Phosphorylated tyrosine 93 of hepatitis C virus nonstructural protein 5A is essential for interaction with host c-Src and efficient viral replication

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Running title: pY93 of NS5A is essential for interaction with c-Src

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ABSTRACT

The hepatitis C virus (HCV) nonstructural protein 5A (NS5A) plays a key role in viral replication and virion assembly, and regulation of the assembly process critically depends on phosphorylation of both serine and threonine residues in NS5A. We previously identified SRC proto-oncogene, non-receptor tyrosine kinase (c-Src) as an essential host component of the HCV replication complex consisting of NS5A, the RNA-dependent RNA polymerase NS5B, and c-Src. Pull-down assays revealed an interaction between NS5A and the Src-homology 2 (SH2) domain of c-Src; however, the precise binding mode remains undefined. In this study, using a variety of biochemical and biophysical techniques, along with molecular dynamics simulations, we demonstrate that the interaction between NS5A and the c-Src SH2 domain strictly depends on an intact, phosphotyrosine binding-competent SH2 domain and on tyrosine phosphorylation within NS5A. Detailed analysis of c-Src SH2 domain binding to a panel of phosphorylation-deficient NS5A variants revealed that phosphorylation of Y93 located within domain 1 of NS5A, but not of any other tyrosine residue, is crucial for complex formation. In line with these findings, effective replication of subgenomic HCV replicons as well as production of infectious virus particles in mammalian cell culture models were clearly dependent on the presence of tyrosine at position 93 of NS5A. These findings indicate that phosphorylated Y93 in NS5A plays an important role during viral replication by facilitating NS5A’s interaction with the SH2 domain of c-Src.

The hepatitis C virus (HCV) is one of the leading causes for chronic liver diseases worldwide and accounts for about 30% of all liver cirrhosis cases and 25% of patients with hepatocellular carcinoma. Approximately 60% of people that are infected with HCV develop a chronic infection. The prevalence of HCV is strongly region-dependent, with HCV infection rates ranging from 0.3 to 22%. Notably, despite ongoing replication with high serum titers a chronic HCV infection predominantly remains asymptomatic for decades, indicating that the virus is able to effectively circumvent host antiviral immunity and persist without severely affecting host cell viability (1).
This is achieved by particular virus-encoded proteins broadly interfering with a variety of different signaling intermediates of the host cell via direct protein-protein interactions to subvert host antiviral effector mechanisms and to ensure propagation of the virus life cycle. All viral proteins are generated from a polyprotein comprising ≈3000 amino acid residues, which is encoded by the positive sense, single-stranded RNA genome of HCV. Co- and posttranslationally, this precursor is processed by viral and host proteases into ten mature viral structural and nonstructural (NS) proteins. Out of the nonstructural proteins, NS3 to NS5B constitute the viral replication machinery. HCV infection triggers a complex reorganization of host cellular membranes, referred to as the membranous web, a subcellular structure comprised of single-, double-, and multi-membrane vesicles (2). The membrane fractions of the membranous web are enriched in viral NS proteins, and even after isolation retain viral replication activity (3,4). The rearrangement of cellular endoplasmic reticulum membranes into the membranous web is hypothesized to be controlled mainly by the viral proteins NS4B and NS5A, while NS5A also plays an important role in RNA replication and viral assembly (5-8).

NS5A is an oligomeric, multifunctional RNA-binding phosphoprotein, which is composed of an amphipathic N-terminal α-helix followed by three domains separated by two low-complexity sequences (LCSs) (9). NS5A domains 1 (D1) and 2 (D2) are important for RNA replication, whereas domain 3 (D3) is associated with viral particle assembly processes (5-7,10). Serine phosphorylation sites between NS5A domains 1 and 2 in the LCS-1 region (11,12) shift the apparent molecular mass from the basally phosphorylated (p56) to the hyperphosphorylated form (p58). The basally phosphorylated form drives the HCV life cycle towards replication, whereas hyperphosphorylation promotes viral assembly (7,13,14). Remarkably, NS5A has been found to interact not only with other viral proteins and RNA, but also with components of the host cell (8,15), which attests to the versatility of this multidomain protein. Purification of full-length NS5A from bacterial culture is very challenging and the full-length product shows drastically reduced stability when compared with the isolated D1 and the D2D3 construct (16). Consequently, structures of only truncated versions of NS5A could be determined, i.e., D1 (17-19) and D2, D3, or D2D3 (20,21). Based on crystal structures, several dimeric arrangements have been suggested for NS5A-D1 (17-19). These structures show similar monomer conformations but differ in their modes of dimerization, indicating that NS5A exposes a number of regions on the surface that facilitate self-association. In most of the reported dimeric forms the presumed RNA binding residues are readily accessible. In contrast to D1, D2 and D3 are highly flexible and act like intrinsically disordered proteins, although they show some local residual structure and a network of long-range interactions that play important roles in viral regulation and host cell interaction (16,21-23). In human hepatoma cell lines expressing an HCV subgenomic replicon (24), formation of a complex involving c-Src, NS5A, and NS5B was found to be a prerequisite for viral replication (25). c-Src belongs to the Src protein family of tyrosine kinases, which are involved in many intracellular signal transduction pathways. Pull-down assays with several recombinant glutathione S-transferase (GST)-c-Src deletion mutants and lysates of Huh-9-13 replicon cells revealed that the Src-homology (SH)2 domain of c-Src was required for direct interaction with NS5A (25). Initially, this finding was surprising, since NS5A contains a well characterized and highly conserved polyproline motif within its LCS-II region, which represents a canonical binding motif for SH3 domains (26). Indeed, the SH3 domain of Bin1 showed strong affinity to NS5A (27-29). Further analysis revealed that in addition to the canonical SH3 binding site, NS5A also displays two non-canonical SH3 binding regions (28,30). NMR studies of the c-Src-SH3:NS5A complex using recombinant and purified proteins revealed a comparatively weak interaction (30). Because the SH2 domain of c-Src is necessary for complex formation with NS5A (25), we postulate that the interaction of NS5A with c-Src mainly involves the SH2 domain rather than the SH3 domain. Canonical SH2 domain binding is mediated through phosphorylation of a tyrosine residue within the SH2 domain-interacting region of the respective protein (31). The presence of a conserved arginine within the SH2 domain is crucial as this arginine coordinates the phosphate group within the SH2 binding pocket (32). While
serine and threonine phosphorylation patterns of NS5A have been investigated thoroughly in the past (9), research on potential tyrosine phosphorylation is limited to two studies dealing with tyrosine-protein kinase Fyn-SH2 binding in B cells and tyrosine phosphorylation by c-Abl in human hepatoma cells, both transfected with a HCV subgenomic replicon (33,34). NS5A comprises several tyrosine residues, Y43, Y93, Y106, Y118, Y129, Y161 and Y181 in D1, Y321 and Y334 in LCS-II, and Y413 in D3 for the HCV consensus genotype 1b. The objective of this study was to identify and characterize NS5A tyrosine residues that are essential for c-Src-SH2 domain interaction.

RESULTS

NS5A from Huh-9-13 lysates associates with c-Src-SH2, but not with the pY-binding deficient R173K mutant

The interaction of NS5A with the SH2 domain of c-Src was investigated by preparing total protein extracts from cell lines harboring the HCV subgenomic replicon (Huh-9-13) and assessing the binding behavior of NS5A to GST-tagged c-Src-ΔSH1 (a construct lacking the kinase domain) and the respective phosphotyrosine (pY) binding deficient substitution mutant, c-Src-ΔSH1 R173K. The recombinantly produced Fyn SH2 domain (along with its R176K mutant) was included as a control because previous studies have shown that NS5A expressed in B-lymphocytes binds to recombinant Fyn-SH2 in a pull-down assay (33). Proteins were covalently immobilized to the beads in equal amounts and subsequently incubated with Huh-9-13 total protein extracts (Figure 1). NS5A was pulled down from extracts by both wild-type Fyn-SH2 and c-Src-ΔSH1. Conversely, negligible amounts of NS5A protein were precipitated using the respective mutants. This behavior strongly suggests a canonical binding mode between NS5A and the SH2 domains. The detection of NS5A in precipitates from SH2 pull-down experiments via immunoblotting was further validated by mass spectrometry analysis. For this an aliquot of the precipitated material was subjected to SDS-PAGE, and four gel slices in the 45–70 kDa region were excised. Prior to the analysis by HPLC-MS/MS, protein fragments were generated by in-gel trypptic digestion. The resulting mass spectrometric data could be assigned to NS5A for several of the peptides and longer protein fragments; peptides representing NS5A yielded a total sequence coverage of ≈50% (Table S1), thus providing clear evidence that the immunoblotting signal was specific and confirming that NS5A was precipitated by c-Src-SH2. Interestingly, peptides of NS5B (molecular mass 65 kDa) were identified in a gel slice comprising the 60 kDa range, with ≈50% coverage of the NS5B sequence (Table S1). This is consistent with NS5A-NS5B interaction models (35) and models describing the c-Src-SH3:NS5B interaction (25). In parallel, we analyzed the precipitated material for SH2-containing proteins that satisfy a sequence coverage of more than 5%. Notably, only c-Src and the tyrosine-protein kinase Yes could be identified by this approach (data not shown).

NS5A tyrosine screening confirms the presence of a canonical SH2 binding motif in NS5A

The identity of the specific pY residue of NS5A responsible for the interaction with c-Src-SH2 was investigated by a peptide ELISA-based assay (Table 1). As demonstrated in Figure 2B, the c-Src SH2 domain without (white squares) and with the SH3 domain (grey squares) showed similar binding behavior towards the various phosphopeptides, demonstrating that the SH2 domain is sufficient for interaction with NS5A. The SH3 domain plays no measurable role in the interaction. pY containing peptides were clearly favored over their non-phosphorylated versions (dark squares). Based on these results, peptides comprising pY93, pY106, pY129, pY161 located in D1 and pY413 located in D3 of NS5A were chosen for further quantitative analysis, because these peptides displayed a relative binding strength at least 20% that of the strongest binder (pY93). We determined their binding affinities to c-Src-ΔSH1, comprising its SH4, Unique, SH3, and SH2 domains (Figure S1), by fluorescence polarization (FP). As depicted in Figure 3 and Figure S2, peptides pY93 (0.7 ± 0.1 μM), pY129 (8.0 ± 0.8 μM), pY161 (2.2 ± 0.3 μM) and pY413con1b (1.8 ± 0.1 μM) displayed $K_d$ (dissociation constant) values in the sub- or low micromolar range, whereas the $K_d$ for pY106 was above 100 μM. Notably, $K_d$ values for the pY93 peptide interaction with c-Src-SH2 (0.5 ± 0.2 μM, Figure
3B) and c-Src-SH3SH2 (0.3 ± 0.1 μM, Figure 3C) were in the same range as with c-Src-ASH1 (Figure 3A), supporting the notion that the SH2 domain rather than the SH3 domain of c-Src is responsible for the interaction.

Tyrosine-phosphorylated D2D3 of NS5A only weakly interacts with c-Src

Y321, Y334 and Y413 of NS5A are, according to the structural model, likely to be surface exposed and thus candidates to mediate SH2 domain interactions when phosphorylated. Out of these, Y321 and Y334 are located in the LCS-II of NS5A, and Y413 is located in D3. Y413 was of particular interest given the results of the screening approach outlined above. The NS5A-D2D3 construct was recombinantly expressed in E. coli TKB-1 cells harboring an ELK tyrosine kinase gene, in order to examine the c-Src-SH2 binding properties towards phosphorylated NS5A-D2D3. ELK is known to mediate tyrosine phosphorylation of overexpressed proteins in a posttranslational and non-specific manner. For clarity, proteins expressed in the E. coli TKB-1 system, and therefore carrying phosphorylated tyrosine residues, are marked by the superscript “ELKpY”. Subsequent to its purification, immobilized NS5A-D2D3ELKpY was incubated with c-Src-ΔSH1 at various concentrations and interaction measured by biolayer interferometry (BLI). Using this approach, the fraction of phosphorylated and therefore possibly c-Src-SH2-binding NS5A-D2D3 in the immobilized material should not be critical. A $K_d$ of 18 ± 4 μM was determined (Figure 4), suggesting that tyrosine residues located in the D2D3 portion of NS5A at most weakly contribute to the pY-SH2 domain interaction between NS5A and c-Src.

Tyrosine-phosphorylated D1 of NS5A displays high affinity for c-Src

Next, we investigated the binding behavior of the purified and tyrosine-phosphorylated NS5A-D1 to c-Src. Y93, Y129 and Y161 within the D1 domain of NS5A are three potential sites for pY-SH2 domain interactions (Figures 2 and 3). When using immobilized c-Src-ΔSH1 as ligand and free NS5A-D1ELKpY as analyte (Figure 5A), a $K_d$ of 0.47 ± 0.1 μM was determined (Figures 5B). These data suggest that the interaction between NS5A and the SH2 domain of c-Src is mediated via tyrosine residues located in the D1 domain of NS5A and that there is at least one SH2 binding site within D1 that displays submicromolar affinity towards the c-Src SH2 domain. In line with these considerations, the non-phosphorylated D1 domain of NS5A was unable to bind immobilized c-Src-ΔSH1 (Figure 5C), nor did NS5A-D1ELKpY interact with c-Src-ΔSH1 R173K (Figure 5D).

Phosphorylation of Y93 is sufficient for binding of NS5A-D1 to c-Src-SH2

NS5A mutants Y93F, Y129F and Y161F, double mutants (Y93/129F, Y93/161F, Y129/161F) and the triple mutant (Y93/129/161F) were prepared to unequivocally identify the pY residue in the NS5A-D1 domain responsible for the interaction with c-Src-SH2. These NS5A-D1 mutants (Figure S1) were purified from ELK overexpressing cells, as described for the wild-type protein. As depicted in Figure 6A and B, loss of high affinity binding to c-Src-SH2 was observed when titrating NS5A-D1ELKpY Y93/129/161F. Thus, the list of candidate sites for the observed binding of D1 to c-Src-SH2 is restricted to Y93, Y129 and Y161. The single tyrosine mutants NS5A-D1ELKpY Y93F, NS5A-D1ELKpY Y129F and NS5A-D1ELKpY Y161F (Figures 6C, D, E; Figure S1; Figures S3A, B, C) were analyzed for their binding properties to immobilized c-Src-ΔSH1. As depicted in Figure 6C, substitution of Y93 to phenylalanine led to a drastic decrease in binding affinity, whereas the other mutations showed no significant effect on binding to c- Src-ΔSH1. Moreover, as shown in Figures 7A and B, the doubly substituted NS5A-D1ELKpY Y129/161F yielded a $K_d$ (0.7 ± 0.2 μM) that was similar to the wild-type protein. As position Y93 is thought to be involved in the binding site for the direct-acting antiviral (DAA) daclatasvir (36,37), we checked for altered binding of NS5A-D1ELKpY to c-Src-ΔSH1 in the presence of daclatasvir. As depicted in Figure S4A, the presence of up to 150 µM daclatasvir did not reduce the amount of NS5A-D1ELKpY bound to c-Src-ΔSH1 and therefore does not appear to affect the binding affinity. Finally, we checked the binding properties of the well-described daclatasvir resistant mutant NS5A Y93H (36) by BLI. To this end, we applied a concentration series of c-Src-ΔSH1 to immobilized NS5A-D1 Y93H. Consistent with our assumption that phosphorylation of Y93 is crucial for c-Src-SH2
binding, this mutant did not show any interaction with c-Src-ΔSH1 (Figure S4B).

Substitution of Y93 does not compromise structural integrity of the NS5A-D1 dimer

In order to assess if the mutations and phosphorylation of Y93 destabilize potential NS5A-D1 dimers, we performed explicit solvent, all-atom molecular dynamics (MD) simulations of 2 µs length. We utilized the non-crystallographic dimer from one of the available NS5A-D1 crystal structures (18) as the starting model into which the mutations as well as the phosphorylation were introduced. Within the time scale of our MD simulations, the wild-type dimer does not dissociate and only shows small local conformational changes, which is reflected by a root-mean-square distance (RMSD) with respect to the starting structure in the 4–7 Å range (Figure S5A). Note that the larger RMSD values originate from pronounced movements of the N- and C-termini (Figure S5A). The c-Src binding, Y93-phosphorylated (Y93pY2) variant features similar RMSD and per-residue root-mean-square fluctuation (RMSF) values, with most of the residues showing no significant increase in RMSF with respect to wild-type NS5A-D1 (Figure S5B). On the contrary, the viable but daclatasvir-resistant Y93H variant shows a statistically significant ($p < 0.05$) but only modest (< 2 Å) increase in the RMSF for many of its residues compared to the wild-type (Figure S5C). This modest increase in RMSF is also reflected by the slightly broader RMSD distribution (4–9 Å). Similar to the wild-type dimer, the dimer of the Y93H variant does not dissociate over the course of the MD simulations and appears to retain its structural integrity. Interestingly, the Y93F mutant, while compromised in terms of function, features dynamics reminiscent of the wild-type and the Y93pY2 variant (Figure S5D). To conclude, the MD simulations suggest that changes in the structural dynamics do not correlate with, and are thus unlikely to underlie, the functional differences between wild-type NS5A-D1 and the variants considered in this study.

The NS5A Y93F mutant severely impairs HCV replication and infectious virion production in mammalian cell culture models

Since phosphorylation of Y93 is clearly indispensable for interaction of NS5A-D1 with the SH2 domain of c-Src in vitro and this interaction is essential for viral replication in the host cell (25), prevention of Y93 phosphorylation should severely impair viral genome replication. In order to test this conjecture, the relevance of Y93 for HCV replication was investigated using the well-established HCV subgenomic replicon system (24) as well as the HCVcc infectious system (38). Point mutations in subgenomic HCV replicons have been shown to modulate their replication (39,40). To test the relevance of NS5A Y93 for viral replication, a subgenomic HCV replicon plasmid and a full-length HCVcc JC1 plasmid encoding the NS5A Y93F mutant were generated. Both wild-type and mutant replicon plasmids were used to transfect Huh-7 cells 24 h before measurement of subgenomic HCV RNA levels by real-time PCR (rtPCR, Figure 8A). The Y93F mutation resulted in significantly reduced HCV RNA compared with wild-type replicon levels (Rep), indicative of drastically impaired replication. Furthermore, Huh-7.5 cells were transfected with both wild-type and mutant HCVcc JC1 plasmids, and virus was collected from the supernatants over a time period of 72 h. Calculating the 50% tissue culture infective dose (TCID$_{50}$) revealed a 100 times lower viral titer for cells transfected with mutant HCVcc JC1 (Figure 8B). Consistently, HCV RNA levels were significantly reduced compared to levels in wild-type JC1-transfected cells (Figure 8C), confirming that mutation of NS5A at Y93 results in impaired viral replication. Taken together, these observations clearly demonstrate a central role of phosphorylated Y93 in binding of NS5A via its D1 domain to the SH2 domain of the cellular tyrosine kinase c-Src.

DISCUSSION

NS5A exists in a basally phosphorylated (p56) and a hyperphosphorylated (p58) form, and the phosphorylation state regulates the various functions of NS5A, which have to occur in a concerted manner during the life cycle of HCV. Many studies have addressed the sites of serine or
threonine phosphorylation as well as the participating kinases, and the related functional consequences (e.g., for viral genome replication or particle assembly) are now being realized (41,42). However, little is known about tyrosine phosphorylation within the HCV nonstructural proteins. Y334 (Con1, Y330 in JFH1) located at the carboxyl terminus of D2 of NS5A has been the only tyrosine phosphorylation site reported, and phosphorylation of this residue was suggested to be required for efficient HCV particle assembly (34). We have shown previously that the interaction of the HCV proteins NS5A and NS5B with host kinase c-Src is a prerequisite for viral replication (25), and that the SH2 domain of c-Src promotes the interaction with NS5A. In this work we investigated and characterized the c-Src-SH2 interaction site of NS5A in detail. The pull-down assays (Figure 1) and peptide ELISA (Figure 2) unambiguously revealed that the interaction follows a canonical mode that depends on the presence of a phosphorylated tyrosine. Peptide mapping results (Figures 2 and 3) combined with data from quantitative binding analysis with posttranslation ally phosphorylated purified protein domains (Figures 4 to 7) led to the identification of several candidate binding sites for c-Src-SH2 located in either D1 or D3 of NS5A. Especially the high-affinity peptides pY93, pY129, and pY413 contain amino acids with similar properties subsequent to the phosphorylated tyrosine (Table 1). While none of these NS5A peptides exactly matches the well-described pYEEI consensus motif for Src-SH2 (43), they do show overlap with the broader consensus “pY-hydrophilic-hydrophilic-I/P” mentioned in the same study. In this context, it is worth noting that also a less restricted consensus motif for c-Src-SH2 binding peptides has been proposed in the more recent literature (44). The observed binding affinities, with a $K_d$ of 0.47 μM for the phosphorylated D1, matched general expectations because SH2-ligand interactions range between $10^{-5}$ and $10^{-8}$ M (45,46). According to our results, D1 showed a roughly 40-fold higher affinity for c-Src-SH2 than D3 upon tyrosine phosphorylation, indicating that a site within D1 is the preferred target for c-Src-SH2. These observations are consistent with our current view that the HCV genome replication process is dependent on the D1 domain of NS5A (5) but also requires c-Src, which promotes formation of the NS5A:NS5B protein complex (25). Finally, our subsequent in-depth analysis of the pre-selected target sites within D1 led to the identification of Y93 as the crucial residue, which, upon phosphorylation, mediates c-Src-SH2 binding (Figures 6 and 7). Given these in vitro protein interaction data, we reasoned that Y93 phosphorylation could be an in vivo mechanistic prerequisite for efficient viral replication activity of the c-Src:NS5A:NS5B complex. To test this hypothesis, we exchanged Y93 with F both in a sub-genomic HCV replicon system and in the HCVcc JC1 infectious system, and found that the mutated systems show drastically decreased replication efficiencies when compared to the respective wild-type systems (Figures 8A and C). Accordingly, the virus titer decreased for the mutated strain JC1 Y93F by a factor of about 100 (Figure 8B). This is very likely based on a drastically decelerated virus production as a consequence of the low intracellular viral RNA levels in the presence of the NS5A Y93F mutant, which therefore can be classified as a “loss of fitness” mutant. Thus, our data support the importance of Y93 for the efficient production of viral RNA and, consequently, of new virions. NS5A is thought to also directly participate in viral assembly (5-8,10). As the Y93F mutant strongly reduces HCV RNA levels, however, we currently cannot draw conclusions regarding independent effects on virion assembly. Further investigation will be needed to address this issue in the future. Interestingly, latest DAAs like daclatasvir and ledipasvir inhibit NS5A via direct binding to D1 with low nanomolar affinity (37), thereby preventing RNA binding to NS5A and inhibiting viral replication. Available drug binding data indicate that Y93 is part of the daclatasvir binding site. However, our in vitro binding assay with phosphorylated NS5A and c-Src-ΔSH1 in the presence of daclatasvir did not reveal competitive inhibition by the compound, suggesting that it may be unable to bind NS5A molecules that display a phosphorylated tyrosine residue at position 93. It is important to realize that the dimeric arrangements derived from available NS5A-D1 crystal structures imply very different accessibilities of pY93 for an approaching SH2 domain. Figure S6 illustrates the expected positions of human c-Src SH2 domains bound to individual NS5A-D1 chains in a canonical fashion,
centering on the \(^{93}\text{pYTTG}^{96}\) segment. Obviously, only the dimer from the asymmetric unit of PDB ID 1ZH1 (left) will allow for binding of two SH2 domains, whereas in the other cases the SH2 domains would either overlap with neighboring NS5A chains (for PDB ID 3FQQ and the AB-dimer from PDB ID 4CL1), thus excluding NS5A dimerization, or clash with the second SH2 domain (CD dimer of PDB ID 4CL1), thus restricting the interaction to only one NS5A chain of a dimer. The 1ZH1-type dimer of NS5A has been previously suggested to recruit viral RNA to the replication complex by virtue of a basic groove located between the two subunits (17,19). Notably, our conceptual model indicates that association with up to two c-Src SH2 domains does not require large rearrangements in this particular NS5A-D1 dimer and should thus be sterically compatible with its presumed function in HCV replication.

Y93 in NS5A is widely conserved in HCV genotypes 1 through 5, consistent with an important function of this residue. Its location at the surface of the molecule, without participation in the hydrophobic core, suggests a role in protein-protein interactions rather than in structural integrity, which is an implicit premise of the mutational experiments described in this work. Indeed, our replica MD simulations did not reveal dissociation of dimers or unfolding of subunits for any of the variants investigated. In concert with biochemical evidence, these observations support the notion that the loss of function observed for the Y93F mutant results from the absence of a critical phosphorylation site.

Taken together, our data point to a complex role of Y93 during viral replication (summarized in Figure 9) because the preference of NS5A for certain binding partners as well as its susceptibility to DAAs like daclatasvir might be regulated through phosphorylation of this residue. Whether the responsible kinase is c-Src or another kinase such as c-Abl, which has been reported to phosphorylate Y334 of NS5A (34), has yet to be defined. Importantly, high-affinity binding of NS5A to c-Src-SH2 via pY93 should result in the loss of autoinhibition in c-Src; the latter is mediated by intramolecular binding of the SH2 domain to the C-terminally located pY530 and, additionally, by interaction of the SH3 domain with an intramolecular proline-rich motif in the SH2-kinase linker, thus forcing the kinase domain into an inactive conformation (reviewed in ref. 47). Therefore, we propose that NS5A binding to c-Src-SH2 via pY93 could lead to a constitutively active c-Src kinase, which facilitates the phosphorylation of tyrosine residues in diverse target molecules, possibly including NS5A. Constitutive activation of cellular kinases by viral proteins, accompanied by changes in cellular signaling cascades, is indeed a common theme. For instance, the accessory protein Nef from human immunodeficiency virus type 1 strongly binds to SH3 domains, particularly that of hematopoietic cell kinase, and this interaction leads to a constitutively active kinase that has severe effects on cellular cytokine signaling and secretion (48,49). Given the remarkable potency of NS5A-targeting DAAs like daclatasvir in the treatment of the HCV infection, specific interference with phosphotyrosine-mediated interactions may be envisaged as a widely-applicable strategy against viral pathogens.

**EXPERIMENTAL PROCEDURES**

**Cultivation of hepatoma cell lines**

The human hepatoma cell lines Huh-7 (50) and Huh-9-13 (51) were cultivated in Dulbecco’s modified Eagle’s medium (DMEM)/nutrient mix F-12 supplemented with 10% (v/v) heat-inactivated fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO₂. Huh-7.5 cells (52) were cultivated in DMEM containing 4.5 g/l glucose and supplemented with 9% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μl/ml nonessential amino acids (modified Eagle medium nonessential amino acids solution: Gibco, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The use of these cell lines is covered by a material transfer agreement with Apath, L.L.C (New York, USA).

**Preparation of Huh-9-13 cell lysates**

At 80% confluence, Huh-9-13 cells were washed with cold Dulbeco’s phosphate buffered saline (Thermo Fisher Scientific, Regensburg, Germany) and harvested in Huh-lysis buffer [20 mM Tris-HCl, 150 mM NaCl, 1% (v/v) Triton X-100, 2 mM EDTA, 50 mM β-glycerol phosphate, 1 mM...
sodium orthovanadate (OV), protease inhibitor cocktail (Roche, Basel, Switzerland), 0.2% (w/v) SDS, 10% (v/v) glycerol and 20 mM sodium pyrophosphate, pH 7.4]. Suspended cells were incubated for 10 min in Huh-lysis buffer, centrifuged for 20 min at 16,000 × g and the supernatant was collected for further analysis or frozen in liquid nitrogen and stored at -80 °C. Calcium phosphate precipitation and protein quantitation were done according to the manufacturer’s instructions (Roti-Quant, Carl Roth, Karlsruhe, Germany).

**Plasmids and mutagenesis of NS5A variants**
The plasmid pFK-I377/NS3-3´ (51) was used for mammalian expression of the subgenomic HCV replicon, and the plasmid pFK-JFH1J6C-846 dg (53) was used for mammalian expression of the HCVcc JC1 strain. As part of the viral replication machinery both plasmids comprise NS5A. The NS5A Y93F mutants were generated using the Quickchange II XL mutagenesis kit (Agilent, Ratingen, Germany), according to the manufacturer’s instructions with the mutagenesis primer pairs “Y93F for” (5’-AGGGGCCCTTGTTCTCCGCGTTAATGGGG-3’) and “Y93F rev” (5’-CCCCTATTAACGCCAGGACCACGGGCCCT-3’) for the subgenomic replicon and “Y93F HCVcc for” (5’-CTGCCCTCCTCGTGAA-GCAATTGATAGGAAAGG-3’) and “Y93F HCVcc rev” (5’-CTTTTCTCATCAATTTGCCCTACGGAGGGCCAG-3’) for the HCVcc JC1 strain.

**Transfection of Huh-7 cells with subgenomic HCV replicon constructs**
Huh-7 cells were transfected using the Lonza (Cologne, Germany) 4D-Nucleofector according to the manufacturer’s instructions. For transfection of Huh-7 cells, cell-specific transfection solution SF and program FF-138 were used. For each transfection, 10⁶ Huh-7 cells and 3 μg plasmid were used. Cells were seeded for 24 h in 6-well plates.

**Virus production in Huh-7.5 cells with HCVcc JC1 constructs**
Generation of HCVcc has been described elsewhere (40). Briefly, plasmid DNA was delivered to Huh-7.5 cells by electroporation. The virus was collected over 72 h and virus stocks were concentrated by PEG precipitation. HCV titers were determined by the TCID₅₀ assay (54) and calculated according to Spearman and Kärber (55).

**Generation and purification of GST-Fyn-SH2 and variants**
A synthetic gene coding for Fyn-SH2 was purchased from GeneArt (Thermo Fisher Scientific, Regensburg, Germany) and incorporated via an in-fusion (Takara Bio, Saint-Germain-en-Laye, France) reaction into the linearized pGEX-6P vector (GE Healthcare, Freiburg, Germany), resulting in pGEX-Fyn-SH2. A R176K Fyn-SH2 mutant was generated by site-directed mutagenesis (Quickchange; Agilent, Ratingen, Germany). The identity and accuracy of all gene constructs was verified by DNA sequencing (MWG Eurofins, Ebersberg, Germany). E. coli BL21 (DE3) cells were transformed with each plasmid. Expression cultures were inoculated from an overnight culture (1:100) into fresh terrific broth media and cultivated at 37 °C under gentle agitation conditions. At OD₆₀₀ = 0.6–0.8, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the culture incubated for a further 4 h. Cells were harvested via centrifugation at 3,500 × g for 10 min and stored at -20 °C. Cells were re-suspended in Fyn lysis buffer [50 mM sodium phosphate, 100 mM Na₂SO₄, protease inhibitor cocktail (Roche, Basel, Switzerland), 0.5% (v/v) Triton X-100, 40 μg/ml DNAse-1, pH 7.0] and homogenized in a cell disruptor at 2,000 bar. After centrifugation at 50,000 × g for 1 h, the supernatant was applied to a GSH affinity column (Protino Glutathione Agarose 4B, Macherey-Nagel, Düren, Germany) connected to an Äkta prime system  (GE Healthcare, Freiburg, Germany) and equilibrated in Fyn affinity buffer [50 mM sodium phosphate, 100 mM Na₂SO₄, protease inhibitor cocktail (Roche, Basel, Switzerland), 0.5% (v/v) Triton X-100, 40 μg/ml DNAse-1, pH 7.0] and homogenized in a cell disruptor at 2,000 bar. After centrifugation at 50,000 × g for 1 h, the supernatant was applied to a GSH affinity column (Protino Glutathione Agarose 4B, Macherey-Nagel, Düren, Germany) connected to an Äkta prime system (GE Healthcare, Freiburg, Germany) and equilibrated in Fyn affinity buffer [50 mM sodium phosphate, 100 mM Na₂SO₄, pH 7.0]. After washing, protein was eluted in the same buffer with 20 mM glutathione added. Afterwards the protein was further purified by size exclusion chromatography (SEC) using a Superdex 75 pg (GE Healthcare, Freiburg, Germany) column connected to an Äkta FPLC system (GE Healthcare, Freiburg, Germany) and equilibrated in 50 mM sodium phosphate, 100 mM Na₂SO₄, 2 mM DTT, pH 7.0. The eluted protein was collected and concentrated using centrifugal concentrators (Sartorius, Göttingen, Germany).
Germany), snap-frozen in liquid nitrogen and stored at -80 °C.

**Generation and purification of GST-c-Src-ΔSH1, -SH3SH2, and -SH2**
The expression plasmid pGEX-c-Src-ΔSH1 described previously (25) was used for overexpression of GST-c-Src-ΔSH1. GST fusion plasmids for the c-Src deletion mutants c-Src-SH3SH2 and c-Src-SH2 were generated by standard PCR cloning with pGEX-c-Src-ΔSH1 as the template. Plasmid pGEX-c-Src-ΔSH1 R173K was generated by PCR using pGEX-c-Src-ΔSH1 as the template, followed by a DpnI digestion and in-fusion recombination.

E. coli BL21 (DE3) cells were transformed with pGEX-c-Src-ΔSH1, pGEX-c-Src-SH3SH2 or pGEX-c-Src-SH2. Bacterial cell cultivation was done as described for GST-Fyn-SH2. After induction by addition of IPTG to a final concentration of 0.25 mM, cells were incubated at room temperature (RT) under gentle agitation (125 rpm) for 12–16 h and then harvested and stored as described for GST-Fyn-SH2. Protein expression was verified using SDS-PAGE with subsequent Coomassie Brilliant Blue (CBB) G250 staining. Cells were re-suspended in Src-lysis buffer [50 mM HEPES-NaOH, 100 mM Na2SO4, 300 mM NaCl, 2 mM DTT, protease inhibitor cocktail (Roche, Basel, Switzerland), 0.5% (v/v) Triton X-100, 40 μg/ml DNase, pH 8.0], homogenized and centrifuged as described for GST-Fyn-SH2. The supernatant was loaded onto a GST column equilibrated in Src-affinity buffer (50 mM HEPES-NaOH, 100 mM Na2SO4, 300 mM NaCl, 2 mM DTT, pH 8.0). After washing, the target protein was eluted in the same buffer with 20 mM glutathione added. The protein was passed over a SEC column (Superdex 75; GE Healthcare, Freiburg, Germany) equilibrated in Src affinity buffer. The eluted protein was collected, concentrated, and stored as described for GST-Fyn-SH2. GST-c-Src-ΔSH1 was incubated with GST-tagged rhinovirus 3C protease (GE Healthcare, Freiburg, Germany) overnight to remove the GST tag. Subsequently, the flowthrough of a GSH affinity chromatography was subjected to SEC to yield the c-Src-ΔSH1 protein with > 95% purity, as judged by SDS-PAGE and CBB staining. The bound GST moiety was eluted from the GSH affinity column with Src affinity buffer containing 20 mM glutathione, passed over the SEC column, and used as a negative control.

**Generation and purification of His6-NS5A-D2D3**
E. coli TKB-1 cells were transformed with the pET28-NS5A-D2D3 vector coding for the 265-residue NS5A-D2D3 fragment (residues 191–447), as described previously (23,30). Bacterial cell cultivation was done as described for GST-Fyn-SH2. At OD600 = 0.6–0.8, cells were induced with IPTG at a final concentration of 0.25 mM and cultivated at 16 °C for another 12–16 h. To activate the indole acrylic acid (IAA) inducible promoter for ELK tyrosine kinase expression, cultures were shifted to 30 °C and supplemented with 10 mg/l IAA, 1 g/l casein hydrolysate and 2 g/l glucose before incubation for another 2 h. Cells were harvested via centrifugation at 3,500 × g for 10 min and stored at -20 °C. Protein purification was performed as described previously (23,28,56), with the addition of 0.2 mM OV in the lysis buffer. After protein purification, the protein was snap-frozen in liquid nitrogen and stored at -80 °C. Protein identity and the presence of phosphorylation were checked by western blotting with an anti-His6-tag mouse monoclonal antibody (clone 27E8, Cell Signalling, Boston, USA) and an anti-phosphotyrosine mouse monoclonal antibody (clone 4G10, Millipore, Darmstadt, Germany), respectively, with a goat-anti-mouse horseradish peroxidase (HRP) conjugated antibody (Jackson ImmunoResearch, Suffolk, United Kingdom) used as the secondary antibody.

**Generation and purification of His6-NS5A-D1**
A synthetic gene coding for NS5A-D1 genotype 1b (residues 33–202) was purchased from GeneArt (Thermo Fisher Scientific, Regensburg, Germany) and incorporated into linearized pET302 N-His Champion vector (Thermo Fisher Scientific, Regensburg, Germany) by an in-fusion (Takara Bio, Saint-Germain-en-Laye, France) reaction to yield pET302-His6-NS5A-D1. All NS5A-D1 Y-to-F or Y-to-H substitution mutants were generated by site-directed mutagenesis using pET302-NS5A-D1 as the template and in-fusion (Takara Bio, Saint-Germain-en-Laye, France) recombination. The E. coli TKB-1 strain was transformed with each of the pET302-His6-NS5A-D1 plasmids. Additionally, the E. coli BL21 (DE3) strain was
transformed with pET302-His$_6$-NS5A-D1 containing the wild-type sequence to obtain the non-phosphorylated NS5A-D1 domain. Bacterial cell cultivation was done as described for GST-Fyn-SH2. At an OD$_{600}$ = 0.6–0.8, cells were induced with IPTG at a final concentration of 0.25 mM and the culture grown for a further 12–16 h at 16 °C. E. coli BL21 cells were then harvested via centrifugation at 3,500 $\times$ g for 10 min and stored frozen at -20 °C. Prior to harvesting, E. coli TKB-1 cell cultures were shifted to 30 °C and treated as described for His$_6$-NS5A-D2D3 to initiate tyrosine modification by ELK. For protein purification, cells were resuspended in NS5A-lysis buffer [100 mM Tris-HCl, 100 mM NaCl, 5 mM imidazole, protease inhibitor cocktail (Roche, Basel, Switzerland), 1% (v/v) Triton X-100, DNAsel, 0.2 mM OV (for ELK tyrosine phosphorylated proteins), pH 8.0], homogenized in a cell disruptor at 2,000 bar and after centrifugation at 50,000 $\times$ g for 1 h the supernatant was applied to a Ni-NTA affinity column (Macherey-Nagel, Düren, Germany) connected to an Äkta prime system (GE Healthcare, Freiburg, Germany) and equilibrated in NS5A affinity buffer (100 mM Tris-HCl, 300 mM NaCl, 5 mM imidazole, pH 8.0). The protein was eluted in the same buffer containing 500 mM imidazole. The protein sample was then passed over a SEC column (Superdex 75 pg; GE Healthcare, Freiburg, Germany) connected to an Äkta prime system (GE Healthcare, Freiburg, Germany) and equilibrated in 25 mM Tris-HCl, 250 mM NaCl, 10% (v/v) glycerol, 2 mM DTT, pH 8.0. The protein peak was collected, concentrated and stored as described for GST-Fyn-SH2.

**Pull-down of NS5A from cell lysates by immobilized c-Src-derived domains**

NHS/EDC agarose beads (GE Healthcare, Freiburg, Germany) were washed with cold 1 mM HCl and incubated with 5 nmol of each purified protein (GST, GST-Fyn-SH2, GST-Fyn-SH2 R176K, GST-c-Src-ΔSH1, GST-c-Src-ΔSH1 R173K, c-Src-ΔSH1) for 4 h at 8 °C. Afterwards, Tris-HCl buffer (pH 8.0) was added to a final concentration of 200 mM and samples were incubated for 30 min at RT. Control beads were also washed with 1 mM HCl and incubated with 200 mM Tris-HCl, pH 8.0. Prepared beads were then centrifuged for 4 min at 500 $\times$ g at 4 °C and the supernatant was discarded. Each bead preparation was washed twice with cold Huh-lysis buffer and was afterwards incubated with Huh-9-13 cell lysate (150 μg total protein) overnight for 12–16 h at 4 °C. Afterwards the beads with bound protein complexes were centrifuged for 4 min at 100 $\times$ g and 4 °C, and washed three times with 1 ml cold Huh-lysis buffer followed by centrifugation, and the supernatant was quantitatively removed. Protein complexes were eluted by adding 20 μl SDS-PAGE sample buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.004% (w/v) bromophenol blue, pH 6.8) and incubating at 95 °C for 10 min. 15 μl of each elution were applied to an SDS-PAGE followed by semi-dry western blotting. For NS5A detection, an anti-NS5A antibody (ab13833; Abcam, Cambridge, United Kingdom) and a secondary goat-anti-mouse HRP conjugated antibody (Jackson ImmunoResearch, Suffolk, United Kingdom) were used.

**Peptides**

A set of peptides (Table 1, no 1–26) covering all tyrosine residues in NS5A in their phosphorylated or non-phosphorylated states in their respective sequence context of HCV genotype Ref.1b.BR.03.BR1427_P1_10-7-03.EF032892 or the consensus of 249 genotype 1b sequences were purchased from JPT (Berlin, Germany) as crude peptides carrying an N-terminal biotinylation tag (purity > 70%). The peptides were used in an ELISA for screening all tyrosines in NS5A for c-Src-SH2 binding. In addition, peptides 6, 8, 12, 14, 26, 27, 28, 29, and 30 were purchased from Caslo (Kongens Lyngby, Denmark) as a set with N-terminal amino hexane fluorescein isothiocyanate (FITC) conjugation, both as purified, resin-synthesized peptides (purity > 95%). All used peptides carried C-terminal amidation to reduce charge and influence of the terminus, thus mimicking the corresponding segments of the native protein.

**ELISA of NS5A-derived peptides with c-Src SH2 domains**

Biotinylated peptides 1–26 (Table 1) with and without tyrosine phosphorylation were bound to
streptavidin coated 96-well plates (Roche, Basel, Switzerland). A threefold excess of the respective biotinylated peptide to streptavidin (90 pmol, n = 3) was applied to ensure comparable numbers of putative SH2 binding sites in each well. After washing three times with water, 200 μl of 20 μM GST-c-Src-SH3SH2 or GST-c-Src-SH2 in HBS buffer (10 mM HEPES-NaOH, 150 mM NaCl, pH 8.0) were added. Plates were incubated for 3 h under gentle agitation at RT followed by three HBS washing steps with 200 μl per well. The anti-GST antibody 26H1 (Cell signalling, Boston, USA) was diluted 1:5,000 in HBS and 200 μl were applied to each well and incubated overnight at 4 °C under gentle agitation conditions. The supernatant was discarded and plates were washed three times with cold HBS prior to incubation with the secondary antibody (goat-anti-mouse HRP conjugated antibody; Jackson ImmunoResearch, Suffolk, United Kingdom). Absorbance data were referenced against blank wells without peptide coupling, but with antibody incubation. Data from three independent wells were averaged and normalized to determine the peptide with the highest binding affinity towards each protein, GST-c-Src-SH3SH2 and GST-c-Src-SH2.

**FP measurements of NS5A-derived peptides with c-Src protein domains**

FITC coupled peptides at 50 nM in HBS (final volume 500 μl) were used for FP in an LS-55 fluorimeter (Perkin-Elmer, Waltham, MA, USA). The excitation monochromator was set at 490 nm (5 nm bandwidth) and the emission monochromator at 520 nm (10 nm bandwidth). c-Src-ΔSH1 was titrated as the analyte under stirring conditions and temperature control at RT. The fluorescence intensity was measured as the average of a 1-min stable signal for parallel ($F_p$) and perpendicular ($F_o$) orientation alternating at 3.7 Hz. The value of $FP$ was calculated using Equation 1. The grating factor $G$ was calculated using free dye. $FP$ was plotted as a function of analyte concentration and fitted using Equation 2 for determination of the dissociation constant.

$$FP = \frac{F_p - G \times F_o}{F_p + G \times F_o} \quad (1)$$

$$y = y_{start} + y_{end} \times \frac{x^n}{K_d^n + x^n} \quad (2)$$

**BLI experiments of c-Src-ΔSH1 and NS5A domains**

BLI experiments with purified c-Src-ΔSH1 and NS5A domains were performed on an OctetRed96 instrument (Forte Bio, Menlo Park, CA, USA). In the first step, the ligand (see respective experiment) was coupled via EDC/NHS coupling to AR2G sensor tips to a signal of 1 nm each. The free tip surface was blocked in 1 M ethanolamine, pH 8.5, and then equilibrated in HBSBLI buffer [HBS supplemented with 0.05% (v/v) Tween 20 and 0.5% (w/v) bovine serum albumin]. Reference sensors were treated the same, but after activation were directly incubated in 1 M ethanolamine, pH 8.5. After coupling, all tips were equilibrated in HBSBLI buffer. For each experiment eight tips were generated for ligand and control groups. The analytes (see respective experiment) were dissolved in HBSBLI buffer generating a dilution series of seven different concentrations per experiment. The eight ligand sensors and reference sensors were placed successively in the analyte solutions and in a buffer-only sample, and association and dissociation phases of 1400 and 300–1400 s, respectively, were recorded. Aligned and referenced ligand sensorgrams were fitted using Equation 3; the resulting $y_{inf}$ values were then plotted against the analyte concentration and fitted using Equation 4 for determining the $K_d$.

$$y = A_1 \times e^{-\frac{x}{t_1}} + A_2 \times e^{-\frac{x}{t_2}} + y_{inf} \quad (3)$$

$$y_{inf} = y_{end} \times \left(\frac{x^n}{K_d^n + x^n}\right) \quad (4)$$

**Total RNA isolation and rtPCR for HCV RNA quantification**

Total cellular RNA was isolated using the RNeasy Miniprep Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. 1 μg of total RNA was reverse-transcribed with Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany) using oligo(dT), which included DNase I digestion. cDNA was diluted fivefold, and 1.2 μl of the diluted cDNA was added as template to a final volume of 25 μl including 1×
SYBR Green PCR master mix (Thermo Fisher Scientific, Regensburg, Germany). PCR controls lacking template or containing cDNA reactions lacking reverse-transcriptase were included to ensure the specificity of the rtPCR. Semi-quantitative PCR results were obtained using the ΔΔCT method (57). The succinate dehydrogenase complex, subunit A (SDHA) gene was used as a control. Threshold values were normalized to SDHA. Data from at least three independent experiments are presented as means with standard deviations (SD). As rtPCR primers, “HCV sense” (5’-AATTATTCTAGGGCGCTGTGG-3’), “HCV antisense” (5’-GAGCTGTGACCCAACCAGGT-3’), “SDHA sense” (5’-AGATGTGTGGTCTCGGTCGAT-3’) and “SDHA antisense” (5’-AGATGTGTGGTCTCGGTCGAT-3’) were used.

MD simulations

The non-crystallographic NS5A-D1 dimer derived from PDB ID 3FQQ (18) and all variants thereof were subjected to all-atom MD simulations. The variants were prepared by capping N- and C-termini with acetyl and N-methyl amide groups, respectively, protonated with PROPKA (58) according to pH 7.4, neutralized by adding counter ions, and solvated in an octahedral box of TIP3P water (59) with a minimal water shell of 12 Å around the solute. The variants were created by deleting the sidechain atoms of Y93 and replacing the sidechain with LEaP (60). Phosphotyrosine was used in its deprotonated state using the parameters by Homeyer et al. (61). The Amber package of molecular simulation software (60) and the ff14SB (62) and GAFF (63) force fields were used to perform the MD simulations. To cope with long-range interactions, the “Particle Mesh Ewald” method (64) was used; the SHAKE algorithm (65) was applied to bonds involving hydrogen atoms. As hydrogen mass repartitionion (66) was utilized, the time step for all MD simulations was 4 fs with a direct-space, non-bonded cut-off of 8 Å, treating the Zn²⁺ ion with the Li-Merz parameters (67). At the beginning, 17500 steps of steepest decent and conjugate gradient minimization were performed; during 2500, 10000, and 5000 steps positional harmonic restraints with force constants of 25 kcal mol⁻¹ Å⁻², 5 kcal mol⁻¹ Å⁻², and zero, respectively, were applied to the solute atoms. Thereafter, 50 ps of NVT (constant number of particles, volume, and temperature) MD simulations were conducted to heat up the system to 100 K, followed by 300 ps of NPT (constant number of particles, pressure, and temperature) MD simulations to adjust the density of the simulation box to a pressure of 1 atm and to heat the system to 300 K. During these steps, a harmonic potential with a force constant of 10 kcal mol⁻¹ Å⁻² was applied to the solute atoms. As the final step in thermalization, 300 ps of NVT-MD simulations were performed while gradually reducing the restraint forces on the solute atoms to zero within the first 100 ps of this step. Afterwards, five independent production runs of NVT-MD simulations with 2000 ns length each were performed. For this, the starting temperatures of the MD simulations at the beginning of the thermalization were varied by a fraction of a Kelvin.

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Author contributions: DW and SH initiated, organized and designed this study. SK contributed to study design, cloned most of the expression vectors, performed the recombinant protein expressions and purifications, the pull-down assays, the ELISA, the FP and BLI measurements and drafted the manuscript. LG contributed to experimental design, protein expression and purification. SS performed the HCVcc infectious system experiments and the HCV replication assays was involved in the cell culture experiments, contributed to study design and writing of the manuscript. CGWG and HG performed and analyzed molecular dynamics simulations and contributed to writing of the manuscript. OHW developed and evaluated structural models illustrating the interaction of NS5A-D1 with c-Src-SH2. JGB contributed to study design and writing of the manuscript. SH, OHW, and DW took primary responsibility for finalizing the manuscript. All authors discussed the data and contributed to the manuscript.

FOOTNOTES

a The abbreviations used are: BLI, biolayer interferometry; CBB, Coomassie Brilliant Blue; c-Src, SRC proto-oncogene, non-receptor tyrosine kinase; DAA, direct-acting antiviral; DMEM, Dulbecco’s modified Eagle’s medium; FP, fluorescence polarization; GST, glutathione S-transferase; HCV, hepatitis C virus; HRP, horseradish peroxidase; IAA, indole acrylic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; $K_d$, dissociation constant; LCS, low-complexity sequence; MD, molecular dynamics; NPT, constant number of particles, pressure, and temperature; NS, non-structural; NVT, constant number of particles, volume, and temperature; OV, orthovanadate; RT, room temperature; rtPCR, real-time PCR; SD, standard deviation; SDHA, succinate dehydrogenase complex, subunit A; SEC, size exclusion chromatography; SH, Src-homology.
Table 1. List of NS5A-derived synthetic peptides used throughout this study. Genotype ref 1b represents Ref.1b.BR.03.BR1427_P1_10-7-03.EF032892 whereas con 1b is a consensus of 249 1b sequences. Bold type signifies phosphorylation.

| No | Name       | Genotype | Position in NS5A (in polyprotein) | Sequence                                                                 |
|----|------------|----------|----------------------------------|--------------------------------------------------------------------------|
| 1  | Y43<sup>ref 1b</sup> | ref 1b   | 36–50 (2013–2027)                | FISCQRGYKGWWRGD                                                          |
| 2  | pY43<sup>ref 1b</sup> | ref 1b   |                                   | FISCQRGYKGWWRGD                                                          |
| 3  | Y43<sup>con 1b</sup> | con 1b   |                                   | FSFCQRYKGWWRGD                                                          |
| 4  | pY43<sup>con 1b</sup> | con 1b   |                                   | FSFCQRYKGWWRGD                                                          |
| 5  | Y93        | ref 1b   | 86–100 (2063–2077)                | GTFPINA<sup>AY</sup>TTGCTP                                              |
| 6  | pY93       | ref 1b   |                                   | GTFPINA<sup>AY</sup>TTGCTP                                              |
| 7  | Y106       | ref 1b   | 99–113 (2076–2090)                | TPSPAPNYSRALWRV                                                         |
| 8  | pY106      | ref 1b   |                                   | TPSPAPNYSRALWRV                                                         |
| 9  | Y118       | ref 1b   | 111–125 (2088–2102)               | WRIAEEVYEVEVRV                                                         |
| 10 | pY118      | ref 1b   |                                   | WRIAEEVYEVEVRV                                                         |
| 11 | Y129       | ref 1b   | 122–136 (2099–2113)               | TRVGDFHYVTGMTTD                                                          |
| 12 | pY129      | ref 1b   |                                   | TRVGDFHYVTGMTTD                                                          |
| 13 | Y161       | ref 1b   | 154–168 (2131–2145)               | DGVRLHR<sup>A</sup>PACKPL                                              |
| 14 | pY161      | ref 1b   |                                   | DGVRLHR<sup>A</sup>PACKPL                                              |
| 15 | Y181<sup>ref 1b</sup> | ref 1b   | 174–188 (2151–2165)               | FQVGLNQYLVSQLP                                                          |
| 16 | pY181<sup>ref 1b</sup> | ref 1b   |                                   | FQVGLNQYLVSQLP                                                          |
| 17 | Y181<sup>con 1b</sup> | con 1b   |                                   | FLVGLNQYLVSQLP                                                          |
| 18 | pY181<sup>con 1b</sup> | con 1b   |                                   | FLVGLNQYLVSQLP                                                          |
| 19 | Y321       | ref 1b   | 314–328 (2291–2305)               | PIWARP<sup>D</sup>YNPLLES                                              |
| 20 | pY321      | ref 1b   |                                   | PIWARP<sup>D</sup>YNPLLES                                              |
| 21 | Y334       | ref 1b   | 327–341 (2304–2318)               | ESWKDPYVPPVVHG                                                          |
| 22 | pY334      | ref 1b   |                                   | ESWKDPYVPPVVHG                                                          |
| 23 | Y413<sup>ref 1b</sup> | ref 1b   | 406–420 (2383–2397)               | KGSDVESYSSMPPLE                                                        |
| 24 | pY413<sup>ref 1b</sup> | ref 1b   |                                   | KGSDVESYSSMPPLE                                                        |
| 25 | Y413<sup>con 1b</sup> | con 1b   |                                   | AGSDVESYSSMPPLE                                                        |
| 26 | pY413<sup>con 1b</sup> | con 1b   |                                   | AGSDVESYSSMPPLE                                                        |
| 27 | Y93Y106    | ref 1b   | 86–113 (2063–2090)                | GTFPINA<sup>AY</sup>TTGCTPSPAPNYSRALWRV                             |
| 28 | pY93Y106   | ref 1b   |                                   | GTFPINA<sup>AY</sup>TTGCTPSPAPNYSRALWRV                             |
| 29 | Y93pY106   | ref 1b   |                                   | GTFPINA<sup>AY</sup>TTGCTPSPAPNYSRALWRV                             |
| 30 | pY93pY106  | ref 1b   |                                   | GTFPINA<sup>AY</sup>TTGCTPSPAPNYSRALWRV                             |
Figure 1. Pull-down analysis of NS5A with wild-type and pY-binding deficient SH2 domains. Huh 9-13 hepatocyte lysates were subjected to pull-down experiments with recombinantly expressed and purified SH2 domain containing constructs as indicated. NS5A was visualized by immunoblotting. Controls were performed with inactivated beads and immobilized GST. NS5A binding to NHS beads and to GST was negligible. Huh 9-13 total protein (1 μg and 2.5 μg) was used as the positive control.
Figure 2. Schematic representation of tyrosine positions in NS5A (A) and screening of NS5A tyrosine residues for binding to c-Src-SH2 (B). Binding of the GST-c-Src constructs to the various immobilized biotinylated peptides was measured colorimetrically. Binding was normalized to the best binder (pY93), which was arbitrarily set to 100. Mean and SD of n = 3 experiments are shown.
Figure 3. Binding affinities of NS5A-derived peptides to c-Src-derived constructs determined by fluorescence polarization. Dissociation constants ($K_d$) of different NS5A peptides towards c-Src-ΔSH1 (A), c-Src-SH2 (B), and c-Src-SH3SH2 (C) are plotted on a logarithmic scale. Raw data and data fits are shown in Figure S2.
Figure 4. Analysis of NS5A-D2D3$^\text{ELKpY}$ binding to c-Src-ΔSH1 via BLI. Immobilized NS5A-D2D3$^\text{ELKpY}$ was incubated with c-Src-ΔSH1 at a wide range of concentrations (A). $K_d$ was determined to be 18 ± 4 μM (in B).
Figure 5. Interaction of phosphorylated and non-phosphorylated NS5A-D1 with wild-type or pY-binding deficient c-Src-ΔSH1. BLI sensorgrams of analyte NS5A-D1\textsuperscript{Elk\ p^{Ty}r} binding to the c-Src-ΔSH1 ligand (A) yield a $K_d$ of 0.47 ± 0.1 μM (B). In (C), the c-Src-ΔSH1 ligand was incubated with non-phosphorylated NS5A-D1. No binding was observed up to 40 μM analyte. In (D), the c-Src-ΔSH1 R173K ligand was incubated with NS5A-D1\textsuperscript{Elk\ p^{Ty}r}. No binding was observed up to 12 μM analyte.
Figure 6. Interaction of NS5A-D1 mutants with immobilized c-Src-ΔSH1 revealing the critical pY for SH2 binding. BLI sensorgrams and fits for different NS5A-D1 variants (analyte) and c-Src-ΔSH1 (ligand) are shown. In (A) c-Src-ΔSH1 was incubated with NS5A-D1<sup>ELK<sub>pY</sub></sup> Y93/129/161F. Plotting of $y_{inf}$ against NS5A-D1<sup>ELK<sub>pY</sub></sup> Y93/129/161F concentration (B) suggests the $K_d$ to be above 40 μM. The remaining panels illustrate c-Src-ΔSH1 interaction with NS5A-D1<sup>ELK<sub>pY</sub></sup> Y93F (C), NS5A-D1<sup>ELK<sub>pY</sub></sup> Y129F (D) and NS5A-D1<sup>ELK<sub>pY</sub></sup> Y161F (E), respectively. $K_d$ values were determined as 1.5 ± 0.5 μM for NS5A-D1<sup>ELK<sub>pY</sub></sup> Y129F and 2.1 ± 0.8 μM for NS5A-D1<sup>ELK<sub>pY</sub></sup> Y161F. No binding of NS5A-D1<sup>ELK<sub>pY</sub></sup> Y93F was observed in the concentration range applied. The raw data sensorgrams and fits are shown in Figure S3.
Figure 7. Analysis of c-Src-ΔSH1 binding to immobilized NS5A-D1ELKpY Y129/161F, confirming that pY93 is critical for SH2 binding. BLI measurements were performed using the NS5A-D1ELKpY Y129/161F mutant as ligand and c-Src-ΔSH1 as analyte. Sensorgrams (A) and fit (B) yield a $K_d$ of $0.7 \pm 0.2 \mu$M.
Figure 8. Substitution of the tyrosine residue Y93 with phenylalanine (F) in NS5A severely impairs HCV replication and virus production in human hepatoma cells. (A) Huh-7 cells were transfected with either wild-type HCV replicon plasmid pFK-I377/NS3-NS3’ (Rep) or the mutated plasmid pFK-I377/NS3-NS3’-Y93F (Rep Y93F). (B, C) Huh-7.5 cells were transfected with either wild-type HCV cc JC1 plasmid pFK-JFH1J6C-846_dg (JC1) or the mutated plasmid pFK-JFH1J6C-846_dg-Y93F (JC1 Y93F). For (A) and (C) total mRNA was prepared by semi-quantitative rtPCR and results were calculated using the ΔΔCT method and SDHA as the control gene. Data are provided as fractions of the respective control cells (Rep or JC1, set as 100) and are depicted as means ± SD of at least three independent experiments. The respective differences were significant ($p = 0.037$ and $p = 0.032$, respectively) as indicated by the one-tailed Mann-Whitney test. For (B) virus was collected from the supernatants and concentrated by PEG precipitation. The TCID$_{50}$ assay was performed and results calculated according to Spearman and Kärber (54). Significant differences ($p = 0.029$) were confirmed using the one-tailed Mann-Whitney test.
**Figure 9. Mechanistic model illustrating formation of the HCV replication complex, with a focus on the role of tyrosine phosphorylation events.** (A) In its basal state, the kinase domain of c-Src is kept in a restrained, inactive conformation by intramolecular interactions of the regulatory SH2 and SH3 domains, which bind to pY530 close to the C-terminus and to a proline-rich motif located in the SH2-kinase linker, respectively (47). (B) Upon NS5A phosphorylation at residue Y93, NS5A-D1 displays a canonical, high affinity binding site for c-Src-SH2; as a result, c-Src is recruited to the replication complex in its activated form, and HCV replication can occur. Other low affinity interactions (21,28) between LCS II or D2 of NS5A and the SH3 domain may also contribute (black dotted arrows). NS5B is complexed through c-Src-SH3 (grey dotted arrow, ref. 25) and NS5A (black broken arrows, ref. 35) interactions. For the sake of clarity the dimerization site of NS5A is indicated without displaying a full dimer situation (refer to Figure S6 for a discussion of steric restraints). DAAAs like daclatasvir can bind to NS5A lacking phosphorylated Y93, but are ineffective in the presence of pY93. Regulatory tyrosine phosphorylation in c-Src is highlighted in yellow, NS5A-D1 phosphorylation at Y93 is marked in red (Abbreviations: AH, amphipathic helix; TD, transmembrane domain).
Phosphorylated tyrosine 93 of hepatitis C virus nonstructural protein 5A is essential for interaction with host c-Src and efficient viral replication
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