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Further studies on hepatitis C virus NS5A–SH3 domain interactions: identification of residues critical for binding and implications for viral RNA replication and modulation of cell signalling

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The NS5A protein of hepatitis C virus has been shown to interact with a subset of Src homology 3 (SH3) domain-containing proteins. The molecular mechanisms underlying these observations have not been fully characterized, therefore a previous analysis of NS5A–SH3 domain interactions was extended. By using a semi-quantitative ELISA assay, a hierarchy of binding between various SH3 domains for NS5A was demonstrated. Molecular modelling of a polyproline motif within NS5A (termed PP2.2) bound to the FynSH3 domain predicted that the specificity-determining RT-loop region within the SH3 domain did not interact directly with the PP2.2 motif. However, it was demonstrated that the RT loop did contribute to the specificity of binding, implicating the involvement of other intermolecular contacts between NS5A and SH3 domains. The modelling analysis also predicted a critical role for a conserved arginine located at the C terminus of the PP2.2 motif; this was confirmed experimentally. Finally, it was demonstrated that, in comparison with wild-type replicon cells, inhibition of the transcription factor AP-1, a function previously assigned to NS5A, was not observed in cells harbouring a subgenomic replicon containing a mutation within the PP2.2 motif. However, the ability of the mutated replicon to establish itself within Huh-7 cells was unaffected. The highly conserved nature of the PP2.2 motif within NS5A suggests that functions involving this motif are of importance, but are unlikely to play a role in replication of the viral RNA genome. It is more likely that they play a role in altering the cellular environment to favour viral persistence.

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INTRODUCTION

Hepatitis C virus (HCV) is a major health issue throughout the world; the World Health Organization estimates that more than 170 million individuals are infected with this virus (WHO, 1999). In 80 % of cases, HCV establishes a chronic infection leading to fibrosis, cirrhosis and ultimately hepatocellular carcinoma. The lack of an efficient *in vitro* culture system for the virus has hampered analysis of the mechanisms of pathogenicity and viral persistence. Experiments using individual HCV protein products and self-replicating subgenomic replicons have established that viral proteins interact with a wide range of cellular factors, modulating cell physiology. In this regard, the non-structural NS5A protein has been the focus of much intensive research (Macdonald & Harris, 2004). Of particular interest for this study was the observation that NS5A contains a number of polyproline motifs (PxxP) that mediate interactions with cellular proteins containing Src homology 3 (SH3) domains. These are found in a diverse range of cellular proteins that are involved in intracellular signalling and cytoskeletal organization (Mayer, 2001) and mediate inter- and intramolecular interactions by binding to proteins that contain PxxP motifs. These motifs can bind to SH3 domains in two opposite orientations, with the position of a basic residue dictating in which orientation the motif binds its cognate SH3 domain. Thus, motifs with the consensus sequence K/RxxPxxP are known as class I motifs, whereas class II motifs have the consensus sequence PxxPxR. The basic residues have been shown to contribute to the interaction by forming a salt bridge with acidic residues within the SH3 domain (Weng *et al.*, 1995). The specificity of SH3 domain interactions has been shown to

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involve sequence variation within the PxxP motif, in both the consensus and the adjacent non-consensus sequences. Furthermore, sequences within the SH3 domains contribute to specificity, in particular residues within the RT and n-Src loops.

One key group of SH3 domain-containing proteins is the Src family of tyrosine kinases (Tatosyan & Mizenina, 2000). These signalling proteins are common targets for viral interference, particularly in the case of viruses that establish a chronic infection. In this regard, one of the best-studied SH3 binding viral proteins is the human immunodeficiency virus type 1 (HIV-1) Nef protein, which has been shown to bind the SH3 domains of the Src family kinases Hck, Lyn, Fyn and Src (Saksela *et al.*, 1995). Indeed, the interaction between Nef and the HckSH3 domain, as determined by surface plasmon resonance (SPR), is amongst the strongest SH3–PxxP interactions reported to date (Lee *et al.*, 1995). Our group and others have reported that NS5A interacts with a variety of SH3 domain-containing proteins, including Grb2, amphiphysin II, PI3K p85 subunit and members of the Src family (Macdonald *et al.*, 2004; Street *et al.*, 2004; Tan *et al.*, 1999; Zech *et al.*, 2003). Interestingly, as is the case for Nef, NS5A shows remarkable selective characteristics: although able to bind a subset of Src kinase SH3 domains, namely Hck, Lck, Lyn and Fyn, it does not bind that of Src itself; nor does it bind to a range of other SH3 domains, including those of the Vav guanine nucleotide-exchange factor, the adaptor proteins Crk and Nck and the Abl tyrosine kinase (Macdonald *et al.*, 2004; Tan *et al.*, 1999). As with other viruses, these interactions with SH3 domain-containing proteins are likely to aid in either virus replication or immune evasion, but, at this stage, their exact functions remain obscure. Molecular characterization of the interaction between NS5A and SH3 domains could therefore not only elucidate the structural basis of SH3 binding specificity, but may also be helpful for development of novel therapeutic strategies aimed at inhibition of NS5A function.

In this study, we have used a semi-quantitative assay to examine the differences in binding of various SH3 domains to NS5A and show that NS5A exhibits a hierarchy of binding affinities to SH3 domains. By using a molecular-modelling approach, we predicted the interaction surface between the PP2.2 motif of NS5A and the FynSH3 domain. Coupled with experimental data, this analysis revealed that residues in the variable region of the RT loop of the SH3 domain do not contact the PP2.2 motif, but play a role in determining the specificity of NS5A-mediated SH3 domain interactions, consistent with additional intermolecular contacts between NS5A and SH3 domains. We also demonstrate a requirement for salt-bridge formation for binding of NS5A to SH3 domains. Finally, we utilize a subgenomic replicon to analyse the potential role of NS5A–SH3 domain interactions in HCV RNA replication and effects on signalling within replicon-harbouring cells.

METHODS

DNA manipulations. To mutagenize NS5A within the 5.1 replicon, the *Nsi*I–*Nsi*I fragment (nt 3682–7122) from pFK5.1 (Krieger *et al.*, 2001) was cloned into LITMUS28 (New England Biolabs), generating pLRM(wt). Mutagenesis of the PP2.2 motif within pLRM(wt) was achieved by using a GeneEditor kit (Promega) and confirmed by sequencing. The *Nsi*I–*Nsi*I fragment was then reintroduced into pFK5.1, generating pFK5.1neo(PA2.2). Other mutations within NS5A were generated by the PCR-overlap method (Higuchi, 1992), using an appropriate template and overlapping internal oligonucleotide primers. Primer sequences are available upon request.

Cell-culture procedures. Cos-7 cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum, 2 mM L-glutamine, 100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1} at 37 °C in a humidified 5 % CO_2 incubator. Huh-7 cells were cultured in minimal essential medium supplemented as for Cos-7 cells, with the addition of 1 % non-essential amino acids. For transient transfection of NS5A-expression vectors, 1×10^6 cells were seeded in 90 mm dishes and incubated for 24 h at 37 °C, prior to transfection with pSG5 constructs expressing NS5A and mutants thereof by using Lipofectin (Invitrogen). Cells were incubated for 6 h, after which they were cultured in growth media for a further 24 h prior to lysis in Glasgow lysis buffer (GLB; Bentham *et al.*, 2003) supplemented with protease inhibitors (Boehringer Mannheim). For the generation of Huh-7 cells harbouring subgenomic replicons, RNA transcripts were generated from pFK5.1, pFK5.1neo(PA2.2) and pBACrepGNDneo$^{T7/NotI}$, and transfected as described previously (McCormick *et al.*, 2004).

Expression and purification of recombinant proteins. Generation and use of the pGEX vectors for bacterial expression of the glutathione *S*-transferase (GST)–SH3 domain fusion proteins was as described previously (Hiipakka *et al.*, 1999; Macdonald *et al.*, 2004). GST–Grb2 was obtained from John Ladbury (University College London, UK). N-terminally His-tagged NS5A (genotype 1b) was expressed by a recombinant baculovirus in Sf9 cells and purified by Ni^{2+}-NTA chromatography. The concentration and integrity of purified proteins were determined by Bradford assay (Bio-Rad), SDS-PAGE and Coomassie staining.

***In vitro* binding assays.** Binding assays were performed as described previously (Macdonald *et al.*, 2004). Briefly, GST–SH3 domains were bound to glutathione–agarose beads overnight at 4 °C. Equal quantities of lysates from cells transiently transfected with the appropriate pSG5 vectors were added to the beads. After 3 h incubation, beads were washed extensively in lysis buffer and bound protein was analysed by SDS-PAGE and immunoblotting with a sheep polyclonal anti-NS5A antiserum. GST alone was used as a negative control.

ELISA protein-interaction assay. Purified GST–SH3 domains (1 μg per well) were coated onto 96-well plates (PS Microplate; Greiner Bio-One) by incubation in 50 μl PBS/0·1 % Tween (PBT) per well overnight at 4 °C. Plates were washed in PBT and blocked in PBT containing 5 % non-fat dried milk for 2 h at room temperature. Plates were washed in PBT and purified His–NS5A or lysate from Cos-7 cells expressing NS5A and mutants thereof, diluted in GLB supplemented with protease inhibitors (50 μl per well), was added for 2 h at 4 °C. After three PBT washes, NS5A was detected by using a sheep polyclonal antiserum for 1 h followed by horseradish peroxidase-conjugated anti-sheep antibody (Sigma) for 1 h. Bound antibody was visualized by using *o*-phenylenediamine (Dako) and quantified at 490 nm with a reference filter at 630 nm, using an MRX microplate reader (Dynex).

Luciferase reporter assays. Cells were transfected with plasmids expressing the appropriate luciferase reporter (0·5 μg) by using Lipofectin (Invitrogen). A Renilla luciferase reporter construct

(pRLTK) was used as an internal control for transfection efficiency. Total DNA was kept constant by adjusting the amount of vector DNA. Cells were grown in reduced serum-containing media (0·5 %) for 24 h and lysed in passive lysis buffer (Promega) prior to analysis. Assays were performed in triplicate and analysed with dual luciferase reagent (Promega) and a luminometer as described previously (Macdonald *et al.*, 2003).

Molecular modelling of the NS5A–SH3 interaction. Modelling of the NS5A PP2.2–FynSH3 domain interaction was carried out by taking the peptide ^{71}Thr-Pro-Gln-Val-Pro-Leu-Arg^{77}, corresponding to the PxxP motif from HIV-1 Nef in complex with FynSH3 (1AVZ; Arold *et al.*, 1997), and mutating it to the equivalent motif of NS5A. Individual mutations were created by using MutantDock, an online computational mutagenesis server (S. J. Campbell & R. M. Jackson, unpublished data), which uses SCWRL to predict the conformation of mutant-residue side-chain positions (Bower *et al.*, 1997) before refining the protein–peptide interaction with MultiDock (Jackson *et al.*, 1998). The mutations required to convert the PP2.2 peptide were done sequentially in all possible orders; this did not, however, significantly change either the final predicted conformation or interaction energies. Results are shown for the model with the most favourable predicted interaction energy. The donor scaffold forms a polyproline helix, therefore mutating the peptide to/from proline did not affect the backbone conformation of the modelled peptide.

RESULTS

Comparison of the binding affinity of SH3 domains for NS5A

Both we (Macdonald *et al.*, 2004) and others (Tan *et al.*, 1999) have used *in vitro* GST-binding assays to study NS5A–SH3 domain interactions; these relied on Western blot detection of bound protein and thus provided only limited quantitative data. To determine the relative affinity of NS5A for these SH3 domains, we therefore established an ELISA assay in which purified GST–SH3 domain fusion proteins were used to capture baculovirus-expressed His–NS5A(1b). As shown in Fig. 1(a), a clear hierarchy of binding affinities was evident: LynSH3 bound most efficiently, followed by Grb2, Fyn, Hck and Lck. Binding to LckSH3 was approximately 10-fold lower than that to LynSH3. We were unable

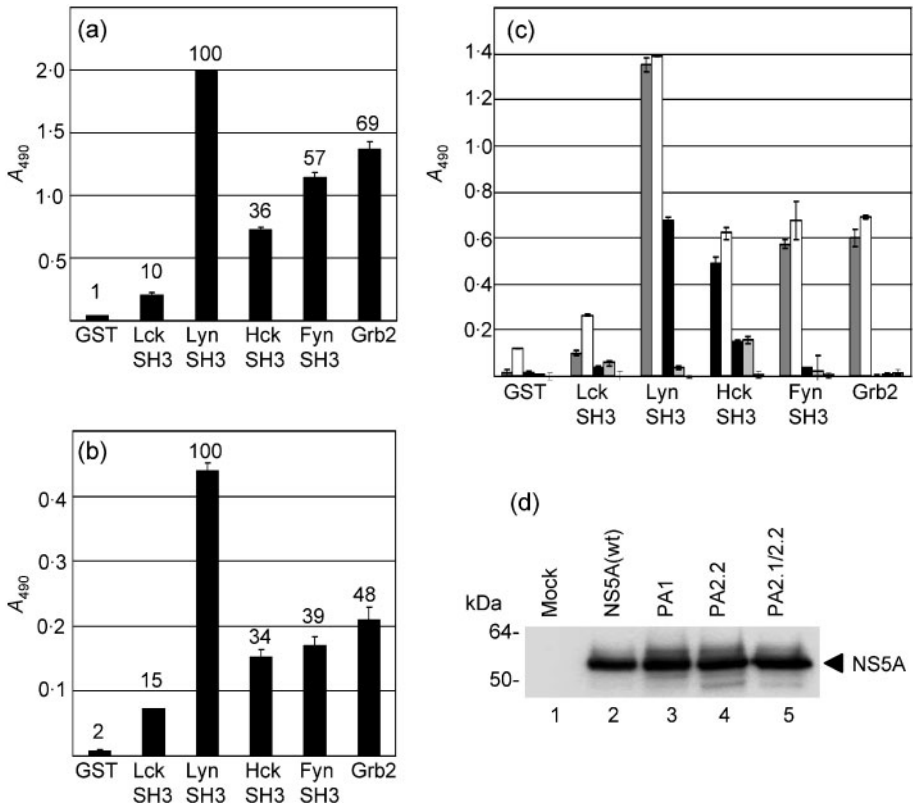

Fig. 1. Use of an ELISA assay to measure NS5A–SH3 domain interactions. The indicated GST-fusion proteins were bound to ELISA plates and subsequently incubated with either (a) N-terminally hexahistidine-tagged NS5A produced in recombinant baculovirus-infected Sf9 cells or (b) lysates from Cos-7 cells transfected with pSG5-NS5A(wt) (Macdonald *et al.*, 2003). Numbers above each bar represent the strength of the signal as a percentage in comparison with the strongest binder (LynSH3), referred to as 100 %. (c) Lysates from Cos-7 cells transfected with either pSG5-NS5A(wt) or the indicated mutants were analysed for binding to the indicated GST-fusion proteins. Dark-grey bars, NS5A(wt); white bars, NS5A(PA1); black bars, NS5A(PA2.2); light-grey bars, NS5A(PA2.1/2.2); hatched bars, mock. (d) Aliquots of the lysates were analysed by Western blotting with a sheep polyclonal anti-NS5A antiserum to confirm similar levels of expression.

to use the ELISA assay to confirm our previous observation that the SrcSH3 domain did not bind to NS5A (Macdonald *et al.*, 2004), due to a high level of non-specific binding of GST–SrcSH3 to the secondary antibody (data not shown). To confirm that the results obtained with purified protein were representative of interactions occurring in the context of cellular proteins, Cos-7 cells were transfected with a pSG5 vector driving the expression of NS5A (from HCV genotype 1a) and the same assay was performed with lysates from these cells. Fig. 1(b) shows that similar results were obtained with this mammalian cell-derived NS5A, although signals were somewhat lower, due to the lower concentrations of NS5A protein present within a cell lysate compared with purified protein. Once again, the hierarchy of binding to NS5A demonstrated higher binding affinity to LynSH3 and Grb2 compared with LckSH3 (Fig. 1b).

Our earlier observations demonstrated that two class II PxxP motifs within NS5A (termed PP2.1 and PP2.2; see Fig. 4a) were absolutely required for binding to SH3 domains (Macdonald *et al.*, 2004); we therefore set out to confirm this by using the ELISA assay system. Lysates from Cos-7 cells transiently transfected with pSG5 vectors expressing NS5A(1a), NS5A(PA1), NS5A(PA2.2) or NS5A(PA2.1/2.2) (Macdonald *et al.*, 2004) were analysed for binding to immobilized GST–SH3 domains. Fig. 1(c) shows that, consistent with our previous data, the PA1 mutation had no effect on binding of NS5A to SH3 domains, whereas both the PA2.2 and PA2.1/2.2 mutants abrogated binding to SH3 domains of Hck, Lck, Fyn and Grb2. Note that although there is a low level of binding of both PA2.2 and PA2.1/2.2 to HckSH3, this is substantially lower than wild-type and is consistent with our previous observation that NS5A(PA2.2) retained low-level binding to full-length Hck (Macdonald *et al.*, 2004). Analysis of the interaction between the NS5A mutants and LynSH3 gave a different pattern: NS5A(PA2.2) retained the ability to interact with LynSH3, although binding was reduced approximately twofold compared with NS5A(wt). In contrast, NS5A(PA2.1/2.2) failed to bind to LynSH3, consistent with our previous observation that LynSH3 bound to either the PP2.1 or PP2.2 motifs (Macdonald *et al.*, 2004). Immunoblot analysis of the lysates used in the ELISA binding assay confirmed that differences observed were not due to variable expression of NS5A (Fig. 1d). These ELISA binding-assay data further characterize the interactions with Src family kinase SH3 domains and Grb2 and suggest that there is a hierarchy of binding, in which NS5A binds strongly to LynSH3 and more weakly to LckSH3. Our data also highlight that NS5A from two HCV genotypes (1a and 1b) behaves in a similar manner and binds the same SH3 domains.

Molecular modelling of the NS5A–SH3 interaction

In an attempt to gain some insight into the structure of the NS5A–SH3 interaction, we exploited the available structure of a proline-rich peptide from HIV-1 Nef interacting with the FynSH3 domain (Arold *et al.*, 1997). The Nef peptide (^{71}Thr-Pro-Gln-Val-Pro-Leu-Arg^{77}) was used as the donor scaffold for computational modelling of PP2.2 (^{350}Pro-Pro-Ile-Pro-Pro-Pro-Arg^{356}). The NS5A–SH3 structures were generated by mutating the Nef peptide residues where they differed from PP2.2. Fig. 2(a) shows the predicted structure of the NS5A–SH3 interface. Figs 2(b) and (c) show the predicted contribution of each residue to the interaction, compared with the corresponding data for the Nef–FynSH3 interaction. The total predicted interaction energies of FynSH3 with the Nef and NS5A peptides are very similar ($-41{\cdot}4$ and $-42{\cdot}7$ kcal mol^{-1}, respectively), most probably due to the conservation of the important residue interactions in the interface. The most important residues in the Nef peptide (Pro72, Pro75 and Arg77) are conserved in NS5A (Pro351, Pro354 and Arg356). They form one face of the polyproline II helix that interacts with the SH3 domain. Only Val74 (Pro353) is not conserved; however, the proline side chain maintains similar van der Waals interactions to valine with FynSH3 (with Trp119, Pro134 and Tyr137). The other residues in the NS5A peptide differ from those in Nef, but are on the other face of the helix projecting away from the SH3 domain. They are not involved in direct interaction with FynSH3 and would appear to contribute little to peptide-binding specificity.

A number of conclusions can be drawn from the model, firstly concerning the role of the SH3 domain RT loop. This loop connects the first and second β-strands, typically consists of 18 aa and is composed of a central, highly variable region flanked by constant stretches of four (N-terminal) and eight (C-terminal) relatively conserved residues with some β-strand character (Larson & Davidson, 2000). These conserved RT-loop residues form part of the third 'specificity pocket' on the SH3 ligand-binding surface that accommodates the PxxP peptide. It has been shown that the non-conserved residues in the central region of the RT loop do not make contacts with the PxxP motif region, but interact elsewhere within the ligand; for example, in the case of the Nef–HckSH3 domain interaction, they interact with multiple non-contiguous residues in Nef that are distant from the PxxP motif – mutations in this region reduce the binding affinity of Nef for the HckSH3 domain (Arold *et al.*, 1998; Lee *et al.*, 1995). These variable residues are indicated in yellow/orange in Fig. 2(a). The modelling data suggested that, as for Nef, these residues (Glu94–Asp99) did not contribute to the PP2.2–SH3 interaction. To test whether these residues might interact with other parts of the NS5A protein, we utilized a panel of HckSH3 and FynSH3 domains containing reciprocal changes within the RT loop (Lee *et al.*, 1995). These changes involved the RTE sequence of FynSH3 (orange residues in Fig. 2a) and resulted in a HckSH3 domain mutant that has a Fyn-like RT loop [HckSH3(RTE)] and a FynSH3 domain mutant that has a Hck-like RT loop [FynSH3(IHH)] (Fig. 3a). The ability of these mutant SH3 domains to bind NS5A (expressed in Cos-7 cells) was then compared with that of wild-type Hck and FynSH3 domains by an *in vitro* binding

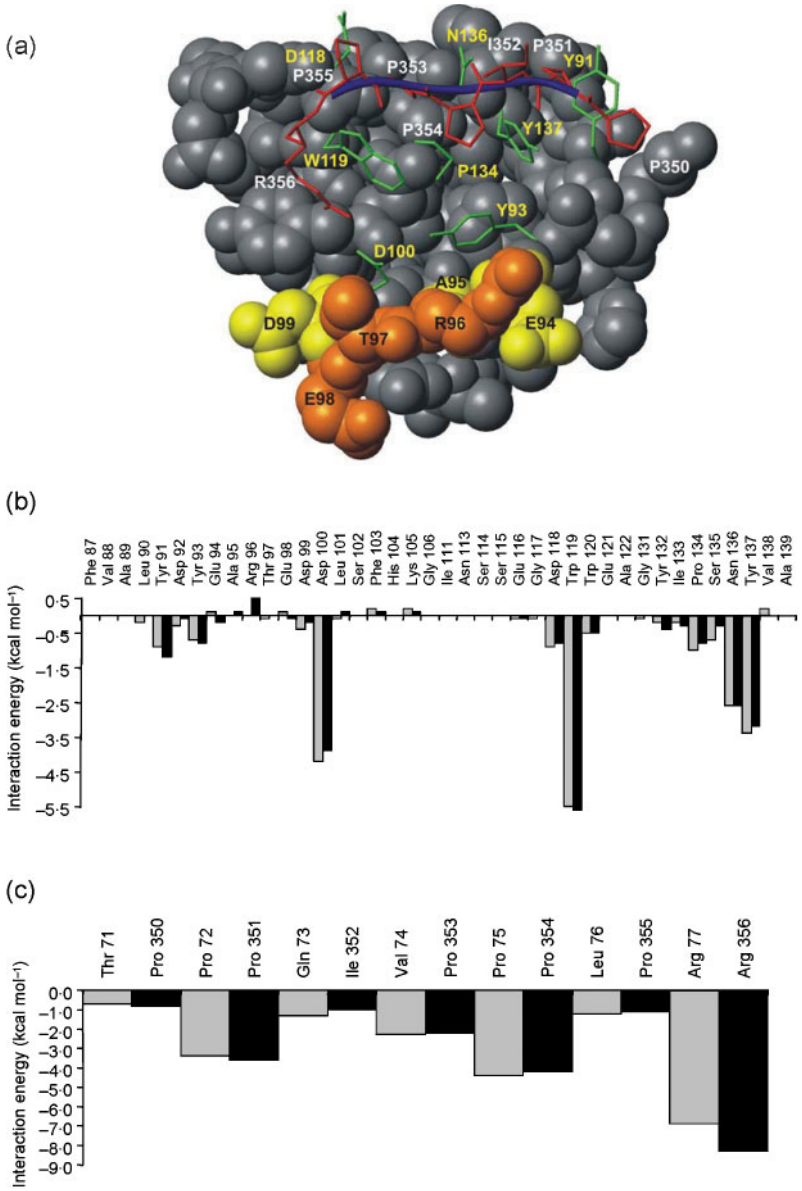

Fig. 2. Molecular modelling of the PP2.2–FynSH3 domain interaction. The donor scaffold for this analysis was derived from Arold *et al.* (1997). The Nef peptide ^{71}Thr-Pro-Gln-Val-Pro-Leu-Arg^{77} binds to FynSH3 with a predicted interaction energy of −41·4 kcal mol^{-1} and required the following mutations to convert the peptide into the NS5A PP2.2 motif: Thr71Pro, Gln73Ile, Val74Pro and Leu76Pro. The total predicted interaction energy of this peptide with FynSH3 is −42·7 kcal mol^{-1}. (a) An image of the interaction between the modelled PP2.2 peptide and the FynSH3 domain. The blue backbone represents the backbone of the donor peptide and red sticks show the atoms of the modelled peptide (residues are labelled in white). The grey Corey–Pauling–Koltun (CPK) representation shows the atoms of the SH3 domain; however, atoms of the residues involved in direct interaction with the modelled peptide are shown as green sticks (these residues are labelled in yellow). The yellow or orange CPK representation shows the atoms of the RT-loop residues (residues labelled in black); those in orange are the amino acids (RTE) mutated in the panel of FynSH3 domain mutants used in Fig. 3. (b) Contribution of each FynSH3 residue to the interaction with either the Nef or NS5A peptides. Grey bars, Nef peptide; black bars, NS5A peptide. (c) Contribution of each residue in either the Nef (Thr71–Arg77; grey bars) or NS5A (Pro350–Arg356; black bars) peptides to the interaction with the FynSH3 domain.

assay. When the Hck RT-loop sequence was changed to the Fyn sequence (RTE), binding of the resultant SH3 domain to NS5A was comparable to that of wild-type FynSH3 (Fig. 3b, lanes 2–4). A reciprocal change within the Fyn RT loop (IHH) resulted in a reduced ability to bind NS5A (comparable to that of the wild-type HckSH3 domain; Fig. 3b, compare lanes 2 and 8). We then tested individual mutant FynSH3 domains to examine the contribution of each of the three Hck-specific amino acids (IHH) in reducing binding to NS5A. Introduction of a single isoleucine or histidine residue had very little effect on the overall binding of Fyn to NS5A (Fig. 3b, lanes 5 and 6); however, introduction of both isoleucine and the first histidine residue provided a phenotype intermediate between Hck and Fyn binding (Fig. 3b, lane 7). These data are consistent with a role for the RT loop in determining the specificity of interactions with NS5A; furthermore, they suggest that sequences outside the PP2.2 motif are involved in interactions with SH3 domains.

Requirement for arginine 356 is consistent with the formation of a salt bridge between NS5A and SH3 domains

Analysis of the structures of SH3 domains complexed with their peptide ligands revealed that there are three binding pockets within the SH3 domain that contribute to binding of the polyproline helix (Mayer, 2001). Pockets 1 and 2 are filled by proline residues from within the polyproline motif, whereas the third binding pocket contains a negatively charged residue (Asp100 in FynSH3) that interacts with an arginine 2 aa C-terminal to the polyproline motif via a salt bridge. Experiments using the HIV-1 Nef protein have highlighted a critical role of this salt bridge in

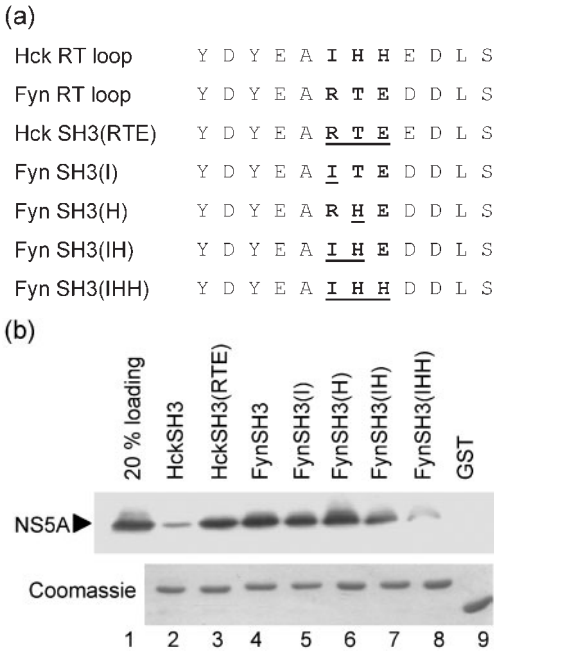

Fig. 3. Involvement of the SH3 domain RT loop in binding to NS5A. (a) Amino acid sequence of the RT loops of HckSH3 and FynSH3 domains. The residues modified in the panel of substitution mutants are indicated in bold and underlined (Lee *et al.*, 1995). The arginine in the RT loop of FynSH3 is residue 96 in the native Fyn polypeptide. All of these SH3 domains were expressed as GST-fusion proteins. (b) A lysate from Cos-7 cells transiently transfected with pSG5-NS5A(wt) was incubated with the indicated GST-fusion proteins, immobilized on glutathione–agarose beads and analysed by Western blotting with a sheep polyclonal anti-NS5A antiserum. Lane 1 shows 20 % of the lysate used in each assay.

stabilizing the interaction; indeed, Nef lacking an arginine at residue 77 is unable to bind to SH3 domains and activate downstream kinases (Manninen *et al.*, 1998). The modelling data predicted that the corresponding arginine in NS5A (Arg356) would play an analogous role (Fig. 2c). Like Nef, it is predicted to make the largest energetic contribution to the interaction from the peptide and we therefore set out to test this experimentally. Interestingly, Arg356 lies at the beginning of a short basic cluster, ending with another arginine (residue 359) (Fig. 4a), and both of these residues are highly conserved throughout HCV genotypes. We created a series of mutations, in two of which alanine was substituted for Arg356, in the context of either an intact PP2.2 or a mutated PA2.2 motif (termed R356A and PA2.2/R356A, respectively). To rule out the possibility that Arg359 could compensate for Arg356 and form a salt bridge with the SH3 domain, we also created a double mutant, again in the context of an intact PP2.2 or a mutated PA2.2 motif (termed R356/359A and PA2.2/R356/359A). GST–SH3 domains were used to precipitate cell lysates expressing these NS5A mutants and bound proteins were analysed by Western blotting with a polyclonal anti-NS5A antiserum. Fig. 4(b) demonstrates that, contrary to NS5A(wt), the R356A mutant failed to interact with the SH3 domains of Src kinases or with Grb2. Weak binding to LynSH3 was observed, consistent with the ability of LynSH3 to bind the PP2.1 motif, although in comparison with NS5A(wt), binding was reduced substantially. The other three mutants (PA2.2/R356A, R356/359A and PA2.2/R356/359A) completely abrogated binding of NS5A to all SH3 domains tested.

We have previously shown that NS5A, by blocking Ras–ERK pathway signalling, was able to inhibit both the basal and epidermal growth factor (EGF)-stimulated activity of the transcription factor AP-1 (Macdonald *et al.*, 2003). We further showed that this was dependent on the PP2.2 motif: wild-type NS5A inhibited the expression of luciferase from an AP-1-responsive reporter construct by ~50 %, whereas the PA2.2 mutant had no effect. These data implied that NS5A–SH3 domain interactions were required for the inhibition of AP-1 activation and we predicted that abrogation of salt-bridge formation would therefore also block this function of NS5A. To test this, we used the luciferase reporter assay to screen all four mutants for the ability to inhibit AP-1-responsive transcription. Consistent with previous data, expression of NS5A(wt) resulted in a 40 % reduction in AP-1-responsive luciferase levels within transfected Cos-7 cells (Fig. 4c); however, as expected, all of the mutations abolished this effect. These data confirm the validity of the molecular modelling and support the hypothesis that salt-bridge formation is critical for binding of NS5A to SH3 domains.

Mutations within the PP2.2 motif of a subgenomic replicon abolish NS5A signalling functions, but replicate to wild-type levels

To examine the role of NS5A–SH3 domain interactions in HCV RNA replication, the PA2.2 mutation was introduced into the FK5.1 culture-adapted HCV subgenomic replicon (Krieger *et al.*, 2001), generating FK5.1(PA2.2). Initially, the ability of FK5.1(PA2.2) to establish itself in cell culture was assessed by transfection into Huh-7 cells, in comparison with both the original 5.1 replicon and a polymerase knockout (GND) control replicon transcript. Whilst no G418-resistant colonies were seen in cells transfected with the GND replicon, transfection of transcripts derived from the FK5.1(PA2.2) clone led to the formation of G418-resistant colonies, demonstrating that this latter replicon is still viable (Fig. 5a). Furthermore, the number of colonies formed after transfection with FK5.1(PA2.2) was similar to that seen after transfection of Huh-7 cells with the original FK5.1 replicon (Fig. 5b), demonstrating that the PP2.2 PxxP motif is dispensable for replicon establishment. We confirmed that the PA2.2 mutation had not reverted during G418 selection by extracting RNA from the FK5.1(PA2.2) replicon cells, amplifying the NS5A coding region by RT-PCR and sequencing five independent clones of the PCR products. No mutations were observed (data not shown);

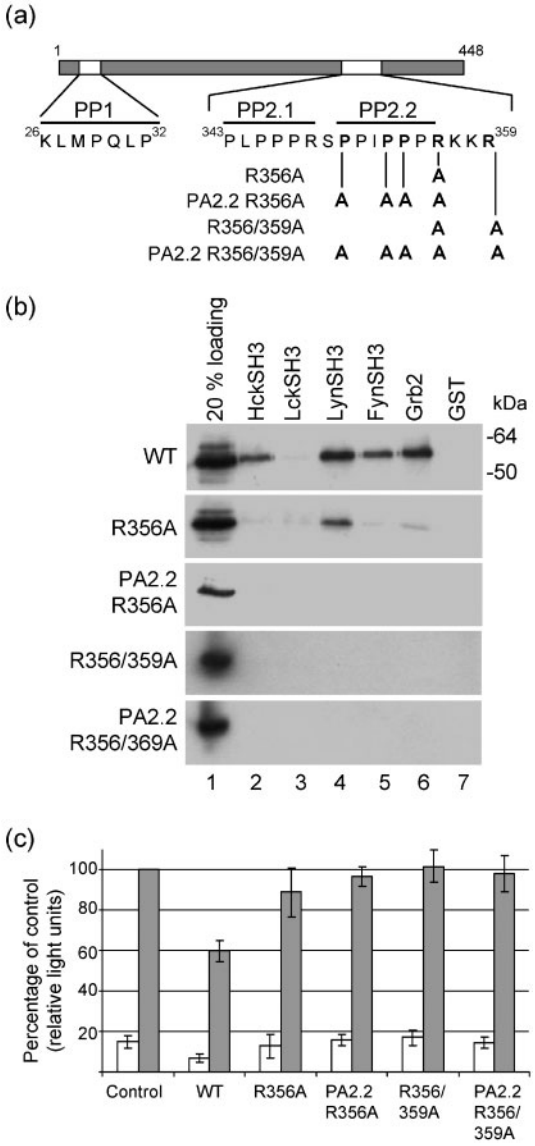

Fig. 4. Arginine residues C-terminal to the PP2.2 motif play a critical role in SH3 domain binding and NS5A function. (a) Schematic of the sequence of the three PxxP motifs in NS5A and the mutants used in this study. Amino acid sequences for each polyproline motif are indicated and the residues subjected to mutagenesis are highlighted in bold. The numbers refer to the NS5A(1a) amino acid sequence; for reference, NS5A starts at residue 1974 of the polyprotein. (b) Mutants were generated by PCR and cloned into pSG5. Lysates from Cos-7 cells transiently transfected with pSG5-NS5A plasmids were incubated with the indicated GST-fusion proteins, immobilized on glutathione–agarose beads and analysed by Western blotting with a sheep polyclonal anti-NS5A antiserum. Lane 1 shows 20 % of the lysate used in each assay. (c) Cos-7 cells were transfected with 1 μg plasmid pAP1-luc, which contains the luciferase reporter under the transcriptional control of three tandem AP-1-binding sites and a minimal TATA-box promoter (Macdonald *et al.*, 2003), with or without co-transfection of pSG5-NS5A plasmids as indicated, and placed in low-serum (0·5 %) growth medium for 18 h. Cells were stimulated by the addition of serum to 10 % and harvested immediately (0 h; white bars) or 6 h later (grey bars). The level of expression of the luciferase reporter was assayed by using a luminometer and normalized for transfection efficiency by using a co-transfected Renilla luciferase control plasmid. Results are means of three independent experiments.

however, we cannot at this stage exclude the possibility of second-site compensatory mutations elsewhere in the replicon.

It was important to confirm that the PA2.2 NS5A protein expressed in the context of the FK5.1 culture-adapted replicon was, as expected, defective for the interaction with cellular SH3 domains and perturbation of cell signalling. To test this, we first analysed the binding of NS5A in lysates of Huh-7 cells harbouring either the FK5.1 or FK5.1(PA2.2) replicons to purified GST–SH3 fusion proteins. As shown in Fig. 6(a), NS5A from the FK5.1 replicon bound to the SH3 domains of Hck, Lck, Lyn, Fyn and Grb2. In contrast, NS5A from the FK5.1(PA2.2) replicon bound only to the LynSH3 domain, consistent with the absence of the PP2.2 motif and the ability of LynSH3 to bind both PP2.1 and PP2.2 (Macdonald *et al.*, 2004).

To further confirm that NS5A expressed in the context of the FK5.1(PA2.2) replicon was defective for cell signalling, cells harbouring either the FK5.1 or FK5.1(PA2.2) replicons were transfected with an AP-1-responsive luciferase reporter construct. As expected, the FK5.1 cells exhibited a 70 % reduction in both basal and EGF-stimulated luciferase expression, consistent with a block to AP-1 activation (Fig. 6b). In the FK5.1(PA2.2) cells, levels of luciferase expression were restored to the levels seen in control cells. We conclude that the PP2.2 PxxP motif in NS5A is required for the perturbation of cell signalling, but plays no role in viral RNA replication.

DISCUSSION

The NS5A protein of HCV has received a high degree of scrutiny over recent years and has been shown to interact with a large number of cellular proteins (Macdonald & Harris, 2004). We (Macdonald *et al.*, 2004; Street *et al.*, 2004) and others (Tan *et al.*, 1999; Zech *et al.*, 2003) have identified a number of SH3 domain-containing proteins that can act as binding partners for NS5A. In this study, we have made a more detailed examination of the binding of NS5A to SH3 domains and analysed the effects of these interactions on viral RNA replication and perturbation of cellular signalling.

By using a semi-quantitative ELISA binding-assay format (Bentham *et al.*, 2003), we compared the binding abilities of various SH3 domains for NS5A. Our data highlight significant differences between the various SH3 domains, with a clear hierarchy of binding affinities over a 10-fold

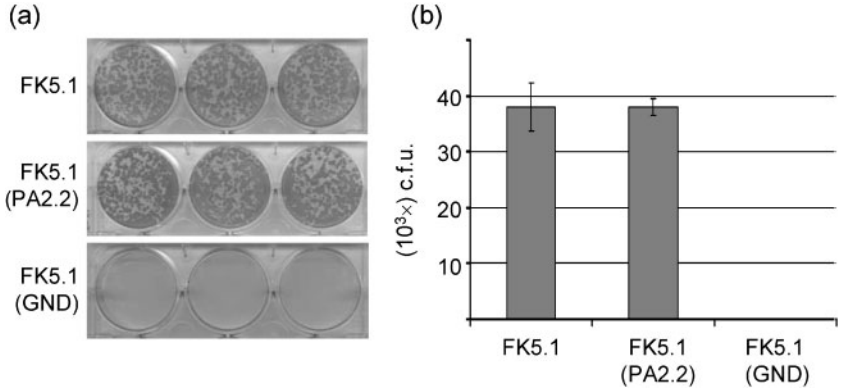

Fig. 5. The PA2.2 mutation does not affect the ability of the HCV subgenomic replicon to establish G418-resistant colonies. (a) Colony-forming assays performed with either parental FK5.1 or the mutant replicon FK5.1(PA2.2). FK5.1(GND) is a point mutation within the active site of the NS5B RNA-dependent RNA polymerase that is unable to replicate. Cells were fixed and stained 14 days post-transfection. (b) Numerical representation of the efficiency of colony formation by parental FK5.1, FK5.1(PA2.2) and FK5.1(GND). Results are means of three independent experiments.

range, from LynSH3 down to LckSH3. The ELISA assay was also used to confirm the previous observation that mutation of the N-terminal class I polyproline motif (PP1) had no effect on binding of NS5A to SH3 domains (Macdonald *et al.*, 2004; Street *et al.*, 2004; Tan *et al.*, 1999). This is consistent with the observation that the PP1 motif resides within the N-terminal amphipathic helix of NS5A, which can act as an endoplasmic reticulum membrane anchor (Brass *et al.*, 2002) and, as such, is unlikely to be available for binding to cytoplasmic proteins. Our data also highlight the differential effects of mutating the PP2.2 motif within NS5A. Previous data have shown that mutating this motif abrogated the interactions between NS5A and the SH3 domains of Hck, Lck, Fyn and Grb2 (Macdonald *et al.*, 2004; Tan *et al.*, 1999), but had no effect on binding to LynSH3. The quantitative nature of the ELISA assay, compared with the GST-binding assay, demonstrated that this mutation reduced binding to LynSH3 twofold (Fig. 2c), whereas the double (PA2.1/2.2) mutant abrogated binding to LynSH3 completely. This is consistent with our previous data demonstrating the ability of LynSH3 to bind either the PP2.1 or PP2.2 motifs (Macdonald *et al.*, 2004).

Molecular modelling of the PP2.2–FynSH3 domain interaction suggested that the central variable region of the RT loop did not interact directly with the polyproline helix. This was consistent with data from other systems (e.g. HIV-1 Nef), showing that this region of the RT loop plays a key role in determining the specificity of binding by interacting with other parts of the ligand – thus, a 12-mer peptide spanning the Nef PxxP motif bound equally (and rather poorly) to Fyn and HckSH3, whereas full-length Nef bound tightly to HckSH3, but not to FynSH3 (Saksela *et al.*, 1995). It was shown previously that replacing the Hck RT loop (EAIHHE) with that of Fyn (EARTED) reduced binding to Nef considerably (Lee *et al.*, 1995). The opposite was true for NS5A – we saw an increase in binding of NS5A to HckSH3 when the RT loop was replaced with that of Fyn, and a corresponding decrease in binding to FynSH3 with an Hck-like RT-loop sequence. These data imply that, as well as the PP2.2 motif, other sequences within NS5A contribute to the interaction. The identity of these sequences remains to be elucidated; however, we predict that these sequences will lie N-terminal to PP2.2, as our data show that a C-terminal deletion from residue 363 was able to bind SH3 domains as well as full-length NS5A (data not shown). Furthermore, we have shown that the N-terminal 270 residues of NS5A are dispensable for SH3 domain binding. A recent study provided evidence for a three-domain structure of NS5A (Tellinghuisen *et al.*, 2004), with the second domain consisting of residues 250–342 and the polyproline region corresponding to a flexible linker between domains 2 and 3. We are thus focusing our attention on domain 2 to identify additional intermolecular contacts between NS5A and SH3 domains.

Molecular modelling also predicted a key role for Arg356 in the stabilization of the PP2.2–SH3 interaction by the formation of a salt bridge with an acidic residue (Asp100 in FynSH3). This prediction was confirmed by experimental analysis. Interestingly, the binding of NS5A to LynSH3 was only abolished when the mutation Arg356–Ala was introduced in the context of PA2.2. The data presented here suggest that, although the proline residues within PP2.2 are dispensable for binding to LynSH3 (as LynSH3 can bind PP2.1), Arg356 is not. It is not clear why binding of LynSH3 to the PP2.1 motif is dependent on Arg356; however, this may be the result of structural constraints. In this regard, secondary-structure predictions suggest that the two class II motifs are highly hydrophilic and are likely to be surface-exposed; furthermore, mutation of the arginines to alanines is predicted to reduce both the hydrophilicity and surface probability of these motifs (data not shown). Further mutagenesis, combined with sensitive

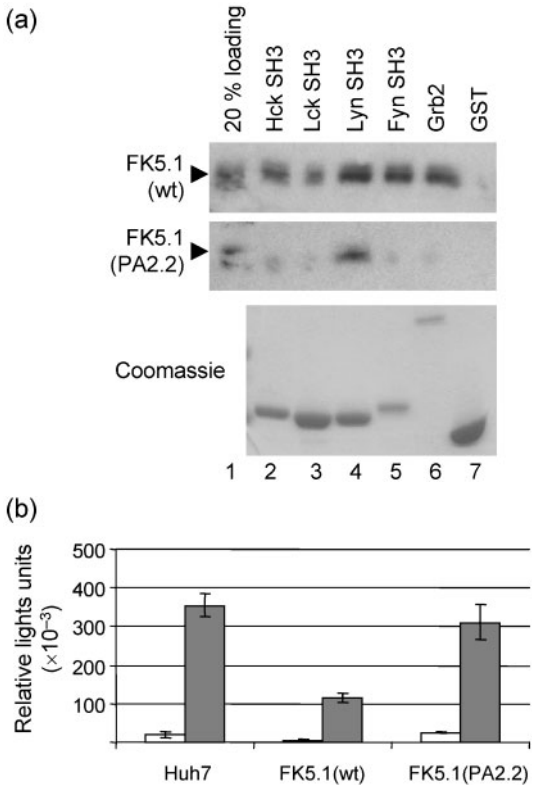

Fig. 6. The PA2.2 mutation abolishes the ability of NS5A to interact with SH3 domains or modulate AP-1 activity in replicon cell lines. (a) Lysate from Huh-7 cells harbouring either the parental FK5.1 or mutant FK5.1(PA2.2) replicons were incubated with the indicated GST-fusion proteins, immobilized on glutathione–agarose beads and analysed by Western blotting with a sheep polyclonal anti-NS5A antiserum. Lane 1 shows 20 % of the lysate used in each assay. A representative SDS-PAGE gel was stained with Coomassie blue to show equivalent loading of GST-fusion proteins. Note that GST–Grb2 contained the entire Grb2 protein and is thus larger than the GST–SH3 domain fusion proteins. (b) Cells were transfected with 1 μg plasmid pAP1-luc (Macdonald *et al.*, 2003) and placed in low-serum (0·5 %) growth medium for 18 h. Cells were either stimulated by the addition of 100 ng EGF ml^{-1} (grey bars) or left unstimulated (white bars), and harvested 6 h later. The level of expression of the luciferase reporter was assayed by using a luminometer and normalized for transfection efficiency by using a co-transfected Renilla luciferase control plasmid. Results are means of three independent experiments.

quantitative methods such as SPR, will be required to fully characterize the interactions between NS5A and SH3 domains; such work is currently under way in our laboratory.

The highly conserved nature of the PP2.2 motif suggested that it was likely to play an important role at some stage during the virus replication cycle. The absence of a robust *in vitro* system that recapitulates the complete infectious cycle of HCV makes this a difficult question to address; however, as a first stage in this process, we used the replicon system to ask whether the PP2.2 motif plays a role in viral genomic RNA replication. Our data show that this motif is dispensable for replicon function: in colony-forming assays, the FK5.1(PA2.2) replicon was quantitatively and kinetically indistinguishable from the parental FK5.1 replicon. Although we demonstrated that the PA2.2 mutation had not reverted during selection, we cannot rule out the possibility of second-site compensatory mutations within the NS3–5B coding sequence that would allow the PA2.2 mutant to replicate. This seems unlikely, however, particularly in light of the observation (Zech *et al.*, 2003) that transient replication of a luciferase reporter-based PA2.2 mutant replicon was only reduced twofold. A detailed analysis of viral RNA replication and protein expression in FK5.1(PA2.2) replicon cells is required to clarify this situation – such studies are under way. It is also possible that the PP2.2 motif might be dispensable for viral RNA replication in Huh-7 cells, a transformed tumour-cell line, but could play a role in the natural site of virus replication (primary human hepatocytes).

In this study, we show that activation of the transcription factor AP-1 is reduced in Huh-7 cells harbouring the FK5.1 replicon, but not the FK5.1(PA2.2) mutant. Given that we have previously shown that ERK activation is reduced in replicon cells (Macdonald *et al.*, 2003), the reduction in AP-1 activation is most likely to be due to the NS5A-mediated inhibition of the Ras-ERK pathway (Georgopoulou *et al.*, 2003; Macdonald *et al.*, 2003). Interestingly, interleukin 1 (IL1) has been shown to reduce replicon replication approximately fivefold via the activation of ERK (Zhu & Liu, 2003); we therefore predict that, by reducing ERK activation, NS5A is able to effect a corresponding resistance to IL1 treatment and, furthermore, that the PA2.2 mutant replicon would exhibit an increased sensitivity to IL1 treatment. Again, these ideas remain to be tested.

In conclusion, we have shown that NS5A–SH3 interactions are not absolutely required for viral RNA replication in Huh-7 cells, but do modulate host-cell signalling pathways; it is likely, therefore, that these interactions may play more subtle roles in either the replication or pathogenesis of HCV infection.

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