Hepatitis C Virus NS5B and Host Cyclophilin A share a common binding site on NS5A*

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Running title: Interaction of NS5B and CypA with NS5A-D2

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Capsule

Background: HCV replication requires the interaction of the viral polymerase NS5B with both viral and host proteins.

Results: We performed the molecular characterization of the interactions between HCV NS5B, NS5A and host CypA.

Conclusions: HCV NS5B and host CypA share a binding site on HCV NS5A

Significance: The NS5A-D2 site which interacts with both the HCV polymerase NS5B and the host CypA might regulate the HCV replication.

Nonstructural protein 5B (NS5B) is essential for Hepatitis C Virus (HCV) replication as it carries the viral RNA-dependent RNA polymerase (RdRp) enzymatic activity. HCV replication occurs in a membrane-associated multiprotein complex in which HCV NS5A and host Cyclophilin A (CypA) have been shown to be present together with the viral polymerase. We used NMR spectroscopy to perform a per residue level characterization of the molecular interactions between the unfolded domains 2 and 3 of NS5A (NS5A-D2 and NS5A-D3), CypA and NS5BΔ21. We show that three regions of NS5A-D2 (residues 250-262 (region A), 274-287 (region B) and 306-333 (region C)) interact with NS5BΔ21 whereas NS5A-D3 does not. We show that both NS5BΔ21 and CypA share a common binding site on NS5A, which contains residues P306 to E323. No direct molecular interaction has been detected by NMR spectroscopy between HCV NS5BΔ21 and host CypA. We show that Cyclosporine A (CsA) added to a sample containing NS5BΔ21, NS5A-D2 and CypA specifically inhibits the interaction between CypA and NS5A-D2 without altering the one between NS5A-D2 and NS5BΔ21. A high quality heteronuclear NMR spectrum of HCV NS5BΔ21 has been obtained and was used to characterize the binding site on the polymerase of NS5A-D2. Moreover these data highlight the potential of using NMR of NS5BΔ21 as a powerful tool to characterize, in solution, the interactions of the HCV polymerase with all kind of molecules (proteins, inhibitors, RNA). This work brings new insights into the comprehension of the molecular interplay between NS5B, NS5A and CypA, three essentials proteins for HCV replication.

Hepatitis C virus (HCV) is a small RNA virus from the Flaviviridae family. Since its discovery in the late 80’s HCV has infected an estimated 130-200 million people worldwide (1). HCV chronic infection is one of the main factors leading to liver cirrhosis and hepatocellular carcinoma and thus represents a serious public health challenge for almost all countries. HCV has been classified into six major genotypes (1 to 6) according to its RNA genomic sequence. Among them, genotype 1 is the most prevalent all over the world except for Africa where genotype 4 represents nearly half of the infections (1). The standard of care to treat HCV infection consists of a combination of ribavirin and pegylated-interferon α (2). This therapeutic strategy suffers...
both from a low efficacy in the case of genotype 1 infection and from non-negligible side-effects for treated patients. The development of more efficient and safer anti-HCV molecules is thus highly expected (3,4). HCV is an obligatory intracellular parasite and requires host cell factors in addition to its own components to achieve its complete life cycle (5). Consequently, anti-HCV drugs may be either direct-acting agents (DAAs) or host-targeting agents. DAAs have the advantage to be highly specific but may elicit resistance mutations selection, thereby limiting their long-time efficacy. The second category of molecules is thought to have a higher genetic barrier for resistance mutations but may interfere with host cellular processes. In both cases, a better molecular characterization of the virus-virus and virus-host interactions mechanisms potentially will lead to more efficient anti-HCV drugs.

The HCV (+)RNA genome encodes for a unique polyprotein precursor (~3000aa) that after processing by viral and host proteases results into ten different proteins divided into structural (Core, E1, E2) and non-structural (p7, NS2, NS3, NS4A, NS4B NS5A and NS5B) ones (5,6). NS3 to NS5B constitute the minimal set of viral proteins that assures the viral RNA replication; all are anchored into the ER host membrane to form the replication complex (RC). NS3, which carries both RNA helicase and serine protease activities, is actually one of the main targets for DAA development, with two compounds (Telaprevir and Bioceprevir) that were last year FDA approved (4). In addition to NS3, NS5B constitutes another promising target for DAA development (2,7).

NS5B is the central protein (65kDa) for the HCV replication process as it carries the RNA-dependent RNA polymerase (RdRp) enzymatic activity (8-10). The last 21 residues at the C-terminus of NS5B are required for ER membrane anchoring (11,12) and in vitro HCV replication (13), but they are not absolutely required for its enzymatic activity in vitro (14,15). NS5B exhibits a similar “right-hand” shape as other RdRps, with thumb, palm and finger subdomains (16-18). NS5B inhibitors are divided into nucleoside inhibitors (NIs), which bind into the active site and are terminator substrates of the RNA polymerase activity, and non-nucleoside inhibitors (NNIs) that bind into one of the allosteric sites of the polymerase. Numerous crystal structures of NS5B in complex with NNIs have led to the identification of at least four major NNI binding sites (reviewed in (2)). However, the conformational changes that follow NNI binding (19-24) can interfere with proper crystal packing, thereby rendering crystallographic results difficult to interpret.

In vivo, HCV RNA replication occurs in a replication complex that next to the ER-derived membrane(s), NS3 to NS5B viral proteins (25) and viral RNA also contains several host factors such as hVAP-33 (26), VAP-B (27) and Cyclophilin A (CypA) (28). The latter is a major host factor required for HCV replication (29). It belongs to the Cyclophilin (Cyp) family of proteins that bind Cyclosporin A (CsA) and exhibit peptidyl-prolyl cis-trans isomerase (PPIase) activity (30). Cyps are able to catalyze the cis-trans isomerization of a peptide bond preceding a proline residue. Cyps have been identified as the main target of the early observed anti-HCV activity of CsA (31) or, more recently, of its non-immunosupressive derivatives (32-34). One of them, Debio-025 (Alisporivir), is currently evaluated in a phase III clinical trial. Watashi et al. initially proposed that Cyclophilin B would be the target of CsA, and proposed a mechanism whereby CypB would be a positive regulator of NS5B by modulating its RNA binding activity (35). Later, several members of the Cyclophilins have been proposed to be involved into the HCV replication (29,31) and NS5B interactions with CypB (35-39) and CypA (28,29,36,40) have been reported. However, more recent evidence points to CypA as the major key player both for HCV replication and CsA sensitivity (29,41). The molecular mechanisms that confer to Cyclophilin inhibitors an anti-HCV activity remain to be identified. In vitro selection for CsA-resistance mutations pointed out three viral non-structural proteins: NS2, NS5A and NS5B as potential Cyclophilin partners (37,41,42). Moreover, mutations located in the second half of NS5A confer the highest level of resistance to Cyclophilin inhibitors (37,43).

NS5A is a 49kDa phospho-protein, with RNA-binding properties (44), that is required for HCV RNA replication and viral particle production (45-47) but for which up to now no enzymatic activity has been identified. The protein...
is involved in several steps of the HCV life cycle, and interacts with almost all other non-structural proteins and with numerous host factors (48,49). NS5A is anchored at the cytoplasmic side of the ER membrane via a N-terminal helix (50) and comprises 3 cytosolic domains (D1, D2 and D3). NS5A-D1 carries most of the RNA-binding properties of the protein. It has been shown that NS5A-D1 may adopt two different hetero-dimeric structures, at least in the context of a crystal (51,52). NS5A-D2 (250-342), which is essential for RNA replication (47), and -D3 (356-447), which is involved in the production and assembly of viral particles (45), have been shown to be natively unfolded (53-57). In previous studies we have shown that both NS5A-D2 (53) and NS5A-D3 (57) establish direct interactions with CypA in vitro and that these domains are substrates for the PPIase activity of CypA. Using NMR spectroscopy we have mapped onto NS5A-D2 the CypA interactions sites and found a correlation with CsA-resistance mutations or even mutations that impair RNA replication (53).

HCV NS5B and NS5A have been shown to interact together both in vivo and in vitro (58-60). Conflicting results have been reported about the functional consequences of this protein-protein interaction. Indeed depending on the experimental setups used it has been shown that NS5A may either stimulate (59,61) or inhibit (59,62,63) the NS5B RdRp activity. At the molecular level, a strategy based on how NS5B mutations affect the NS5B-NS5A pull-down efficacy identified four discontinuous regions of NS5B (139-145, 149-155, 365-371 and 382-388) as essential for NS5A binding (60). Using truncated versions of NS5A, two regions of this protein (105-162 and 277-334) have been shown to be independently essential for the interaction with NS5B (59). Nevertheless, the absence of a structure of the complex leaves open many questions about the functional consequences of this interaction.

HCV NS5B, NS5A and host CypA are three essential components of the viral replication complex, and as such form three attractive targets for anti-HCV drugs. However, conflicting results have been reported as for the role of CypA for the proper association of these three proteins into the replication complex (28,40). Here we report NMR spectroscopy analyses, at a per-residue level, of the molecular interactions between NS5A domains 2 and 3, NS5B and CypA. We show that both HCV NS5B and host CypA share a common binding site on NS5A, and use NMR of the NS5B enzyme to get a first localization of the NS5A binding site on NS5B.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Non-labeled NS5B** – Expression of recombinant NS5B from HCV JFH-1 strain and fused to a C-terminal 6xHis-tag was performed in *E. coli* BL21(DE3) with the pET21-NS5B plasmid that was kindly provided by Dr. S. Bressanelli (CNRS, Gif-sur-Yvette, France) (64). Cells were grown at 37°C in Luria-Bertani (LB) medium supplemented with 1% Glucose. When the culture reached OD600~0.5 the temperature was lowered to 23°C and the protein production was induced with 0.5mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) overnight. Cells were harvested by centrifugation and resuspended in 40 ml of lysis buffer (20mM Tris-Cl pH7.5; 500mM NaCl; 1% Triton X100; 10% glycerol; Proteases inhibitors (Complete EDTA-free, Roche). Cells were lysed using lysozyme and sonications. NS5B containing fractions were selected following SDS-PAGE analysis pooled and dialyzed against buffer A (300 mM NaH2PO4/Na2HPO4 (NaPi) pH 6.8, 50 mM NaCl, 1 mM EDTA and 4 mM DTT). The protein was concentrated up to ~100 µM with a Vivaspin15 concentrator (cutoff, 30 kDa) (Satorius Stedim Biotech), filtered (0.22 µm) and stored at 4°C.

**Expression and Purification of Non-labeled and 15N-labeled NS5A-D2** – Expression and purification of recombinant 15N-labeled NS5A-D2 from HCV JFH-1 strain were done as described previously (53). The resulting recombinant domain 2 of HCV NS5A (residues 248-341) has extra M- and -LQHHHHHHH extensions at the N and C termini, respectively.
purification of recombinant CypA were performed as described previously in (53).

Expression and Purification of 15N-labeled NS5A-D3 (JFH-1) – Expression and purification of recombinant 15N-labeled domain 3 of NS5A (NS5A-D3) from HCV JFH-1 strain were done as described previously in (57). The recombinant NS5A-D3 (JFH-1) comprises residues 355–464 of HCV NS5A protein and has extra M- and –LQHHHHHHH sequences at N and C termini.

NMR analyses of the interactions between NS5A-D2, CypA and NS5B – NMR spectra on 15N-NS5A-D2, 15N-NS5A-D3 and 15N-CypA were acquired at 298K on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic triple resonance probe head (Bruker). All the NMR samples were prepared in buffer A (300 mM NaH2PO4/Na2HPO4 (NaPi) pH 6.8, 50 mM NaCl, 1 mM EDTA and 4 mM DTT) with TMSP (sodium 3-trimethyl-silyl-[2,2,3,3-d4]propionate) as proton chemical shift reference and 5% D2O. Spectra were acquired and processed with TopSpin 2.1 software (Bruker). Non-labeled NS5BΔ21 was mixed with either 15N-NS5A-D2, 15N-NS5A-D3 or 15N-CypA at two different molar ratios (1:1 [50µM] and 5:1 [100:20µM]) and 1H,15N-HSQC spectra were acquired. The potential interactions were assessed by comparing the heteronuclear spectra of those labeled proteins in absence and in presence of NS5B. Two parameters were analyzed: line broadening and chemical shift perturbations. Line broadening was assessed by measuring the maximal peak intensities rather than integrals due to partial signal overlap in the proton dimension. The 1H,15N-chemical shift perturbations were calculated using Equation 1 in which δΔ(1HN) and δΔ(15N) are the chemical shift perturbations in the proton and nitrogen dimensions, respectively.

\[
\delta \Delta = |\delta \Delta(1HN)| + 0.2 \times |\delta \Delta(15N)| \quad \text{(Eq. 1)}
\]

Assignment of the NS5A-D2, NS5A-D3 and CypA spectra was taken from our previous studies (53,57).

Surface Plasmon Resonance analyses of the NS5BΔ21–NS5A-D2, CypA–NS5BΔ21 and CypA–NS5A-D2 interactions – All materials and chemicals came from Biacore (GE Healthcare Europe Gmbh, Uppsala, Sweden). Analyses were performed at 25°C on a Biacore 3000 system. HBS-P (Hepes 10 mM, pH 7.4, NaCl 150 mM, Surfactant P20 0.005 %) was used both as running buffer at 10 µL/min and as dilution buffer for analytes. To assess the NS5BΔ21–NS5A-D2 interaction His-tagged recombinant NS5BΔ21 was covalently coupled onto a Biacore CM5 (carboxymethylated) sensor chip using an amine-coupling kit (Biacore) according to the manufacturer’s instructions. NS5BΔ21 was immobilized at a concentration of 4 µg/mL in a sodium acetate 10 mM buffer, pH 5 and at a 5 µL/min flow rate of HBS. Covalent binding resulted in a signal of 2400 RU (resonance units). To assess the CypA–NS5A-D2 interaction the same procedure was applied to immobilized-CypA at 8µg/ml with a resulting signal of 2200 RU. A flow cell was submitted to a similar treatment but in absence of protein. This control flow cell was used as a control to assess bulk effects and non-specific binding of the NS5A-D2. The NS5A-D2 was injected at concentrations ranging from 12.5 to 200 µM in HBS-P at a 10 µL/min flow rate during 4 min. Dissociation was detected during 11 min with 10 µL/min flow of HBS-P. Regeneration was done by injecting 10 µl of 10 mM NaOH with a 10 µL/min flow rate. Results are represented as sensograms expressing the kinetics of NS5A-D2 binding (resonance units) to respectively immobilized NS5BΔ21 and CypA. Experiments have been performed at least 3 times. To assess the CypA–NS5BΔ21 interaction NS5BΔ21 was injected at concentrations ranging from 12.5 to 90 µM in HBS-P supplemented with NaCl up to 300mM at a 10 µL/min flow rate during 4 min. Data were analyzed using BiaEvaluation 3.1 software and both dissociation constant (Kd) and Req were calculated from kinetics parameters using method based on the Langmuir’s 1:1 binding model.

Expression and Purification of 15N,2H-labeled NS5BΔ21 (JFH-1) – To produce the 15N,2H-labeled NS5BΔ21 BL21(DE3) cells carrying the pET21-NS5BΔ21 plasmid were grown in deuterated M9-based semi-rich medium (M9 medium in 99.8% D2O with 15NH4Cl (1g/liter), 2H7-D-glucose (3 g/liter) and 2H,15N-rich medium (Isogro 15N-D, Isotec) (0.5g/liter). To reach A600~1.2 the cells were grown at 37°C then the temperature was lowered to 23°C and protein expression was induced with 0.5mM IPTG (in D20) overnight.
Purification of $^{15}$N,$^2$H-labeled NS5B$_{\Delta 21}$ was performed in a similar way that for the non-labeled protein.

**NMR data collection on $^{15}$N,$^2$H-NS5B$_{\Delta 21}$** - NS5B NMR spectra were acquired at 305K on a Bruker 900MHz spectrometer equipped with cryogenic probe. All $^{15}$N,$^2$H-NS5B$_{\Delta 21}$ samples were in buffer A. The $^{15}$N,$^2$H-NS5B$_{\Delta 21}$ concentration was 90µM when alone and 70µM in a 1:1 molar ratio when in presence of NS5A-D2. For the interaction of NS5B with a small molecule, a thiophene-2-carboxylique (TCA) inhibitor (NNI-1 in (65)) was lyophilized and then redissolved with a 90µM $^{15}$N,$^2$H-NS5B$_{\Delta 21}$ sample in buffer A supplemented with 3% (v/v) DMSO- $d_6$. For all samples $^1$H,$^{15}$N TROSY spectra (66) were acquired on 300µL samples in 5mm Shigemi tubes with 4096 and 320 complex points in the direct and indirect dimensions respectively and with 128 scans.

The PyMOL software was used for molecular graphics (DeLano, W.L. The PyMOL Molecular Graphics System (2002) on the World Wide Web http://www.pymol.org).

**RESULTS**

**Interaction of NS5B with NS5A** - HCV NS5B is the central enzyme for HCV replication as it carries the RNA dependent RNA polymerase activity (8-10). NS5A is a multi-domain protein that has been shown to be essential both for HCV replication and infectious particle production (45-47) but for which no precise molecular function has been evidenced. NS5B and NS5A are two central components of the replicase, where HCV RNA replication occurs, and have been shown to interact together. Here we use NMR spectroscopy to gain a very precise view, at a per residue level, of the direct molecular interaction between the domains 2 and 3 of NS5A (NS5A-D2 and NS5A-D3) and the HCV polymerase NS5B$_{\Delta 21}$ lacking the 21 C-terminal residues that correspond to its in vivo membrane anchor. All proteins have been produced in _E. coli_ and then purified to almost homogeneity as judged by coomassie stained SDS-polyacrylamide gels.

We previously assigned the spectra of the D2 and D3 domains of HCV NS5A when isolated in solution (53,56,57,67). To monitor by NMR spectroscopy the effects induced by NS5B$_{\Delta 21}$ on the spectrum of NS5A-D2 (or NS5A-D3), we mixed at equimolar concentrations (i.e. 50µM) $^{15}$N-labeled NS5A-D2 (or NS5A-D3) with unlabeled polymerase and recorded $^1$H,$^{15}$N-HSQC spectra. $^1$H,$^{15}$N-chemical shift perturbations and/or signal broadening thereby should serve as indicators of a physical interaction. In the $[^{15}$N]-NS5A-D2 spectrum in presence of the polymerase, we observed numerous NMR peaks that were broadened up to near disappearance (Fig. 1 and Suppl. Fig. 4), but chemical shift perturbations were limited (see supplemental Figs. 2 and 5).

Hence, _in vitro_ NS5B$_{\Delta 21}$ establishes a direct physical interaction with NS5A-D2. Broadening of the NS5A-D2 peaks, which are usually very sharp due to the unfolded nature of this domain, is indeed expected upon interaction with the high molecular weight NS5B$_{\Delta 21}$ (65kDa). Based on our previous assignment of the NS5A-D2 spectrum, we could plot on a per residue basis the ratio of the peak intensities in the absence or presence of NS5B$_{\Delta 21}$, and as such identified three different regions of NS5A-D2 that interact with the polymerase (Fig. 1A and 1C and Suppl. Fig. 5). Two peptides of roughly ten residues form the first anchoring points (residues 250-262 (region A) and residues 274-287 (region B)). A larger zone of interaction, spanning residues 306 to 333 forms the third zone of interaction (region C). Together, these three regions span roughly half of the NS5A-D2 domain, underscoring the extensive physical interaction between the D2 domain and NS5B$_{\Delta 21}$. When looking at the physicochemical properties of the defined regions, we notice that the two first N-terminal ones are negatively charged, with 3 Asp + 1 Glu and 4 Glu residues for regions A and B, respectively. Region C is different, as it carries besides the 3 negatively charged residues also 4 positively charged residues (4 Arg). Although we previously showed that the D2 domain is essentially unfolded, and that this property is conserved across HCV genotypes (53-55,67), we re-analyzed the experimental $^{13}$C NMR chemical shifts corresponding to the $C_\alpha$ and $C_\beta$ atoms of NS5A-D2 (BMRB accession number 16165, (53)) with the SSP (Secondary Structure Propensities) program (68). This novel algorithm detected a residual secondary structure in the three
interaction regions of NS5A-D2 (Fig. 1B and 1C). The SSP positive score for region A corresponds to a helical tendency, the negative score for region B rather points to a \(\beta\)-strand or extended tendency, and region C shows a non-regular structural propensity.

As the NMR spectrum of \([^{15}\text{N}]\)-NS5A-D2 in presence of NS5B\(_{A21}\) suffered from excessive line broadening, thereby preventing a titration experiment, we used surface plasmon resonance (SPR) to measure an affinity constant for the complex. The purified NS5B\(_{A21}\) was immobilized on a sensor chip and then increasing concentrations of NS5A-D2, from 12.5 to 200\(\mu\)M, were injected in the device (Fig. 2). Analysis of the corresponding sensogram showed that the signal response, at equilibrium, was initially dose-dependent and then saturated, which corresponds to a specific interaction between NS5A-D2 and NS5B\(_{A21}\). The Scatchard plot of these data is linear and thus data are consistent with the Langmuir model (see Suppl. Fig. 3). The measured association (\(k_a\)) and dissociation (\(k_d\)) rate constants allowed the determination of an equilibrium dissociation constant (\(K_d\)) of 21x10\(^{-6}\) M (see Fig. 2) for the interaction between NS5B\(_{A21}\) and NS5A-D2.

We then used the same strategy to evaluate a potential interaction between the domain 3 of NS5A and NS5B\(_{A21}\). \([^{15}\text{N}]\)-labeled NS5A-D3 was mixed in a 1:1 ratio (50\(\mu\)M) with unlabeled NS5B\(_{A21}\) and peak intensities of the \(^1\text{H},^{15}\text{N}\)-HSQC spectra were compared to the ones of \([^{15}\text{N}]\)-NS5A-D3 in absence of the polymerase. In contrast with what has been observed for NS5A-D2, no significant peak broadening or even chemical shift perturbations were detectable on the NS5A-D3 spectrum in presence of the polymerase (Fig. 3A and Suppl. Fig. 2). We only observed a slight decrease for the peaks intensities of \([^{15}\text{N}]\)-NS5A-D3 with NS5B\(_{A21}\) that was nearly uniform all along the primary sequence of NS5A-D3 and that was probably due to an increased viscosity of the NMR sample when NS5B\(_{A21}\) was added as the total protein concentration increased from 1mg/ml (50\(\mu\)M of the 10kDa NS5A-D3) to near 4.3mg/ml (50\(\mu\)M of both NS5A-D3 and the 64.4kDa NS5B\(_{A21}\)). These data showed that \textit{in vitro} there is no direct molecular interaction between NS5A-D3 and the HCV NS5B\(_{A21}\), and confirm the specificity of the previously detected NS5A-D2:NS5B\(_{A21}\) interaction.

\textbf{Interaction of NS5B with Cyclophilin A – As CypA is an essential host factor for HCV replication and as interaction with the NS5B RdRp has been repeatedly reported (28,29,36,40), we equally compared the \(^1\text{H},^{15}\text{N}\)-HSQC spectra of purified \([^{15}\text{N}]\)-CypA alone or in presence of NS5B\(_{A21}\) in a 1:1 ratio (50\(\mu\)M each). As for the individual domains of NS5A, we looked for chemical shift perturbations and/or signal broadening in the CypA spectrum. We again detected the overall 10\% reduction of peak intensities that we previously ascribed to the increased NMR sample viscosity (Fig. 3B), but did not detect any region of CypA that underwent specific signal broadening when NS5B\(_{A21}\) was added. The analysis of the \(^1\text{H}\) and \(^{15}\text{N}\) chemical shift perturbations also did not reveal significant modification of the CypA NMR spectrum in presence of NS5B\(_{A21}\) (see Suppl. Fig. 2). These data hence show that \textit{in vitro}, there is no direct interaction between the HCV NS5B\(_{A21}\) polymerase and the human CypA. We repeated the experiment with an excess of unlabeled NS5B\(_{A21}\) over \([^{15}\text{N}]\)-CypA in a 5:1 molecular ratio (100\(\mu\)M:20\(\mu\)M), but could still not detect any significant perturbation of the NMR spectrum (see Suppl. Fig. 2). Even a weak affinity between both proteins thereby can be excluded. As our NMR results were in contradiction with previous reports in literature this potential interaction was further investigated using SPR (Suppl. Fig. 4B) but again we did not detect a specific interaction between NS5B\(_{A21}\) and CypA \textit{in vitro}.

\textbf{Interplay between NS5B, CypA and NS5A-D2 – As we showed in this study that NS5A-D2 interacts with NS5B\(_{A21}\) and as we had previously shown that NS5A-D2 also directly interacts with CypA (53), we set up an experiment with the three proteins present together. Firstly, we analyzed the perturbations on the \(^1\text{H},^{15}\text{N}\)-HSQC spectrum of \([^{15}\text{N}]\)-NS5A-D2 induced by non-labeled CypA alone. In agreement with our previous findings...
and confirmed very recently with a peptide-immobilized spot assay (69), two different regions of NS5A-D2 were broadened in presence of CypA (Fig. 1D). The first one comprises residues G304-P323 whereas the second one corresponds to residues G337-P341 located at the C-terminal end. One interaction region, centered at P315, is hence shared between NS5B_{A21} (Fig. 1C) and CypA (Fig. 1D), whereas the two N-terminal A and B regions are NS5B_{A21} specific (Fig. 1B), and the C-terminus of the domain is solely recognized by CypA (Fig. 1D). The common binding region corresponds to the most conserved residues of NS5A-D2 over all genotypes, with 5 residues (P310, W312, A313, P315 and P319) that are strictly conserved (see ref. (53)), and further corresponds to the location where Csa-A resistance mutations were identified (37,70). To further probe this common binding motif in NS5A-D2, we prepared a new NMR sample with the three different proteins in a 1:1:1 molecular ratio (i.e. ([^{15}N]-NS5A-D2 : NS5B_{A21} : CypA at 43µM each), and looked at the NMR spectral perturbations on NS5A-D2. In the HSQC [^{15}N]-NS5A-D2 spectrum of the ternary mixture (Fig. 1E), we observed the sum of the individual effects caused by either the addition of NS5B_{A21} (Fig. 1C) or the addition of CypA (Fig. 1D) alone. Indeed when the NS5A-D2 peak intensities in the experiment with both NS5B_{A21} and CypA (Fig. 1E) were normalized to the ones when only NS5B_{A21} (Fig. 1C) was added, we obtained the intensity profile (Fig. 1F) identical to the one whereby only CypA (Fig. 1D) was added. Addition of Csa, a CypA inhibitor, into the [^{15}N]-NS5A-D2 : NS5B_{A21} : CypA ternary sample restored the pattern of the NS5A-D2 peak intensities to that when only NS5B_{A21} was added (Fig. 1G). Thus Csa inhibits the interaction of CypA with NS5A-D2, but has no influence on the molecular interaction between the polymerase NS5B_{A21} and NS5A-D2. Our data hence do not favor the formation of a stable ternary complex between NS5A-D2, NS5B_{A21} and CypA, but rather point to a system where NS5A-D2 can interact either with NS5B_{A21} or with CypA with comparable affinities. For those NS5A-D2 residues in the P306-E323 region that allowed to observe a chemical shift difference as they were not broaden beyond detection in the presence of NS5B_{A21} and CypA, we found intermediate frequencies compared to the ones in the spectrum with NS5B_{A21} or CypA alone. Moreover, the minor peaks of the [^{1}H,^{15}N]-HSQC spectrum of NS5A-D2, which correspond to residues in the vicinity of a Proline in cis conformation (53), can be used as a further probe for CypA accessibility as they tend to disappear rapidly from the spectrum upon addition of even a catalytic amount of CypA, because of exchange broadening (53). Despite the fact that regions A (residues 250-262) and B (residues 274-287) of NS5A-D2 seem to be only involved in the binding of the polymerase (Fig. 1C and 1D), we still observe an effect of CypA in these locations in the sample where both NS5B_{A21} and CypA were present. Indeed, minor peaks in the A and B NS5B_{A21} specific regions still disappear from the NS5A-D2 spectrum when CypA is added (see Suppl. Fig. 6), demonstrating that CypA can still access the regions A and B of NS5A-D2 in the presence of NS5B_{A21}. These same minor peaks, assigned to residues in region B that neighbor a proline in cis conformation, are however only weakly affected by the addition of NS5B_{A21} (see Suppl. Figs. 6 and 7), indicating in this case a trans-isomers specificity for the NS5A-D2–NS5B_{A21} interaction.

NMR analysis of NS5B – Numerous structures of NS5B in complex with nucleotides or with inhibitors have been solved by X-ray crystallography. However, molecular data that lead to the mapping of NS5B binding sites for interacting proteins and even for RNA molecules are mostly lacking. NMR spectroscopy on the purified polymerase NS5B_{A21} could be useful in the characterization of such interactions, but evidently faces the technical hurdle stemming from its high molecular weight (65kDa). Recent technical developments, however, combining extensive protein deuteration and novel transverse relaxation optimized pulse schemes have shown promising results on systems of up to 1 MDa (71). We thus produced the doubly labeled [^{15}N,^{2}H]-NS5B_{A21}, and although the biomass of the bacteria grown in D_{2}O minimal medium was reduced, we did obtain enough purified protein for several NMR experiments (see Suppl. Fig. 1). The purified [^{15}N,^{2}H]-NS5B_{A21} was kept almost two weeks in protonated buffer at 4°C to allow the
amid deuterons to back-exchange with protons from water. Then we acquired a \(^{1}H,^{15}N\)-TROSY spectrum (66,72) on a 90µM sample of \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) using a high-field 900MHz NMR spectrometer equipped with a cryogenic probe. The resulting heteronuclear NMR spectrum of the HCV polymerase was of high quality, but evidently showed a large number of correlation peaks (Fig. 4). An automatic peak-picking procedure using TopSpin software (Bruker) led to the detection of nearly 490 peaks in the \(^{1}H,^{15}N\)-TROSY spectrum (Fig. 4). This number of peaks is lower than the 578 residues (including His-tag) in the NS5B\(_{\Delta 21}\) construct we used, mainly due to signals overlaps, but still important regarding the coverage of the primary sequence of the polymerase, i.e. more than 80%. The assignment of the NS5B\(_{\Delta 21}\) spectrum is ongoing in our laboratory, but will require intensive biochemistry (through different labeling strategies) and NMR spectroscopy optimization. Nevertheless, as a first application of the spectrum, we tested if the interaction with NS5A-D2 could now be monitored directly on the \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) NMR spectrum. We hence mixed \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) with unlabeled NS5A-D2 at equimolar concentrations (70 µM) and compared the resulting \(^{1}H,^{15}N\)-TROSY spectrum with that of isolated \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\). In the presence of NS5A-D2, several peaks in the \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) spectrum underwent line broadening (Fig. 5, A and B and Suppl. Fig. 9A), thereby confirming our previous findings on a direct physical interaction between NS5B\(_{\Delta 21}\) and NS5A-D2. Only a subset of peaks in the \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) spectrum was affected, meaning that the NS5A-D2 binding site on the polymerase is rather localized, and arguing against important conformational changes that would occur upon NS5A-D2 binding. In order to get information about the NS5A-D2 binding site on the HCV polymerase, but in the absence of the assignment of the TROSY spectrum, we used an indirect strategy. We first analyzed the NMR spectral perturbations that were induced on the \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) spectrum following addition of a TCA inhibitor (Fig. 5, C and D and Suppl. Fig. 9B), for which the binding site onto NS5B is known as its structure in complex with the polymerase has been solved by crystallography (20,65) (see Suppl. Fig. 10). Then we compared these perturbations with the ones that were induced in the \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) spectrum upon addition of NS5A-D2 (Fig. 5, A and B). Some spectral perturbations on the \([^{15}N,^{2}H]\)-NS5B\(_{\Delta 21}\) spectrum were common upon addition of both NS5A-D2 and TCA inhibitor, while others were specific to one or the other binding partner. These data suggest that the NS5A-D2 binding site on NS5B\(_{\Delta 21}\) overlaps at least partially with that of the TCA inhibitor. NMR of NS5B might hence represent a highly valuable tool to study interactions mechanisms of this RNA dependent RNA polymerase with different ligands or proteins.

**DISCUSSION**

The replication of HCV together with the polyprotein processing constitute main targets for development of anti-HCV drugs. As the replication process requires the formation of a membrane-associated multiprotein complex its impairment can be done by hitting several individual targets, which can be viral or even host factors. The drugs that are currently in development and that act through the inhibition of HCV replication target either the HCV RdRp NS5B, NS5A or CypA, an essential host factor. These three proteins are essential components of the replicase, and have all been reported to interact two by two. Here we characterized these molecular interactions, at a per residue level, by NMR spectroscopy (Fig. 6).

Through the analysis of the chemical shift perturbations in the NS5A-D2 NMR spectrum that were induced following addition of unlabeled NS5B\(_{\Delta 21}\) we identified three regions in NS5A-D2 that are involved in the interaction with the polymerase (see Fig. 1, A and C). These NS5B-binding sites correlate with the regions in the unfolded domain 2 of NS5A that exhibit some residual secondary structure propensity (see Fig 1, B and C). NS5A-D2 thereby joins the growing list of unfolded proteins that interact with relevant biological partners through their residual structural elements (73,74). Using the same NMR strategy, we showed that the unfolded domain 3 of NS5A does not contribute to the binding of NS5B (see Figs. 3A and 6). Our results thereby complete the ones from Shirotal et al. who had performed a rough identification of the NS5B-binding site on
NS5A using internal deletion mutants (59). They identified, in genotype 1b, two discontinuous regions of NS5A, residues 105-162 and 277-334, which are both required for proper interaction with NS5B. NS5A residues 277-334 in genotype 1b correspond to residues 273-330 in the genotype 2a we used in this study. As it has been later shown that region 105-162 belongs to the folded domain 1 of NS5A its deletion may abolish the overall 3D structure of this domain and render the previous result difficult to interpret. The second described region 273-330 (genotype 2a numbering) encompasses two different binding regions, 274-287 and 306-333, that we identified at a per residue level in this work (see Fig. 1C). Shirota et al. have reported that the regions 105-162 and 273-330 (genotype 2a numbering), located into domain 1 and domain 2 of NS5A respectively, are independently essential for the binding of NS5B (59). We do not confirm this conclusion, as we showed that the isolated domain 2 of NS5A (residues 248 to 341) is sufficient to interact with the polymerase. However, our NMR assay does not allow distinguishing whether regions A to C of NS5A-D2 interact at the same time with the same NS5B molecule, or if individual regions A, B and C interact at distinct moments with different NS5B molecules. Nevertheless, the Scatchard plot of the SPR data is consistent with a 1:1 Langmuir model (see Suppl. Fig. 3). Considering the conservation level of the NS5A-D2 primary sequence among all HCV genotypes, the NS5B-interacting regions A, B and C correspond to regions with less variability and even contain some strictly conserved residues (A257, N258; D278; P310,W312, A313, P315, P319, W325 and Y330) (see Suppl. Fig. 11 and ref. (53)). This indicates that the 3 regions we identified in NS5A-D2 are functionally important for the virus; one possibility is that these regions are required for the proper interaction of NS5A-D2 with NS5B and hence for an efficient replication mechanism.

The NS5A-D2 region (273-330, genotype 2a numbering), identified by Shirota et al. to be required for NS5B binding (59), was further characterized in two subregions. Alanine substitutions in residues 273 to 305 (genotype 2a numbering) partially impaired the level of replication, whereas residues 306 to 330 are essential as their substitutions led to absence of RNA replication (58). However the absence of replication when NS5A-D2 residues 306 to 330 were alanine-substituted may not be directly related to an impaired NS5A-NS5B interaction but may also be linked to the functional interaction with host CypA. Indeed we showed here that NS5A-D2 residues G304-E323 and G337-P341 constitute CypA-binding sites (see Fig. 1D). This result is identical with what we initially described (53) and is coherent with the tandem cyclophilin-binding site of NS5A that has recently been reported by Grisé et al. (69). We show here that HCV NS5B and host CypA share a common binding site on NS5A, which contains residues P306 to E323 (see Fig. 1, C and D). Caution in the interpretation of replication assays with mutations and/or deletions in this particular NS5A region is thus required, as the observed effect may arise either from a modification of the NS5A-NS5B interaction and/or of the NS5A-CypA interaction (Fig. 6). We did not detect any direct physical interaction between CypA and NS5B (Fig. 6), in contradiction with several previous reports (28,29,36,40). The absence of chemical shifts perturbations in the CypA spectrum upon addition of NS5B (see Fig. 3B) was confirmed by the reverse experiment, whereby the NMR spectrum of NS5B did not appreciably change upon addition of CypA (data not shown). Although this apparent discrepancy may arise from the different constructs or genotypes that were used, a more plausible hypothesis is that the described CypA-NS5B interaction was indirect and mediated by a third interacting partner. Indeed, to our knowledge, no studies have been done in vitro with only the two purified proteins but rather with cell lysate pull-down experiments (29,40), crude replication complex preparations pull down experiments (28), or in in vitro translation reactions (36) that at least contain RNAs. Without interaction between NS5B and CypA, the interplay between HCV NS5B, NS5A and host CypA, three essential viral replicase components, may be driven by the common binding site on NS5A-D2 that we identified. When we mixed NS5A-D2 with both NS5B\textsubscript{21} and CypA in the same tube, we observed in the NS5A-D2 NMR spectrum the sum of the spectral perturbations (see Fig. 1E) that were individually observed by the addition of either NS5B\textsubscript{21} (see Fig. 1C) or CypA (see Fig. 1D). Our NMR results are in favor of a model where NS5A-D2 would interact with similar
affinities either with CypA or with NS5B (Fig. 6). We previously estimated, by NMR spectroscopy, the affinity corresponding to the CypA-NS5A-D2 interaction at 64µM (53) and in this work, using SPR, we measured a $K_D$ of $\sim$18µM (see Suppl. Fig. 4A). The fact that these two values were determined by different methods, in solution or at surface, may explain the relative variation. For the NS5B-NS5A-D2 interaction, using SPR, we measured a $K_D$ of $\sim$21µM (see Fig. 2). Thus the interaction strengths between NS5A-D2 and either CypA or NS5B are indeed quite similar. Moreover, we show that CsA only disrupts the molecular interaction between CypA and NS5A-D2 without interfering with the NS5A-D2-NS5B interaction (Fig. 6). Grisé et al. recently described that they have preliminary data indicating the same findings (69). Chatterji et al have shown that CsA treatment has no influence on the association of NS5A and NS5B with the replication complex but rather depletes it from CypA (75). Their findings and our data are in favor of a model where the association of NS5A and NS5B into the replication complex would be mediated by direct interaction between these two viral proteins and that CypA incorporation in this functional complex would be done via direct interaction between these two viral proteins and that CypA incorporation in this functional complex would be done via its interaction with domain 2 of NS5A. This model is strengthened by our observation that CypA and NS5B have different cis-trans isomer specificities for NS5A-D2 binding in region B (see Suppl. Figs. 6 and 7).

In this study we report the first high quality heteronuclear NMR spectrum of HCV NS5B (see Fig. 4 and Suppl. Fig. 8), and show that this spectrum can be used to monitor molecular interactions directly onto the polymerase (see Fig. 5 and Suppl. Fig. 9). Indeed we detected shifts and/or broadenings for a limited number of peaks in the NMR spectrum of a doubly labeled $[^1H,^{15}N]$-NS5B$_{\Delta 21}$ in presence of unlabeled NS5A-D2 (see Fig. 5, A and B and Suppl. Fig. 9B). These effects, which corresponds to fast exchange, are in agreement with the $K_D$ of $\sim$21µM that we measured by SPR. The limited number of peaks that were affected indicates that NS5B does not undergo a global conformational change upon NS5A-D2 binding. This possibility has often been proposed to explain the effect, positive or negative, of NS5A-D2 on the RdRp enzymatic activity of NS5B (61,62). Currently, we have only started to assign the NMR peaks of NS5B$_{\Delta 21}$, but it is already clear that even a partial assignment will require intensive biochemistry and NMR spectroscopy efforts. Nevertheless, we used an indirect strategy and showed that the NS5A-D2 binding site on NS5B partially overlaps with the binding site of a TCA inhibitor (see Fig. 5 and Suppl. Fig. 9), which is known to bind into the thumb site 2 of the polymerase (2,20,65) (see Suppl. Fig. 10). NMR hence should allow the direct mapping of the residues involved into various molecular interactions without using deletion or resistance mutants that may interfere with the global structure of the polymerase. Direct solution NMR spectroscopy on NS5B$_{\Delta 21}$ should also allow the study of compounds that induce relatively large conformational changes upon binding and that may interfere with crystal packing (19,20,23). Finally, the NMR spectrum of NS5B$_{\Delta 21}$ that we obtained may be used to characterized low affinity (over the micromolar range) interactions mechanisms as it is the case with NS5A-D2.

In conclusion, we report here the first direct high-resolution characterization of the molecular interactions between HCV NS5A, NS5B and host CypA. We show that NS5B and CypA share a binding site on NS5A-D2-NS5B interaction (Fig. 6). Further studies will be required to make a link between the interactions we observed in-vitro and their potential effects on the RdRP activity of the HCV polymerase. The NMR spectrum of HCV NS5B$_{\Delta 21}$ furthermore constitutes a powerful tool to investigate its potential interactions with various interaction partners such as other proteins, small inhibitors or even RNAs.
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FOOTNOTES

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4 The abbreviations used are: aa, amino acid; CsA, Cyclosporin A; Cyp, cyclophilin; HCV, hepatitis C virus; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; NS5A, nonstructural protein 5A; NS5A-D2, recombinant protein representing aa 248-341 of NS5A from JFH-1 HCV strain, with a N-terminal Methionine and a C-terminal LQHHHHHH extension; NS5A-D3, recombinant protein representing aa 355-464 of NS5A from JFH1 HCV strain, with a N-terminal Methionine and a C-terminal LQHHHHH extension; NS5BΔ21, recombinant protein representing aa 1-570 of NS5B from JFH1 HCV strain, with a C-terminal SHHHHHH extension; PPIase, peptidyl-prolyl cis/trans isomerase; RdRp, RNA-dependent RNA polymerase.
FIGURE LEGENDS

FIGURE 1. NMR analyses of the NS5A-D2 interactions with NS5B Δ21 and CypA. A, The NS5A-D2 sequence from JFH-1 HCV strain (genotype 2a) that was used in this study is shown with its numbering according to the full-length NS5A protein. B, Secondary Structure Propensity (SSP) (68) analyses of the 13Cα and 13Cβ NMR chemical shifts of NS5A-D2 (53). Positive SSP scores indicate α-helical tendency whereas negative scores indicate β-strand or extended tendencies. C to G, NMR analyses of the molecular interactions between NS5A-D2 and either NS5B Δ21 (1:1) (C), or CypA (1:1) (D), or both NS5B and CypA (1:1:1) in absence (E and F) or in presence of CsA (G). For each NS5A-D2 residue the intensity of its corresponding peak in a 1H,15N-HSQC spectrum is shown as a black bar and has been normalized compared to the intensity observed for NS5A-D2 alone (C to E) or for NS5A-D2 in presence of NS5B Δ21 (F and G). The positions of proline residues are indicated by grey points as they do not have proton amide and thus do not have corresponding signal in HSQC experiments. In case of signal overlaps the intensities of the corresponding residues were divided by the number of peaks concerned.

FIGURE 2. Surface Plasmon Resonance analysis of NS5A-D2 interacting with NS5B Δ21. NS5B Δ21 was immobilized on a CM5 sensorchip and NS5A-D2 was injected at different concentrations (12.5 to 200 µM). Association was studied during 4 mins, and dissociation during 11 mins. The data shown correspond to one representative experiment among four independent repetitions. The sensorgrams, which show the kinetics of specific binding of NS5A-D2 at various concentrations to immobilized NS5B Δ21 (expressed as response unit (RU)), were obtained by subtracting unspecific binding of NS5A-D2 to the sensor chip control cell. The upper right insert shows the parameter results (kₐ, k₈ and K_D; ±SD, standard deviation) that have been determined using BiaEvaluation software 3.1 with a Langmuir 1:1 model over four independent experiments.

FIGURE 3. NMR analyses of the potential interactions of NS5A-D3 and CypA with NS5B Δ21. 15N-NS5A-D3 (A) or 15N-CypA (B) were mixed with unlabeled NS5B Δ21 (1:1) then for each residue of these proteins the NMR intensity of its corresponding HSQC peak was measured and normalized to that in absence of the polymerase. Grey points indicate proline residues that do not have correlation signal in HSQC NMR experiment as they do not have amide proton.

FIGURE 4. 1H,15N TROSY NMR spectrum of HCV NS5B Δ21. The spectrum was acquired on a 900MHz NMR spectrometer on a 2H,15N-labeled NS5B Δ21 sample at 90µM (305K).

FIGURE 5. Interaction of [2H,15N]-NS5B Δ21 with NS5A-D2 or TCA inhibitor. Subset regions of overlaid 1H,15N TROSY NMR spectra of NS5B Δ21 in absence (blue spectra) (A to D) or in presence of either NS5A-D2 (red spectrum in A and B) or a TCA inhibitor (red spectrum in C and D). Both experiments in C and D contained 3% (v/v) DMSO-d₆ in the sample buffer. The NMR peaks on which spectral perturbations were observed with both NS5A-D2 and TCA, with only NS5A-D2 and with only TCA inhibitor were respectively labeled with •, ▼ and ◆ symbols.

FIGURE 6. NMR analyses of the interactions between HCV NS5A and NS5B proteins and host CypA. NS5A (in light grey) is shown as a dimer anchored to the cytoplasmic side of the endoplasmic reticulum (ER) membrane via a N-terminal amphipatic helix (AH). Domain 1 of NS5A (D1) is shown as cartoon from the PDB entries 1ZH1 and 1R7C. The disordered domain 2 and 3 of NS5A are represented by manually drawn lines. NS5B (in black) is shown as cartoon from the PDB entry 3I5K. Its C-terminal membrane anchoring helix is represented by a 20 residues alpha-helix. Human CypA and CsA are respectively shown as cartoon and sticks from PDB entry 1CWA. The direct in vitro molecular
interactions between these proteins were investigated by NMR spectroscopy and summarized in this figure. NMR detected interactions are represented by solid arrows whereas absence of interactions are represented by dotted lines. The interactions that were found to be inhibited by CsA are highlighted with a ⊘ symbol.
Figure 1

NSSA-D2

A

B

C

D

E

F

G

residues
Figure 2
Figure 4
Figure 5
Figure 6
Hepatitis C Virus NS5B and Host Cyclophilin A share a common binding site on NS5A
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