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

Received for publication, January 22, 2019, and in revised form, March 11, 2019 Published, Papers in Press, March 12, 2019, DOI 10.1074/jbc.RA119.007656

Stefan Klinker1, Sabine Stindt2, Lothar Gremer1, Johannes G. Bode1, Christoph G. W. Gertzen1, Holger Gohlke1, Oliver H. Weiergräber1, Silke Hoffmann1, and Dieter Willbold1

From the 1Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, 40204 Düsseldorf, the 2Department of Gastroenterology, Hepatology and Infectiology, Heinrich-Heine-Universität Düsseldorf, 40225 Düsseldorf, the 3Institute of Complex Systems: Structural Biochemistry (ICS-6), Forschungszentrum Jülich, 52425 Jülich, the 4John von Neumann Institute for Computing (NIC) and Jülich Supercomputing Centre (JSC), Forschungszentrum Jülich, 52425 Jülich, and the 5Institute for Pharmaceutical and Medicinal Chemistry, Heinrich-Heine-Universität-Universität Düsseldorf, 40225 Düsseldorf, Germany

Edited by Charles E. Samuel

The hepatitis C virus (HCV) nonstructural protein 5A (NS5A) plays a key role in viral replication and virion assembly, and the regulation of the assembly process critically depends on phosphorylation of both serine and threonine residues in NS5A. We previously identified SRC proto-oncogene, nonreceptor 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. Pulldown 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 Tyr-93 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 Tyr-93 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)2 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 interferring 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 post-translationally, this precursor is processed by viral and host proteases into 10 mature viral structural and nonstructural (NS) proteins. 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 composed of single-, double-, and multimembrane vesicles (2). The membrane fractions of the membranous web are enriched in viral NS proteins, and even after isolation they 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, whereas 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 isopropyl β-D-thiogalactopyranoside; LCS, low-complexity sequence; MD, molecular dynamics; NS, nonstructural; NVT, constant number of particles, volume, and temperature; OV, orthovanadate; rPCR, real-time PCR; SDHA, succinate dehydrogenase complex, subunit A; SEC, size exclusion chromatography; SH, Src-homology; RMSD, root-mean-square deviation; PDB, Protein Data Bank; RMSF, root-mean-square fluctuation; EDC, 1-ethyl-3-(dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; Fyn, FYN proto-oncogene, Src family tyrosine kinase; c-Abl, ABL proto-oncogene 1, non-receptor tyrosine kinase; ELK, EPH receptor B1.
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 (pS6) to the hyperphosphorylated form (pS8). The basally phosphorylated form drives the HCV life cycle toward 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 the bacteria 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. Pulldown assays with several recombinant GSH 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, because 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 noncanonical 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). Although serine and threonine phosphorylation patterns of NS5A have been investigated thoroughly in the past (9), research on potential

**pTyr-93 of NS5A is essential for interaction with c-Src**

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 (pTyr)-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 pulldown assay (33). Proteins were covalently immobilized to the beads in equal amounts and subsequently incubated with Huh-9-13 total protein extracts (Fig. 1). NS5A was pulled down from extracts by both WT Fyn–SH2 and c-Src–ΔSH1. Conversely, negligible amounts of NS5A protein were precipitated with the pTyr-binding–deficient R173K mutant.

**Results**

NS5A from Huh-9-13 lysates associates with c-Src–SH2 but not with the pTyr-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 (pTyr)-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 pulldown assay (33). Proteins were covalently immobilized to the beads in equal amounts and subsequently incubated with Huh-9-13 total protein extracts (Fig. 1). NS5A was pulled down from extracts by both WT 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 pulldown experiments via immunoblotting was further validated by MS 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 tryptic 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 $\approx50\%$ (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 $\approx50\%$ 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 YES proto-oncogene 1, Src family tyrosine kinase 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 pTyr residue of NS5A responsible for the interaction with c-Src–SH2 was investigated by a peptide ELISA-based assay (Table 1). As demonstrated in Fig. 2B, the c-Src SH2 domain without (white squares) and with the SH3 domain (gray squares) showed similar binding behavior toward the various phosphopeptides, demonstrating that the SH2 domain is sufficient for interaction with NS5A. The SH3 domain plays no measurable role in the interaction. pTyr-containing peptides were clearly favored over their nonphosphorylated versions (Fig. 2B, dark squares). Based on these results, peptides comprising pTyr-93, pTyr-106, pTyr-129, and pTyr-161 located in D1 and pTyr-413 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 (pTyr-93). We determined their binding affinities to c-Src–SH1, comprising its SH4, Unique, SH3, and SH2 domains (Fig. S1), by fluorescence polarization (FP). As depicted in Fig. 3 and Fig. S2, peptides pY93 (0.7 ± 0.1 μM), pY129 (8.0 ± 0.8 μM), pY161 (2.2 ± 0.3 μM), and pY413 (1.8 ± 0.1 μM) displayed $K_d$ (dissociation constant) values in the sub- or low micromolar range, whereas the $K_d$ value for pY106 was above 100 μM. Notably, $K_d$ values for the pY93 peptide interaction with c-Src–SH2 (0.5 ± 0.2 μM, Fig. 3A) and c-Src–SH3SH2 (0.3 ± 0.1 μM, Fig. 3C) were in the same range as with c-Src–ΔSH1 (Fig. 3A), supporting the notion that the SH2 domain rather than the SH3 domain of c-Src is responsible for the interaction.

<table>
<thead>
<tr>
<th>No</th>
<th>Name</th>
<th>Genotype</th>
<th>Position in NS5A (in polyprotein)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Y43&lt;sup&gt;ref lb&lt;/sup&gt;</td>
<td>ref lb</td>
<td>36–50 (2013–2027)</td>
<td>FISCOQRYKGVWRGD</td>
</tr>
<tr>
<td>2</td>
<td>pY43&lt;sup&gt;ref lb&lt;/sup&gt;</td>
<td>ref lb</td>
<td>86–109 (2063–2077)</td>
<td>GTFFINAYTGPCTP</td>
</tr>
<tr>
<td>3</td>
<td>Y43&lt;sup&gt;con lb&lt;/sup&gt;</td>
<td>con lb</td>
<td>99–113 (2076–2090)</td>
<td>TPSAFNYSRALWRV</td>
</tr>
<tr>
<td>4</td>
<td>pY43&lt;sup&gt;con lb&lt;/sup&gt;</td>
<td>con lb</td>
<td>111–125 (2088–2102)</td>
<td>WRvAAEYEVTRVG</td>
</tr>
<tr>
<td>5</td>
<td>Y93</td>
<td>ref lb + con lb</td>
<td>122–136 (2099–2113)</td>
<td>TRvGDFHYVTMTTD</td>
</tr>
<tr>
<td>6</td>
<td>pY93</td>
<td>ref lb + con lb</td>
<td>154–168 (2131–2145)</td>
<td>DVrLHryAPACKPL</td>
</tr>
<tr>
<td>7</td>
<td>Y106</td>
<td>ref lb</td>
<td>174–188 (2151–2165)</td>
<td>FvGvLqyLVGQLP</td>
</tr>
<tr>
<td>8</td>
<td>pY106</td>
<td>ref lb</td>
<td>314–328 (2291–2305)</td>
<td>FvARFDYFNFLLES</td>
</tr>
<tr>
<td>9</td>
<td>Y118</td>
<td>ref lb</td>
<td>327–341 (2394–2318)</td>
<td>EsvRdFyvFvVvHg</td>
</tr>
<tr>
<td>10</td>
<td>pY118</td>
<td>ref lb</td>
<td>406–420 (2383–2397)</td>
<td>KcDvEYSSNPPLE</td>
</tr>
<tr>
<td>11</td>
<td>Y129</td>
<td>ref lb</td>
<td>86–113 (2063–2090)</td>
<td>GtFFINAYTGPCTPSAFNYSRALWRV</td>
</tr>
<tr>
<td>12</td>
<td>pY129</td>
<td>ref lb</td>
<td>154–168 (2131–2145)</td>
<td>DVrLHryAPACKPL</td>
</tr>
</tbody>
</table>

*Table 1*

List of NS5A-derived synthetic peptides used throughout this study

Genotype ref lb represents ref1b.BR.03.BR1427_P1_10-7-03.EF032892, whereas con lb is a consensus of 249 lb sequences. Bold type signifies phosphorylation.
Tyrosine-phosphorylated D2D3 of NS5A only weakly interacts with c-Src

Tyr-321, Tyr-334, and Tyr-413 of NS5A are, according to the structural model, likely to be surface-exposed and thus candidates to mediate SH2 domain interactions when phosphorylated. Of these, Tyr-321 and Tyr-334 are located in the LCS-II of NS5A, and Tyr-413 is located in D3. Tyr-413 was of particular interest given the results of the screening approach outlined above. The NS5A–D2D3 construct was recombinantly expressed in *Escherichia coli* TKB-1 cells harboring a gene encoding the kinase domain of EPH receptor B1 (ELK) to examine the c-Src–SH2 binding properties toward phosphorylated NS5A–D2D3. ELK is known to mediate tyrosine phosphorylation of overexpressed proteins in a post-translational and nonspecific 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 interactions 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 $18 \pm 4 \mu M$ was determined (Fig. 4), suggesting that tyrosine residues located in the D2D3 portion of NS5A at most weakly contribute to the pTyr–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. Tyr-93, Tyr-129, and Tyr-161 within the D1 domain of NS5A are three potential sites for pTyr–SH2 domain interactions (Figs. 2 and 3). When using immobilized c-Src–ΔSH1 as ligand and free NS5A–D1ELKpY as analyte (Fig. 5A), a $K_d$ of $0.47 \pm 0.1 \mu M$ was determined (Fig. 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 toward the c-Src SH2 domain. In line with these considerations, the nonphosphorylated D1 domain of NS5A was unable to bind immobilized c-Src–ΔSH1 (Fig. 5C), nor did NS5A–D1ELKpY interact with c-Src–ΔSH1 R173K (Fig. 5D).

Figure 2. Schematic representation of Tyr-containing peptides derived from 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 (pTyr-93), which was arbitrarily set to 100. Mean and S.D. 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 toward 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 Fig. S2.

pTyr-93 of NS5A is essential for interaction with c-Src

Expressed in *Escherichia coli* TKB-1 cells harboring a gene encoding the kinase domain of EPH receptor B1 (ELK) to examine the c-Src–SH2 binding properties toward phosphorylated NS5A–D2D3. ELK is known to mediate tyrosine phosphorylation of overexpressed proteins in a post-translational and nonspecific 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 interactions 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 $18 \pm 4 \mu M$ was determined (Fig. 4), suggesting that tyrosine residues located in the D2D3 portion of NS5A at most weakly contribute to the pTyr–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. Tyr-93, Tyr-129, and Tyr-161 within the D1 domain of NS5A are three potential sites for pTyr–SH2 domain interactions (Figs. 2 and 3). When using immobilized c-Src–ΔSH1 as ligand and free NS5A–D1ELKpY as analyte (Fig. 5A), a $K_d$ of $0.47 \pm 0.1 \mu M$ was determined (Fig. 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 toward the c-Src SH2 domain. In line with these considerations, the nonphosphorylated D1 domain of NS5A was unable to bind immobilized c-Src–ΔSH1 (Fig. 5C), nor did NS5A–D1ELKpY interact with c-Src–ΔSH1 R173K (Fig. 5D).

Figure 2. Schematic representation of Tyr-containing peptides derived from 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 (pTyr-93), which was arbitrarily set to 100. Mean and S.D. 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 toward 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 Fig. S2.
Phosphorylation of Tyr-93 is sufficient for binding of NS5A–D1 to c-Src–SH2

NS5A mutants Y93F, Y129F, and Y161F, double mutants (Y93F/Y129F, Y93F/Y161F, and Y129F/Y161F) and the triple mutant (Y93F/Y129F/Y161F) were prepared to unequivocally identify the pTyr residue in the NS5A–D1 domain responsible for the interaction with c-Src–SH2. These NS5A–D1 mutants (Fig. S1) were purified from ELK-overexpressing cells, as described for the WT protein. As depicted in Fig. 6, A and B, loss of high-affinity binding to c-Src–SH2 was observed when titrating NS5A–D1ELKpY Y93F/Y129F/Y161F. Thus, the list of candidate sites for the observed binding of D1 to c-Src–SH2 is restricted to Tyr-93, Tyr-129, and Tyr-161. The single tyrosine mutants NS5A–D1ELKpY Y93F, NS5A–D1ELKpY Y129F, and NS5A–D1ELKpY Y161F (Fig. 6, C–E and Figs. S1 and S3, A–C) were analyzed for their binding properties to immobilized c-Src–SH1. As depicted in Fig. 6C, substitution of Tyr-93 to phenylalanine led to a drastic decrease in binding affinity, whereas the other mutations showed no significant effect on binding to c-Src–SH1. Moreover, as shown in Fig. 7, A and B,
Figure 6. Interaction of NS5A–D1 mutants with immobilized c-Src–ΔSH1 revealing the critical pTyr for SH2 binding. BLI sensograms and fits for different NS5A–D1 variants (analyte) and c-Src–ΔSH1 (ligand) are shown. A, c-Src–ΔSH1 was incubated with NS5A–D1^{ELKpY} Y93F/Y129F/Y161F. Plotting of $y_{\inf}$ against NS5A–D1^{ELKpY} Y93F/Y129F/Y161F concentration (B) suggests the $K_d$ to be above 40 $\mu$M. The remaining panels illustrate c-Src–ΔSH1 interaction with NS5A–D1^{ELKpY} Y93F (C), NS5A–D1^{ELKpY} Y129F (D), and NS5A–D1^{ELKpY} Y161F (E), respectively. $K_d$ values were determined as 1.5 ± 0.5 $\mu$M for NS5A–D1^{ELKpY} Y129F and 2.1 ± 0.8 $\mu$M for NS5A–D1^{ELKpY} Y161F. No binding of NS5A–D1^{ELKpY} Y93F was observed in the concentration range applied. The raw data sensograms and fits are shown in Fig. S3.

Figure 7. Analysis of c-Src–ΔSH1 binding to immobilized NS5A–D1^{ELKpY} Y129F/Y161F, confirming that pTyr-93 is critical for SH2 binding. BLI measurements were performed using the NS5A–D1^{ELKpY} Y129F/Y161F mutant as ligand and c-Src–ΔSH1 as analyte. Sensorgrams (A) and fit (B) yield a $K_d$ of 0.7 ± 0.2 $\mu$M.
**pTyr-93 of NS5A is essential for interaction with c-Src**

The doubly substituted NS5A–D1ELKpY Y129F/Y161F yielded a $K_d$ (0.7 ± 0.2 μM) that was similar to the WT protein. As position Tyr-93 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 Fig. S4A, the presence of up to 150 μM daclatasvir did not reduce the amount of NS5A–D1ELKpY bound to c-Src–ΔSH1, and therefore it 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 Tyr-93 is crucial for c-Src–SH2 binding, this mutant did not show any interaction with c-Src–ΔSH1 (Fig. S4B).

**Substitution of Tyr-93 does not compromise structural integrity of the NS5A–D1 dimer**

To assess whether the mutations and phosphorylation of Tyr-93 destabilize potential NS5A–D1 dimers, we performed explicit solvent, all-atom molecular dynamics (MD) simulations of 2 μs length. We utilized the noncrystallographic 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 WT dimer does not dissociate and only shows small local conformational changes, which is reflected by a root-mean-square deviation (RMSD) with respect to the starting structure in the 4–7 Å range (Fig. S5A). Note that the larger RMSD values originate from pronounced movements of the N and C termini (Fig. S5A). The c-Src binding, Tyr-93-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 WT NS5A–D1 (Fig. 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 with the WT (Fig. S5C). This modest increase in RMSF is also reflected by the slightly broader RMSD distribution (4–9 Å). Similar to the WT 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, although compromised in terms of function, features dynamics reminiscent of the WT and the Y93pY2 variant (Fig. 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 WT NS5A–D1 and the variants considered in this study.

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

Because phosphorylation of Tyr-93 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 Tyr-93 phosphorylation should severely impair viral genome replication. To test this conjecture, the relevance of Tyr-93 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 Tyr-93 for viral replication, a subgenomic HCV replicon plasmid and a full-length HCVcc JC1 plasmid encoding the NS5A Y93F mutant were generated. Both WT and mutant replicon plasmids were used to transfected Huh-7 cells 24 h before measurement of subgenomic HCV RNA levels by real-time PCR (rtPCR, Fig. 8A). The Y93F mutation resulted in significantly reduced HCV RNA compared with WT replicon levels.
pTyr-93 of NS5A is essential for interaction with c-Src

indicative of drastically impaired replication. Furthermore, Huh-7.5 cells were transfected with both WT 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 (Fig. 8B). Consistently, HCV RNA levels were significantly reduced compared with levels in WT JC1-transfected cells (Fig. 8C), confirming that mutation of NS5A at Tyr-93 results in impaired viral replication. Taken together, these observations clearly demonstrate a central role of phosphorylated Tyr-93 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 hyper-phosphorylated (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 non-structural proteins. Tyr-334 (Con1, Tyr-330 in JFH1) located at the C 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 (35) 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 pulldown assays (Fig. 1) and peptide ELISA (Fig. 2) unambiguously revealed that the interaction follows a canonical mode that depends on the presence of a phosphorylated tyrosine. Peptide mapping results (Figs. 2 and 3) combined with data from quantitative binding analysis with post-translationally–phosphorylated purified protein domains (Figs. 4–7) 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 pTyr-93, pTyr-129, and pTyr-413 contain amino acids with similar properties subsequent to the phosphorylated tyrosine (Table 1). Although 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 the 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 depends 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 Tyr-93 as the crucial residue, which, upon phosphorylation, mediates c-Src–SH2 binding (Figs. 6 and 7). Given these in vitro protein interaction data, we reasoned that Tyr-93 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 Tyr-93 with Phe both in a sub-genomic HCV replicon system and in the HCVcc JC1 infectious system, and we found that the mutated systems show drastically decreased replication efficiencies when compared with the respective WT systems (Fig. 8, A and C). Accordingly, the virus titer decreased for the mutated strain JC1 Y93F by a factor of about 100 (Fig. 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 Tyr-93 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 work will be needed to address this issue in the future. Interestingly, latest DAAAs 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 Tyr-93 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 pTyr-93 for an approaching SH2 domain. Fig. 6 illustrates the expected positions of human c-Src SH2 domains bound to individual NS5A–D1 chains in a canonical fashion, centering on the “pYTTG” segment. Obviously, only the dimer from the asymmetric unit of PDB code 1ZH1 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 code 3FQQ and the AB dimer from PDB code 4CL1), thus excluding NS5A dimerization, or clash with the second SH2 domain (CD dimer of PDB code 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.
Tyr-93 in NS5A is widely conserved in HCV genotypes 1–5, 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 Tyr-93 during viral replication (summarized in Fig. 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 Tyr-334 of NS5A (34), has yet to be defined. Importantly, high-affinity binding of NS5A to c-Src–SH2 via pTyr-93 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 HIV 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 humified atmosphere with 5% CO₂. Huh-7.5 cells (52) were cultivated in DMEM containing 4.5 g/liter glucose and supplemented with 9% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 μl/ml non-

---

**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 pTyr-530 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 Tyr-93, 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 (gray dotted arrow) (25) and NS5A (black broken arrows) (35) interactions. For the sake of clarity, the dimerization site of NS5A is indicated without displaying a full dimer situation (refer to Fig. S6 for a discussion of steric restraints). DAAs like daclatasvir can bind to NS5A lacking phosphorylated Tyr-93 but are ineffective in the presence of pTyr-93. Regulatory tyrosine phosphorylation in c-Src is highlighted in yellow; NS5A–D1 phosphorylation at Tyr-93 is marked in red (abbreviations used are: AH, amphipathic helix; TD, transmembrane domain).
essential amino acids (modified Eagle’s medium nonessential amino acids solution: Gibco, ThermoFisher Scientific, Waltham, MA). The use of these cell lines is covered by a material transfer agreement with Apath, LLC (New York).

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

At 80% confluence, Huh-9-13 cells were washed with cold Dulbecco’s PBS (ThermoFisher Scientific, Regensburg, Germany) and harvested in Huh-lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% (v/v) Triton X-100, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate (OV), protease inhibitor mixture (Roche Applied Science, 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 and 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. 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’-AGGGGCCCCGTGTTCCGCGTTAATGGGG-3’) and “Y93F rev” (5’-CCCCATTAACCGCGGAGACCACCGGCCCCT-3’) for the subgenomic replicon and “Y93F HCVccc for” (5’-CTGGGCCTCCGTGAAGCAATTGATAGAAAGG-3’) and “Y93F HCVccc rev” (5’-CCTTTTCTATCATACATTGCTTTCA-CGGAGGCCAG-3’) for the HCVccJC1 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 of plasmid were used. Cells were seeded for 24 h in 6-well plates.

**Virus production in Huh-7.5 cells with HCVcccJC1 constructs**

Generation of HCVccc 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 established protocols (55).

**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 recombinaction. *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 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) G-250 staining. Cells were resuspended in Src-lysis buffer (50 mM HEPES-NaOH, 100 mM Na2SO4, 300 mM NaCl, 2 mM DTT, protease inhibitor mixture (Roche Applied Science), 0.5% (v/v) Triton X-100, 40 μg/ml DNase I, pH 7.0) and homogenized in a cell disruptor at 2000 bar. After centrifugation at 50,000 × g for 1 h, the supernatant was applied to a GSH affinity column (Protein G Agarose 4B, Macherey-Nagel, Düren, Germany) connected to an Äkta prime system (GE Healthcare) and equilibrated in GSH affinity buffer (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), snap-frozen in liquid nitrogen, and stored at −80 °C.

**pTyr-93 of NS5A is essential for interaction with c-Src**

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 recombinaction. *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 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) G-250 staining. Cells were resuspended in Src-lysis buffer (50 mM HEPES-NaOH, 100 mM Na2SO4, 300 mM NaCl, 2 mM DTT, pH 7.0). The eluted protein was collected and concentrated using centrifugal concentrators (Sartorius, Göttingen, 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 recombinaction. *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 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) G-250 staining. Cells were resuspended in Src-lysis buffer (50 mM HEPES-NaOH, 100 mM Na2SO4, 300 mM NaCl, 2 mM DTT, pH 7.0). The eluted protein was collected and concentrated using centrifugal concentrators (Sartorius, Göttingen, Germany), snap-frozen in liquid nitrogen, and stored at −80 °C.
pTyr-93 of NS5A is essential for interaction with c-Src

was eluted in the same buffer with 20 mM GSH added. The protein was passed over an SEC column (Superdex 75; GE Healthcare) 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) overnight to remove the GST tag. Subsequently, the flow-through of a GST 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 GST affinity column with Src affinity buffer containing 20 mM GSH, passed over the SEC column, and used as a negative control.

**Generation and purification of His<sub>6</sub>–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 A<sub>600</sub> = 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 promotor for ELK tyrosine kinase expression, cultures were shifted to 30 °C and supplemented with 10 mg/liter IAA, 1 g/liter casein hydrolysate, and 2 g/liter glucose before incubation for another 2 h. Cells were harvested via centrifugation at 3500 × 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-His<sub>6</sub>–affinity buffer containing 20 mM GSH, passed over the SEC column (Superdex 75; GE Healthcare) equilibrated in Src affinity buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM imidazole, protease inhibitor mixture (Roche Applied Science), 1% (v/v) Triton X-100, DNase, 0.2 mM OV (for ELK tyrosine-phosphorylated proteins), pH 8.0) and homogenized in a cell disruptor at 2000 bar, and after centrifugation at 50,000 × g for 1 h, the supernatant was applied to a nickel-nitriotriacetic acid–affinity column (Macherey-Nagel, Düren, Germany) connected to an Äkta prime system (GE Healthcare) 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; GE Healthcare) connected to an Äkta FPLC system (GE Healthcare) 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.

**Pulldown of NS5A from cell lysates by immobilized c-Src–derived domains**

N-hydroxysuccinimide (NHS)/1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC)-agarose beads (GE Healthcare) 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, and c-Src–ΔSH1) for 4 h at 8 °C. Afterward, Tris-HCl buffer, pH 8.0, was added to a final concentration of 200 mM, and samples were incubated for 30 min at room temperature. 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 × g at 4 °C, and the supernatant was discarded. Each bead preparation was washed twice with cold Huh-lysis buffer and was afterward incubated with Huh-9-13 cell lysate (150 µg of total protein) overnight for 12–16 h at 4 °C. Afterward, the beads with bound protein complexes were centrifuged for 4 min at 100 × g at 4 °C and washed three times with 1 ml of cold Huh-lysis buffer followed by centrifugation, and the supernatant was quantitatively removed. Protein complexes were eluted by adding 20 µl of 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) bromphenol 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, UK) and a secondary goat anti-mouse HRP–conjugated antibody (Jackson ImmunoResearch, Suffolk, UK) were used.

**Peptides**

A set of peptides (Table 1, no. 1–26) covering all tyrosine residues in NS5A in their phosphorylated or nonphosphorylated 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 IPT (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, and 26–30 were purchased from Caslo (Kongens Lyngby, Denmark) as a set with N-terminal biotinylation and a second 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 Applied Science). A 3-fold 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 room temperature followed by three HBS washing steps with 200 µl per well. The anti-GST antibody 26H1 (Cell Signaling, Boston, MA) was diluted 1:5000 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, UK). After three washing steps, wells were incubated with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), and the absorbance was directly measured at 405 nm over 2 h in a M200pro or a M1000pro plate reader (Tecan, Männedorf, Switzerland). 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 toward each protein, GST–c-Src–SH3SH2 and GST–c-Src–SH2.

**FP measurements of NSSA-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 (PerkinElmer Life Sciences). 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 room temperature. The fluorescence intensity was measured as the average of a 1-min stable signal for parallel (F∥) and perpendicular (F⊥) 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.

\[
FP = \frac{F_1 - G \times F_⊥}{F_1 + G \times F_⊥} \quad \text{(Eq. 1)}
\]

\[
y = y_{\text{start}} + y_{\text{end}} \times \frac{x^\alpha}{K_d + x^\alpha} \quad \text{(Eq. 2)}
\]

**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). 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) BSA). 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 sensograms were fitted using Equation 3; the resulting yint values were then plotted against the analyte concentration and fitted using Equation 4 for determining the Kd.

\[
y = A1 \times e^{\frac{x}{t_1}} + A2 \times e^{\frac{x}{t_2} + y_{\text{inf}}} \quad \text{(Eq. 3)}
\]

\[
y_{\text{int}} = y_{\text{end}} \times \left( \frac{x^\alpha}{K_d + x^\alpha} \right) \quad \text{(Eq. 4)}
\]

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

Total cellular RNA was isolated using the RNasy 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 5-fold, and 1.2 µl of the diluted cDNA was added to a plate to a final volume of 25 µl including 1× SYBR Green PCR Master Mix (ThermoFisher 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 ∆∆CT 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 (S.D.). As rtPCR primers, “HCV sense” (5′-AATTAT-TCTAGGGCCGTGTG-3′), “HCV antisense” (5′-GACCTGT-GACCCAACCCAGGT-3′), “SDHA sense” (5′-AGATGT-GGTGCCTCCTCGGTGAT-3′) and “SDHA antisense” (5′-AGATGT-GGTGCCTCCTCGGTGAT-3′) were used.

**MD simulations**

The noncrystallographic NS5A–D1 dimer derived from PDB code 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.
pTyr-93 of NS5A is essential for interaction with c-Src

The variants were created by deleting the side-chain atoms of Tyr-93 and replacing the side chain with LEaP (60). Phosphoryrosine 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 reparationing (66) was utilized; the time step for all MD simulations was 4 fs with a direct-space, non-bonded cutoff of 8 Å, treating the Zn$^{2+}$ ion with the Li-Merz parameters (67). At the beginning, 17,500 steps of steepest descent and conjugated gradient minimizations were performed; during 2500, 10,000, and 5000 steps positional harmonic restraints with force constants of 25 and 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. Afterward, five independent production runs of NVT-MD simulations with 2000 ns length each were performed. For this, the starting temperatures at the beginning of the thermalizations were varied by a fraction of a Kelvin.


**Acknowledgments**—We thank Olga Valdau for generation of the c-Src–SH2 and c-Src–SH3SH2 expression vectors. We are grateful to Anja Stefanski (Molecular Proteomics Laboratory of the Biologisch-Medizinisches Forschungszentrum, Heinrich-Heine-Universität Düsseldorf, Germany) and Marc Veltser (Mass Spectrometry Service Unit, University Hospital Bonn, Germany) for MS measurements. H. G. is grateful to the Jülich Supercomputing Centre at the Forschungszentrum Jülich for computing time on the supercomputers JURECA and JUWELS (NIC Project ID: HKF7) and to the Zentrum für Informations- und Medientechnologie (ZIM) at the Heinrich Heine Universität Düsseldorf for computational support.

**References**


SH3 interaction sites in an intrinsically disordered fragment of the hepatitis C virus protein NS5A. J. Mol. Biol. 420, 310–323 CrossRef Medline
pTyr-93 of NS5A is essential for interaction with c-Src

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

Stefan Klinker, Sabine Stindt, Lothar Gremer, Johannes G. Bode, Christoph G. W. Gertzen, Holger Gohlke, Oliver H. Weiergräber, Silke Hoffmann and Dieter Willbold

doi: 10.1074/jbc.RA119.007656 originally published online March 12, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA119.007656

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 65 references, 22 of which can be accessed free at
http://www.jbc.org/content/294/18/7388.full.html#ref-list-1