The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity

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The hepatitis C virus (HCV) non-structural NS5A protein has been shown to associate with a variety of cellular signalling proteins. Of particular interest is the observation that a highly conserved C-terminal polyproline motif in NS5A was able to interact with the Src-homology 3 (SH3) domains of the adaptor protein Grb2. As it has previously been shown that specific polyproline motifs can interact with a range of SH3 domains, we investigated whether NS5A was capable of interacting with other SH3 domain-containing proteins. We show here that NS5A interacts with the SH3 domains of members of the Src family of tyrosine kinases: a combination of in vitro binding assays and co-immunoprecipitation experiments revealed an interaction between NS5A and Hck, Lck, Lyn and Fyn, but interestingly not Src itself. Mutational analysis confirmed that the polyproline motif responsible for binding to Grb2 also bound to the SH3 domains of Hck, Lck, Lyn and Fyn. Furthermore, a previously unidentified polyproline motif, adjacent to the first motif, was also able to mediate binding to the SH3 domain of Lyn. Using transient transfections and Huh-7 cells harbouring a persistently replicating subgenomic HCV replicon we demonstrate that NS5A bound to native Src-family kinases in vivo and differentially modulated kinase activity, inhibiting Hck, Lck and Lyn but activating Fyn. Lastly, we show that signalling pathways controlled by Src-family kinases are modulated in replicon cells. We conclude that the interactions between NS5A and Src-family kinases are physiologically relevant and may play a role in either virus replication or pathogenesis.

INTRODUCTION

Hepatitis C virus (HCV) is the major aetiological agent of transfusion-associated chronic viral hepatitis. An estimated 2% of the population worldwide is currently infected with HCV; more than 80% of these persons are predicted to develop chronic hepatitis with up to 30% progressing to develop cirrhosis and 5% developing liver tumours (Lavanchy et al., 1999). Treatment is limited to the combination of IFN-α with the nucleoside analogue ribavirin, which is effective in less than 30% of patients.

The genome of HCV is a 9–5 kb positive-sense RNA molecule that is translated via a cap-independent mechanism to generate a 3000 residue polyprotein, subsequently cleaved by a combination of host cell and viral proteases into structural and non-structural proteins. The NS5A protein is one of six non-structural proteins of HCV that are likely to form an RNA replication complex (Bartenschlager & Lohmann, 2000). Aside from this role, an increasing body of evidence suggests that NS5A may also interfere with host cell functions. Indeed, NS5A has been demonstrated to interact with and inhibit the IFN-induced kinase PKR (Gale et al., 1998) and has also been shown to interact with the cell cycle regulatory machinery, promoting anchorage-independent growth in NIH3T3 murine fibroblasts and tumour formation in nude mice (Ghosh et al., 1999); NS5A can repress transcription of the cell cycle repressor gene p21^{WAF1}, while upregulating PCNA expression, effects that may be mediated by reported physical associations of NS5A with p53 (Majumder et al., 2001) and a novel transcription factor SRCAP (Ghosh et al., 2000).

Recently, it was shown that NS5A was capable of perturbing mitogenic signalling pathways by interacting with the adaptor protein Grb2 (Tan et al., 1999). This interaction was mapped to a highly conserved polyproline motif near the C terminus of NS5A, which was shown to bind to the SH3 domains of Grb2. SH3 domains are widely distributed among signalling proteins. They share a common structure but individual SH3 domains exhibit different binding specificities mediated both by amino acid sequences within the RT loop of the SH3 domain and sequences flanking the polyproline motifs of the binding protein (Mayer, 2001). Two classes of polyproline motif are defined by the location...
of a conserved basic residue: in Class I motifs the basic residue is situated at the N terminus, whereas Class II motifs contain a C-terminal basic residue. The position of the basic residue dictates the binding orientation of the motif. One key group of SH3 domain-containing proteins is the Src family of non-receptor tyrosine kinases (Tatosyan et al., 2000), nine proteins that share a common domain structure and mode of regulation. Interestingly, these kinases are a common target for virus interference with cell signalling, particularly in the case of viruses that establish chronic infections (Collette & Olive, 1997). In this regard HCV has not been shown to interact with Src-family kinases and we set out to investigate whether the polyproline motifs in NS5A might mediate such an interaction.

Here we demonstrate that two closely spaced Class II polyproline motifs near the C terminus of NS5A were able to bind to isolated SH3 domains of members of the Src kinase family in vitro. Co-immunoprecipitation analysis coupled with in vitro kinase assays demonstrated that NS5A could interact with, and modulate the activity of, native Src-family kinases in vivo. Importantly, using Huh-7 derived cells harbouring subgenomic HCV replicons, we reveal that expression of NS5A in the context of the other non-structural proteins is able to differentially regulate the activity of endogenous Src-family kinases and perturb Src-family regulated signalling pathways. These data suggest that, in common with other viruses such as human immunodeficiency virus (HIV) and Epstein–Barr virus (EBV) that can establish persistent or chronic infections, HCV is able to interact with and modulate the activity of Src-family kinases.

**METHODS**

**DNA manipulations.** NS5A sequences from HCV genotypes 1a (H77) (Yanagi et al., 1997), 1b (J4) (Yanagi et al., 1998), 2a (J6) (Yanagi et al., 1999) (kindly provided by Jens Buhk, NIH, Bethesda, MD, USA) and BVDV CP-7 (Meyers et al., 1997) (kindly provided by Gregor Meyers, Tübingen, Germany) were amplified by PCR and cloned into the expression vector pSG5 (Green et al., 1996) using an appropriate template and overlapping internal oligonucleotide primers. All constructs were verified by dideoxy sequencing. Coding sequences for Src-family kinases were subcloned into pSG5 from cDNA clones of Hck, Lck, Fyn and c-Src (kindly provided by Mark Marsh, UCL, UK) and Lyn (kindly provided by Kalle Saksela, University of Tampere, Finland).

**Tissue culture.** Cos-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 IU penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. Huh-7 cells were cultured as for Cos-7 cells with the addition of 1% non-essential amino acids. The generation of Huh-7 cells harbouring an HCV replicon has been described elsewhere (Macdonald et al., 2003).

**Immunoprecipitation and Western blotting.** Cells were transfected with pSG5 vectors expressing Src-family kinases alone or in conjunction with pSG5.NS5A vectors using Lipofectin (Invitrogen). After 24 h cells were lysed in modified RIPA buffer (150 mM NaCl, 50 mM Tris/HCl pH 8.0, 1% NP-40, 0.1% SDS, 5 mM EDTA, 1 mM Na₂VO₄, 1 mM NaF, 500 nM cantharidin, 500 nM calyculin and 10% glycerol) and equal amounts of protein (0.5 mg) precipitated with anti Src-family kinase monoclonal antibodies and protein-G beads (Santa Cruz). Immunoprecipitates were washed three times in lysis buffer and resolved by SDS-PAGE. Western blotting was performed with a sheep polyclonal anti-NS5A antiserum or commercially available antibodies to Src-family kinases, phosphotyrosine (PY69), tyrosine-phosphorylated STAT3 (all Santa Cruz) or STAT3 (Upstate) using standard techniques. Immunoblots were visualized by using an enhanced chemiluminescence system.

**Kinase assays.** To assay for Src-family kinase activity cells were lysed as above, and protein was precipitated by using appropriate monoclonal antibodies and protein-G beads. After three washes with lysis buffer and two with kinase buffer (20 mM HEPES/KOH pH 7.4, 10 mM MgCl₂, 1 mM NaF, 1 mM Na₂VO₄, 500 nM cantharidin), immunoprecipitates were incubated with kinase buffer containing 2 µg acid-denatured enolase (Sigma) and 5 µCi [³²P]ATP for 15 min at 30 °C. The reactions were terminated by the addition of Laemmli loading buffer, boiled for 5 min at 90 °C and resolved by SDS-PAGE.

**Expression and purification of GST fusion proteins.** Vectors for bacterial expression of the GST–SH3 fusion proteins were obtained from Kalle Saksela (University of Tampere, Finland) and used as described (Hiipakka et al., 1999). GST–Grb2 was obtained from John Ladbury (UCL, UK). Expression and purification of the GST fusion proteins were carried out by standard methods as described (Smith & Johnson, 1988).

**In vitro GST–SH3 binding assay.** GST–SH3 fusion proteins were bound to GA-beads at 4 °C for 1 h. Equal quantities of lysates from cells transiently transfected with the appropriate pSG5 vectors, or [³⁵S]methionine-labelled in vitro transcription/translation reactions (Promega TNT) programmed with pSG5.NS5A(BVDV) were incubated with beads in cell lysis buffer. After 3 h of incubation, beads were washed extensively in lysis buffer. Proteins were analysed by SDS-PAGE followed by autoradiography or Western blotting using an NS5A-specific antibody. GST alone was used as a negative control.

**Luciferase assay.** Cells were seeded into six-well dishes 1 day prior to transfection and subsequently transfected using Lipofectin (Invitrogen) as previously described (Macdonald et al., 2003). For Fyn co-transfections, the final concentration of DNA was kept constant with the addition of empty expression vector as appropriate. After transfection cells were incubated in reduced serum growth media (0.5% FCS) at 37 °C overnight prior to analysis. Where appropriate cells were treated with 10 µM PD98059 or 0.5 µM PD2 (both from Calbiochem) 1 h prior to stimulation with growth media containing 10% serum. Quantification of relative light units (RLU) was done as previously described (Macdonald et al., 2003).

**RESULTS**

**HCV NS5A binds to the SH3 domain of Src-family tyrosine kinases via a C-terminal proline rich region in vitro**

It had been previously shown that a class II polyproline motif located near the C terminus of NS5A interacts with the SH3 domains of the adaptor protein Grb2 (Tan et al., 1999). Because polyproline motifs are able to bind to specific subsets of SH3 domains we set out to determine whether NS5A could interact with other SH3 domain-containing proteins. Accordingly, we used a panel of GST
fusion proteins containing the SH3 domains derived from the Src-family kinases Hck, Lck, Lyn, and Src itself, or the guanine nucleotide exchange factor Vav, to precipitate lysates from Cos-7 cells transiently transfected with plasmids driving expression of NS5A from HCV genotypes 1a, 1b and 2a. Precipitated proteins were analysed by Western blotting with a sheep polyclonal anti-NS5A antiserum. Fig. 1(a) confirms that, as previously shown, NS5A(1b) was able to interact with a GST–Grb2 fusion protein (lane 8) but did not bind to a GST–SrcSH3 fusion protein (lane 9). NS5A(1b) also interacted with the SH3 domains of Hck, Lck, Lyn and Fyn (lanes 2–5), but failed to bind to either of the two SH3 domains of Vav (lanes 6 and 7) or GST alone (lane 10). This pattern of specificity was conserved in NS5A from HCV genotypes 1a and 2a. Precipitated proteins were analysed by Western blotting with a sheep polyclonal anti-NS5A antiserum.

Fig. 1. HCV NS5A binds to the SH3 domains of Src-family kinases in vitro. (a)–(c) Lysates from Cos-7 cells transiently transfected with pSG5.NS5A plasmids were incubated with the indicated GST fusion proteins immobilized on glutathione–agarose beads. Precipitates were analysed by SDS-PAGE and Western blotting with a sheep polyclonal anti-NS5A antiserum. (d) Because of a lack of suitable immunological reagents a coupled in vitro transcription/translation system (Promega T N T) was programmed with pSG5.NS5A(BVDV) and the reaction products incubated with the indicated GST-fusion proteins. Precipitates were analysed by SDS-PAGE and autoradiography. (e) 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.

Visual alignment of amino acid sequences revealed the presence of two conserved polyproline regions within NS5A: an N-terminal class I polyproline motif and a C-terminal proline-rich region containing two class II polyproline motifs. We focussed our analysis on these two regions of NS5A and for clarity these motifs will be referred to as PP1 (class I), PP2.1 – a previously unidentified class II motif only completely conserved in HCV genotypes 1a and 1b, and PP2.2 – the highly conserved motif previously shown to bind Grb2 (Tan et al., 1999) (Fig. 2a). In order to define the role played by each of these motifs in binding to Src-family kinase SH3 domains we generated four NS5A(1a) mutants in which specific proline residues were substituted by alanine: PA1 (Pro29/32-Ala), PA2.1 (Pro343/345/346-Ala), PA2.2 (Pro350/353/354-Ala) and PA2.1/2.2.

GST–SH3 fusion proteins were used to precipitate lysates from Cos-7 cells transfected with pSG5 vectors expressing these NS5A mutants. Mutation of either PP1 (PA1, Fig. 2b), or the poorly conserved PP2.1 motif (PA2.1, Fig. 2c), had no effect on the ability of NS5A(1a) to bind to the SH3 domains of either Src-family kinases or Grb2. As expected, mutation of PP2.2 (PA2.2, Fig. 2d) abrogated binding to Grb2 and the SH3 domains of Hck, Lck and Fyn; surprisingly, however, this mutant retained the ability to bind Lyn SH3. This raised the possibility that either the PP2.1 or PP2.2 motifs could bind Lyn SH3, a hypothesis confirmed by the observation that the double mutant (PA2.1/2.2) failed to bind any of the SH3 domains tested (Fig. 2e). As controls, NS5A(1a) and all the mutants analysed bound to a GST–PKR fusion protein, suggesting that introducing these mutations had not altered the overall folding of the protein (data not shown). These data demonstrate that NS5A interacts with the SH3 domains of Hck, Lck and Fyn via a previously identified C-terminal class II polyproline motif. In contrast Lyn SH3, at least in the case of NS5A(1a), is able to bind both the previously characterized motif and a newly identified polyproline motif within the C terminus of NS5A.

in vitro was conserved within the Flaviviridae family, NS5A from the pestivirus bovine viral diarrhoea virus (BVDV) was expressed using an in vitro transcription/translation system (due to lack of suitable antibodies for immunodetection). No interaction was observed between BVDV NS5A and SH3 domains in vitro (Fig. 1d), consistent with a lack of distinguishable polyproline motifs within BVDV NS5A. Control experiments confirmed that in vitro translated HCV NS5A bound to SH3 domains as expected (data not shown); thus the lack of binding of BVDV NS5A was not due to the different expression system. Equal loading and integrity of the various GST fusion proteins was verified by Coomassie staining (representative gel shown in Fig. 1e). These data demonstrate that the ability of NS5A to interact with SH3 domains is not restricted to Grb2, as NS5A is able to interact with SH3 domains derived from members of the Src family of tyrosine kinases, although interestingly not with the SH3 domain of Src itself.
**NS5A interacts with and modulates Src-family kinase activity in vivo**

These data clearly showed that NS5A was able to interact with the SH3 domains of Src-family kinases. In order to determine whether NS5A was also able to interact with the full-length, native kinases we co-transfected Cos-7 cells with pSG5 vectors expressing both Src-family kinases and either NS5A(wt) or defined proline motif mutants (PA2.2 or PA2.1/2.2). As the interaction with Hck, Lck or Fyn SH3 domains was abolished by the PA2.2 mutation we used the NS5A(PA2.2) mutant as a control in co-transfections with either Hck, Lck or Fyn. Src-family kinases were subsequently immunoprecipitated and their activity analysed by *in vitro* kinase assay, measuring both autophosphorylation and phosphorylation of the exogenous substrate, enolase. Immunoprecipitates were also analysed by Western blotting for NS5A to determine whether the two proteins could form a stable complex *in vivo*. The top two Western blots in each panel of Fig. 3 confirm that transfected cells expressed the appropriate Src-family kinase and NS5A. Panels (a) and (b) clearly show that the kinase activity of both Hck and Lck was inhibited by NS5A(wt) but unaffected by NS5A(PA2.2); the corresponding Western blots show that NS5A(wt) was able to bind to both Hck and Lck, but that NS5A(PA2.2) failed to bind to either kinase (although a very low level of binding was observed for Hck). In contrast, panel (c) shows that the activity of Fyn was stimulated by NS5A(wt) but unaffected by NS5A(PA2.2); again this modulation of kinase activity corresponded with the ability of NS5A(wt) to co-precipitate with Fyn. In agreement with the inability of the Src SH3 domain to bind to NS5A (Fig. 1), NS5A did not co-precipitate with Src, and the presence of either NS5A(wt) or NS5A(PA2.2) had no effect on Src activity (panel d).

In contrast to Hck, Lck and Fyn, panel (e) demonstrates that Lyn co-precipitated with both NS5A(wt) and NS5A (PA2.2). Furthermore, both of these proteins modestly reduced the activity of Lyn. This observation is consistent with the data in Fig. 2 showing that both the PP2.1 and PP2.2 motifs could bind Lyn SH3. To confirm that binding of NS5A to full-length, native, Lyn was mediated by these motifs we co-transfected cells with Lyn and the NS5A (PA2.1/2.2) mutant, which we had previously shown was unable to bind the isolated Lyn SH3 domain. As expected NS5A(PA2.1/2.2) failed to co-precipitate with Lyn and had no effect on the activity of Lyn.

Taken together, these data demonstrate that NS5A is able to modulate the activity of Src-family kinases *in vivo*; this effect correlates with the ability of NS5A to bind to Src-family kinases *in vivo*. The correlation between the binding of NS5A to native Src-family kinases, and the binding to isolated SH3 domains, points to a critical role for polyproline:SH3 domain binding in mediating these interactions.

**HCV replicon cells exhibit differential activity of endogenous Fyn and Lyn in response to mitogenic stimulation**

To further address the physiological significance of the NS5A:Src-family kinase interaction, we employed the Huh-7 pFK-Igsneo/NS3-3'5.1 cell clone (Krieger et al., 2001) as a model system. These cells carry a selectable self-replicating culture-adapted subgenomic HCV RNA and express the NS5A protein in the context of the...
non-structural polyprotein. Preliminary experiments had shown that Huh7 cells express both Fyn and Lyn, in contrast, we were unable to detect significant levels of either Hck or Lck in these cells (data not shown); thus we focused our analysis on Fyn and Lyn. Cells expressing the HCV replicon (FK5.1) or a control Huh-7 cell line stably expressing HCV NS5A and Src kinases

Fig. 3. NS5A binds to Src-family kinases and modulates phosphotransferase activity in vivo. Cos-7 cells were transfected with pSG5 vectors driving the expression of Src-family kinases in the presence of pSG5.NS5A(wt), pSG5.NS5A(PA2.2), pSG5.NS5A(PA2.1/2.2) or empty pSG5 expression vector as indicated. Cells were incubated in growth medium for 18 h and then lysed in lysis buffer. Src-family kinases were immunoprecipitated with monoclonal antibodies bound to protein G–agarose; precipitates were then either analysed by Western blotting with a polyclonal antiserum directed against NS5A, or subjected to in vitro kinase assays where kinase activity was analysed by measuring in vitro autophosphorylation and phosphorylation of acid-denatured enolase in the presence of [γ-32P]ATP. In vitro kinase reactions were separated by SDS-PAGE and visualized by autoradiography. The position of the autophosphorylated kinase is indicated by the open triangles (△); phosphorylated enolase is indicated by filled triangles (●). Lysates were analysed for equal expression of Src-family kinases and NS5A by Western blot analysis (top two panels of each section).
neomycin phosphotransferase (HuH-7neo) were grown in reduced serum growth medium to suppress mitogenic signalling pathways, and then stimulated with mitogens (EGF, insulin and PMA). Lysates were immunoprecipitated with antibodies to either Fyn or Lyn and subjected to either in vitro kinase assays (Fig. 4b, e) or Western blotting for Fyn (Fig. 4a), Lyn (Fig. 4d) or NS5A (Fig. 4c, f). The addition of mitogens stimulated the autophosphorylation and activation of both kinases. In FK5.1 cells, EGF- or insulin-stimulated Fyn activity was significantly higher than in control HuH-7neo cells, consistent with the transient transfection data in Fig. 3(c). In contrast EGF- or insulin-stimulated Lyn activity was reduced in FK5.1 cells, again consistent with the data in Fig. 3(e). However, for both Fyn and Lyn, PMA treatment appeared to partially override any replicon specific modulation of kinase activity (lanes 7 and 8). Importantly, Fig. 4(c, f) also shows that NS5A forms a stable complex with both Fyn and Lyn in FK5.1 cells, confirming the data in Fig. 3(c, e). These data demonstrate that in a physiologically relevant context NS5A both interacts with and differentially modulates Src-family kinases.

### Perturbation of Src-family kinase mediated signalling events in HCV replicon cell lines

At this stage in the study a key question that remained to be answered was whether the modulation of Src-family kinase activity by NS5A had any physiological consequences for the cell. Src kinases regulate a bewildering array of cell signalling events so in order to provide evidence for such physiological relevance we investigated a previously documented effect of NS5A, namely activation of the transcription factor STAT3. We chose STAT3 activity for two reasons: firstly, because STAT3 is a substrate for Src-family kinases including Hck, Lyn and Fyn (Schreiner et al., 2002), and secondly NS5A-expressing HuH-7 cells were recently shown to exhibit elevated tyrosine phosphorylation of STAT3 (Gong et al., 2001). The mechanism of this activation was not examined, although it was indirectly linked with altered calcium homeostasis. We reasoned that, as Fyn is expressed in HuH7 cells, NS5A-mediated activation of STAT3 in cells harbouring the subgenomic replicon would result in the concomitant activation of STAT3.

To test this hypothesis HuH-7neo and FK5.1 cells were transfected with a β-casein promoter luciferase reporter construct (responsive to STAT3) (Berchtold et al., 1997) and treated with inhibitors of Src-family kinases (PP2) or the Ras–ERK pathway (PD98059). As shown in Fig. 5(a), in comparison to HuH-7neo, FK5.1 cells exhibited a fivefold elevation in STAT3 activity (bars 1 and 2), in both cell lines this activity was inhibited by PP2 (bars 3 and 4) but unaffected by PD98059 (bars 5 and 6), suggesting that STAT3 activity in HuH7 cells is regulated by Src kinases but not by the Ras–ERK pathway. Further proof of this activation was shown by co-transfection with pSG5-Fyn, which resulted in a significant increase in STAT3 activity. In control HuH-7neo cells the addition of exogenous Fyn resulted in a sevenfold increase in STAT3 activity (compare bars 1 and 7); however, in FK5.1 cells, this increase was only twofold (compare bars 2 and 8), consistent with the higher activation of Fyn in FK5.1 cells.
basal STAT3 activity observed in FK5.1 cells. Again, the increase in STAT3 activation following Fyn transfection was partially inhibited by PP2 (bars 9 and 10), but unaffected by PD98059 (data not shown). These data were supported by Western blot analysis of STAT3 tyrosine phosphorylation (Fig. 5b), showing that levels of STAT3 phosphorylation correlate very closely with transcriptional activity. Overall expression (Fig. 5c) and serine phosphorylation of STAT3 (data not shown) were unaltered. To confirm that the activity of Fyn also correlated with STAT3 activity we analysed the autophosphorylation of Fyn – as previously shown in this paper (Figs 3 and 4) and by others (e.g. Briggs et al., 2000) this correlates with kinase activity. Fyn was therefore immunoprecipitated and analysed by Western blot using a phosphotyrosine-specific antibody (PY69). This analysis confirmed that levels of Fyn autophosphorylation (Fig. 5d) correlated with both the activity and tyrosine phosphorylation of STAT3. These data are thus consistent with the hypothesis that NS5A binds to and activates Fyn, thereby stimulating Fyn-mediated tyrosine phosphorylation of STAT3.

**DISCUSSION**

Src-family tyrosine kinases are a common target for viral interference with cellular signalling pathways (Collette & Olive, 1997). Viral protein interactions with Src-family kinases are mediated predominantly by two types of molecular recognition event: firstly the interaction of phosphotyrosine residues with SH2 domains, for example EBV LMP2A association with Lyn (Miller et al., 1995), and secondly the interaction of polyproline motifs with SH3 domains, as exemplified by the association of HIV-1 Nef with Hck, Lck, Lyn, Fyn and Src (Briggs et al., 2000; Saksela et al., 1995). Our data clearly place NS5A in this second category, as it is able to bind the SH3 domains of Hck, Lck, Lyn and Fyn. Interestingly, as previously shown, NS5A was unable to bind to the Src SH3 domain; neither did NS5A bind either of the SH3 domains of Vav. The molecular basis for this binding specificity is currently under investigation. NS5A from various genotypes of HCV exhibited a similar binding profile of SH3-binding specificity, but BVDV NS5A did not interact with any of the SH3 domains tested. However, we cannot rule out the possibility that BVDV NS5A might interact with host cell signalling proteins via distinct mechanisms.

Our data demonstrate that NS5A is not only able to interact with native Src-family kinases in cell culture, but also to differentially modulate their activity – in the case of Hck, Lck and Lyn activity was inhibited, whereas the activity of Fyn was stimulated. This situation is reminiscent of two other viral proteins that interact with Src-family
kinases: the HIV-1 Nef protein and the murine polyoma-virus middle-T antigen. Nef binds to the SH3 domains of Hck, Lck, Fyn, Lyn and Src; it activates Hck, suppresses Fyn and has little effect on the other three kinases (Briggs et al., 2000). Middle-T binds to the catalytic domains of Src, Yes and Fyn; binding to Src and Yes activates kinase activity whereas middle-T has no effect on Fyn (Ichaso et al., 2001). Regulation of Src-family kinase activity is mediated by a number of factors including intramolecular interactions involving the SH2/SH3 domains. It is conceivable that there are subtle differences in the regulation of individual Src-family kinases that are exploited by N55A to effect differential modulation; alternatively, there may be other intermolecular interactions between N55A and Src-family kinases that contribute to the observed effects – in this regard it is pertinent that Hck exhibited some binding to the N55A(PA2.2) mutant, suggesting that other determinants may contribute to binding.

A key question that remains to be answered is: what are the physiological consequences of N55A–Src-family interactions for virus replication and pathogenesis? Although providing evidence for this is hampered by the absence of a robust in vitro replication system, as a first stage in answering this question we analysed both kinase activity and signalling pathways regulated by Src-family kinases in Huh-7 cells harbouring a subgenomic replicon. Activity of both Fyn and Lyn was dysregulated in these cells in comparison to control cells, consistent with data from transient transfections.

Lastly, our data demonstrate that N55A-mediated activation of both endogenous and exogenous Fyn in replicon cells results in the activation of the transcription factor STAT3. This observation sheds light on the mechanism of STAT3 activation by N55A reported by other groups (Gong et al., 2001), as it has been shown that STAT3 is a substrate for multiple Src-family kinases, including Fyn. Increasingly hepatocellular carcinoma (HCC) is associated with HCV infection (Zoulim et al., 2003) and in this regard it is of interest that constitutive STAT3 activation is associated with many tumours including HCC. Usually these tumours exhibit dysregulation of STAT3 activity (Yoshikawa et al., 2001), and we speculate therefore that indirect activation of STAT3 by N55A might contribute to the development of HCC in HCV-infected individuals.

Clearly, given that N55A both interacts with and modulates the activity of Fyn, the two proteins must co-localize within replicon cells. Although we do not have any direct evidence for co-localization, it is pertinent that many studies have demonstrated that N55A associates predominantly with the cytosolic face of the ER membrane (Brass et al., 2002). Fyn has also been reported to associate with, and phosphorylate, the ER membrane-located IP3-receptor (Jayaraman et al., 1996), providing indirect evidence for co-localization of N55A and Fyn.

In conclusion, Src-family kinases regulate a multitude of signalling pathways, including the arrangement of the actin cytoskeleton, the response to growth factor stimulation and, importantly, the response to cytokine stimulation. Although we have identified a specific effect of N55A on a signalling pathway controlled by Src-family kinases, the differential modulation of individual Src-family kinases by N55A is likely to have complex effects on the final response of infected cells to a variety of signalling events. Thus it may be possible that by varying the activity of each individual kinase, the role of N55A is to subvert and re-route the response of the cell to best suit HCV replication. Further speculation must await the results of further experimentation in this area.

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