the contrary, only two of the mutant virus replicates, both corresponding to the T53A mutant 15 days post-transfection, gave a viral titer value above the cut-off value (Figure 7A). T53A TCID50/ml values were $4.64 \times 10^3$ and $2.43 \times 10^3$.

To rule out that the virus collected from the supernatant of the cells transfected with the mutant T53A was a product of mutation reversal, we purified the viral RNA, subjected it to an RT-PCR reaction and sequenced the DNA product. The result allowed
FIGURE 6 | Electromobility shift assays. (A) Gel showing retardation of a labeled RNA (10,000 cpm each lane) by increasing concentrations of WT NS5B. Concentration of WT NS5B is indicated at the top (nM). The protein concentration chosen for the following experiments is indicated by a blue box. (B) Gel showing retardation of a labeled RNA by WT and S282E NS5B proteins at increasing concentrations (30, 130, 230, 330, 430, and 530 mM) of NaCl. Free labeled probe (RNA) and retarded products (NS5B:RNA) are indicated.

us to confirm that the T53A change was present in the rescued virus (Figure 7B).

DISCUSSION

Previous work in our laboratory has shown that the cellular kinase Akt interacts with and phosphorylates the HCV polymerase NS5B (Valero et al., 2016). Now, we have identified phosphorylated positions, and analyzed the effect these modifications have on RDRP activity and virus replication. We identified positions S29 (or much less likely S27), T53, S269 (or much less probable T267) and S282 as substrates of the cellular Akt kinase (Figure 1). Coverage of the NS5B proteomic analysis was 70% of the sequence, leaving the possibility that some other phosphorylatable positions have not been identified. Previous work has shown that Akt is important for virus replication in HCV-infected cells in culture (13 and references therein), suggesting that the HCV-Akt relationship is important in vivo and supporting the in vitro results described in the present work. Residue T267 is not conserved among genotypes (Figure 8A), and mutations at this position rendered the highest RDRP activity values among all mutants analyzed in this study, and even better than WT in the case of the T267E mutant. Residue T53 has not been described so far in relation to NS5B activity nor HCV replication. S29 and S282 residues have been previously described as important for both RDRP activity and viral replication. S29 has already been described previously as substrate of other important kinases in the HCV replicative cycle (Han et al., 2014; Hernandez et al., 2015). Furthermore, S29 is the homologous position of T33 of norovirus, which is also phosphorylated by Akt (Eden et al., 2011). These antecedents indicate us that phosphorylation of S29 seems to be very
important for the replicative cycle of HCV (Han et al., 2014). Finally, S282 is a residue involved in substrate recognition by interacting with other amino acids (D225) and ribonucleotide substrates (Figure 8B; Appleby et al., 2015). Residue S282 has also been related to sofosbuvir and ribavirin resistance (Aloia et al., 2012; Ji et al., 2015; Kulkarni et al., 2016; Fourati et al., 2019). Therefore, with the exception of position T267, the positions identified in this work that are modified by Akt are critical for the RDRP activity of the NS5B protein as any change greatly affects RDRP activity.

S29 is located in the fingertips subdomain and is part of the residues involved in fingers-thumb interaction (Cai et al., 2005; Figures 3, 8B). Previous in vitro work focused on this NS5B region showed that fingers-thumb interactions were key for RDRP activity, binding of RNA and for the transition from de novo initiation to primer extension (Chinnaswamy et al., 2008, 2010). In addition, viruses carrying the S29A mutation have serious replication defects in cell culture (Han et al., 2014), which is consistent with the low levels of RDRP activity we have found (Figures 4, 5). The S29E change decreases the distance with R503, a residue that is at binding distance and is also part of the fingers-thumb interaction region (Figure 8B). The distance between S29 and R503 is between 3.3 and 3.8 Å, while the distance between the most stable rotamer of mutant S29E and R503 is between 3.4 and 2.6 Å. In addition, the phosphorylation of S29 introduces a negative charge in that location, and, considering the proximity of the R503 residue (∼3 Å), the interactions of this area could be distorted. This NS5B region must undergo large conformational changes for allowing the transition from de novo synthesis to primer extension (Chinnaswamy et al., 2008, 2010; Appleby et al., 2015). Mutations in these positions (e.g., phosphorylation of S29) could prevent these conformational changes or even impair a proper folding of the NS5B protein to carry out the replication of the genome.

Position S282 is part of the NS5B catalytic pocket (Figure 8C). This residue is part of the motif B next to D225 and is essential for the accurate positioning of NTP and the subsequent formation of the phosphodiester bond. The crystalline structure of NS5B in the presence of ADP shows that S282 has a hydrogen bond with the ribose of the nucleotide (Figure 8C; Appleby et al., 2015). By introducing the mutation S282E to mimic the phosphorylation in that position, all the rotamers including the more stable...
ones lost that hydrogen bond (Figure 8A). Previously, it has been described that changes S282T/G/C/R are associated with resistance to sofosbuvir (Lam et al., 2012; Perales et al., 2015; Chen et al., 2016; Gane et al., 2017). Changes at this position, even the most subtle (S282T) that could be also phosphorylated by Akt, give rise to viruses with very low replication efficiency (Suda et al., 2019; Figure 7). The crystalline structure of the NS5B protein with sofosbuvir located in its active center has been determined (Appleby et al., 2015). This structure showed that S282 is displaced to avoid steric hindrance (compare the protein structures with ADP in cyan and with sofosbuvir in orange in Figure 8C). In addition, the S282E mutation does not allow the hydrogen bond with the ribose ring of the incoming nucleotide in any of the structures, neither in the presence of ADP nor sofosbuvir, as the WT protein does. The lack of an interaction network together with the displacement of protein motifs could be related to the results shown in Figures 4–7.

Position T53 is the least studied of the above-mentioned phosphorylatable residues. It is fully conserved in all HCV genotypes. The amino acid T53 is probably involved in maintaining the structure of the Δ1 loop and fingertips through interactions with other amino acids such as W6, with which it establishes hydrogen bonds (Figure 8D). Other hydrogen bonds involved would be those formed by residues S1:R56, M2:R56, S3:V52, S3:F54, and E17:S42. Notably, the Δ1 loop S42 residue is a substrate of the serine kinase PRK2, and mutations at this position also affect RDRP activity and HCV replication (Han et al., 2014). Therefore, phosphorylation of S42 and T53, together with S29, could be destabilizing the interactions that allow the maintenance of an active NS5B structure. The large number of interactions between positions 1–6 and 52–56 could be responsible for maintaining the structure of this region of the NS5B protein and thus for the replication of the T53A mutant (Figure 7), albeit at much lower levels than the parental virus.

In summary, the positions described in this work are phosphorylated in vitro by Akt, a Ser/Thr kinase involved in many cellular processes. The positions identified are critical for NS5B RDRP activity, and Ser/Thr to Glu changes at these positions (imitating phosphorylation) as well as changes to Ala result in loss of HCV polymerase activity. Furthermore, mutants that mimic phosphorylation are unable to replicate in cell culture. The reason why HCV polymerase is a substrate for cellular kinases is currently unclear. The inactive NS5B protein product of phosphorylation might be able to interact with other viral or cellular proteins, thus modulating the viral and cellular replicative cycle. These lines of research are currently being explored in our laboratory.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RS and AM: conceptualization, formal analysis, and writing—original draft preparation. RS, LA-G, BP, PC-C, ER, CG-C, IG, AA, and AM: investigation. RS, CP, ED, and AM: methodology. RS, LA-G, PC-C, and AM: writing—review and editing. CP, ED, and AM: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

The proteomic analysis: Protein Identification by LC-MS/MS was carried out in the “CBMSO PROTEIN CHEMISTRY FACILITY” that belongs to ProteoRed, PR3-ISCIII, supported by Grant PT17/0019. The work at UCLM was funded by MINISTERIO DE CIENCIA E INNOVACIÓN, Grant Nos. SAF2016-80451-P and PID2019-106068GB-I00 and UNIVERSIDAD DE CASTILLA- LA MANCHA Grant Nos. G120163501, G120174100, and 2019-GRIN-27080. BP is supported by predoctoral contract 2020-PREDUCLM-16195 from UCLM. The work at CBMSO and IIS-FJD was supported by grants SAF2017-87846-R and BFU2017-91384-EXP from Ministerio de Ciencia, Innovación y Universidades (MIUCI), PI18/00210 from Instituto de Salud Carlos III, and S2018/BAA-4370 (PLATESA2 from Comunidad de Madrid/FEDER). CP was supported by the Miguel Servet program of the Instituto de Salud Carlos III (CPII19/00001), cofinanced by the European Regional Development Fund (ERDF). CG-C was supported by the predoctoral contract PRE2018-083422 from MIUCI. CIBERehd (Centro de Investigación en Red de Enfermedades Hepáticas y Digestivas) was funded by the Instituto de Salud Carlos III. Institutional grants from the Fundación Ramón Areces and Banco Santander to the CBMSO are also acknowledged. The team at CBMSO belongs to the Global Virus Network (GVN).

ACKNOWLEDGMENTS

The Huh7.5 cell line was kindly supplied by Bartenschlager (University of Heidelberg). Dr. Sandra Franco is acknowledged for valuable discussions about generation of HCVp0 wt and mutant viruses. Dr. Piet de Groot is acknowledged for critical reading the manuscript. José Joaquín Lorenzo was acknowledged for technical assistance.

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