

Dimerization-driven interaction of hepatitis C virus core protein with NS3 helicase

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Hepatitis C virus (HCV) infects over 130 million people causing a worldwide epidemic of liver cirrhosis and hepatocellular-carcinoma. Because current HCV treatments are only partially effective, molecular mechanisms involved in HCV propagation are actively being pursued as possible drug targets. Here, we report on a new macromolecular interaction between the HCV capsid core protein and the helicase portion of HCV non-structural protein 3 (NS3h), confirmed by four different biochemical methods. The protease portion of NS3 is not required. Interaction between the two proteins could be disrupted by two types of specific inhibitors of core dimerization, the small molecule SL201 and core106, a C-terminally truncated core protein. Cross-linking experiments suggest that the physical interaction with NS3h is probably driven by core oligomerization. Moreover, SL201 blocks the production of infectious virus, but not the production of a subgenomic HCV replicon by hepatoma cells. Time-of-addition experiments confirm that SL201 has no effect on entry of the virus. These data underline the essential role of core as a key organizer of HCV particle assembly, confirm the importance of oligomerization, reveal the interaction with viral helicase and support a new molecular understanding of the formation of the viral particle at the level of the lipid droplets, before its migration to the site of release and budding.

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INTRODUCTION

Hepatitis C virus (HCV) infects nearly 2.2% of the world population and is a common cause of chronic liver disease (Alter, 2007; Lavanchy, 2009). No vaccine is available, and the only current treatment (combination therapy of pegylated interferon with ribavirin) has limited efficacy and serious side effects (Sakamoto & Watanabe, 2009; Tan *et al.*, 2002). HCV, an enveloped positive-sense ssRNA virus (Choo *et al.*, 1989), belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The 9.6 kb HCV genome encodes a single 3000-residue precursor polyprotein cleaved by host-cellular and viral enzymes into three structural proteins (core, the envelope glycoproteins E1 and E2) and seven non-structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) of diverse functions (Moradpour *et al.*, 2007). Core, the capsid protein, is essential for infectious particle assembly and release (Miyanari *et al.*, 2007; Penin *et al.*, 2004; Shavinskaya *et al.*, 2007; Strosberg *et al.*, 2010). This protein of 191 residues consists of three distinct domains: (i) D1, an N-terminal hydrophilic domain of 117 aa; (ii) D2, a more hydrophobic domain of about

60 aa; and (iii) the C-terminal D3 which contains approximately the last approximately 14 mostly hydrophobic residues cleaved off during core maturation and which serve as the signal peptide for the downstream protein E1 (Boulant *et al.*, 2006). D1 is mostly responsible for self-association, for binding RNA and for interaction with several host cellular proteins (Angus *et al.*, 2010; Chen *et al.*, 2008; Yan *et al.*, 2007), whereas D2 is responsible for coating of lipid droplets (LDs) and for the folding of the N-terminal part (Appel *et al.*, 2008; Boulant *et al.*, 2005; Masaki *et al.*, 2008). Core dimers and higher-order complexes associate with the viral RNA to form the nucleocapsid and associate with E1 and E2 to form the infectious particles (Boulant *et al.*, 2005; Klein *et al.*, 2004; Kunkel *et al.*, 2001; McLauchlan, 2000; McLauchlan *et al.*, 2002). Recent studies suggest that several steps of virus assembly take place near the LDs and their associated membranes (McLauchlan, 2009a; McLauchlan *et al.*, 2009b; Miyanari *et al.*, 2007; Rouille *et al.*, 2006; Strosberg *et al.*, 2010). The interaction of NS5A with core and its recruitment to the LDs (McLauchlan *et al.*, 2002; Shi *et al.*, 2002) appear to be essential for virus assembly (Miyanari *et al.*, 2007) and a prerequisite for HCV particle production (Masaki *et al.*, 2008). In addition to the central role of core and that of NS5A, several recent studies suggest that other NS proteins,

Two supplementary figures and a supplementary table are available with the online version of this paper.

such as p7, NS2 and NS4B as well as NS3, may also be important in viral assembly (Jones *et al.*, 2007, 2009; Ma *et al.*, 2008; Murray *et al.*, 2007, 2008; Steinmann *et al.*, 2007; Yi *et al.*, 2007, 2009).

NS3 displays a serine protease activity at the N terminus (aa 1–166), and helicase and nucleoside triphosphatase activities in the remainder of the 631 residues of the protein (Fig. 1a) (Gallinari *et al.*, 1998). The NS3 protease activity becomes fully active by association with NS4A. The helicase portion of NS3 (NS3h) folds and functions independently of the protease domain. Recent studies with the HCV strain 2a J6/JFH-1 in infected cells suggest the involvement of NS3h at an early step of viral assembly, independently of its essential roles in polyprotein processing and in viral replication (Ma *et al.*, 2008). However, the physical interaction between HCV core and HCV helicase was not demonstrated and a precise role for NS3 in virus assembly and release remains unclear.

Several independent methods were used here to show that HCV core protein indeed interacts directly with NS3h. Moreover, inhibitors previously found to disrupt core oligomerization and production of infectious particles (Kota *et al.*, 2009, 2010; Wei *et al.*, 2009) abolish the core–NS3 interaction *in vitro*, suggesting that dimerization of core protein is important for the binding of helicase. Collectively, these data underline the essential role of core as a key organizer of HCV particle assembly.

RESULTS

Purification of core106, core169 and NS3h

The first N-terminal 106 residues of HCV core (core106) were chosen as the minimum domain necessary to homodimerize (Kota *et al.*, 2009; Matsumoto *et al.*, 1996; Nolandt *et al.*, 1997) and to mimic the hydrophilic domain D1. The first 169 residues (core169) were selected to represent mature core composed of domains D1+D2 (Boulant *et al.*, 2005; Kota *et al.*, 2009). Both core106 and core169 homo/heterodimerize in a similar fashion, at comparable concentrations. Proteins were expressed as such

or as fusion proteins with glutathione S-transferase (GST) or Flag at their N terminus (Kota *et al.*, 2009) and tagged with eight His residues at their C terminus (Fig. 1a, upper panel). GST-tagged HCV NS3 protein lacking the NS3 residues 1–166 (i.e. the protease domain) (here named NS3h or GST-NS3h), unwinds both DNA and RNA duplexes at rates comparable to those seen with the full-length NS3–NS4A complex (Frick *et al.*, 2004). The protein was produced as an N-terminally GST-fused and a C-terminally 8-His-tagged protein (Fig. 1a, lower panel). The proteins were purified to apparent homogeneity from *Escherichia coli* extracts, by using immobilized metal ion affinity chromatography and followed, for GST-NS3h, by glutathione-bead purification. The identity and homogeneity of the proteins were verified by SDS-PAGE followed by Coomassie blue staining (Fig. 1b, lanes 1–3) and immunoblotting (Fig. 1b, lanes 4–6), revealing expected bands for core106, core169 (both contain a C-terminal 8-His) and GST-NS3h at 15, 20 and 80 kDa, respectively. (Of the 80 kDa for GST-NS3h, 50 kDa correspond to the helicase domain and 30 kDa are contributed by the GST protein and His-tag.)

Core interacts directly with NS3h

Recent studies suggest the involvement of NS3h at an early step of viral assembly (Ma *et al.*, 2008) and describe colocalization of NS3 with core in LDs (Miyanari *et al.*, 2007). However, the physical interaction between core and NS3 proteins was not demonstrated. An indirect sandwich ELISA was used here for the initial characterization of the interaction between affinity-purified HCV core and NS3h proteins (Fig. 2a). When GST-NS3h was included in wells coated with either core106 or core169, a strong signal was observed revealing the interaction with the capsid protein. An irrelevant GST-fusion protein (GST-IRP), used as a negative control, gave a signal similar to the background in the assay, confirming that the interaction between NS3h and either core106 or core169 was specific (Fig. 2a).

To measure the strength of the core–NS3h interaction, we adapted a previously developed Amplified Luminescent Proximity Homogeneous Assay (AlphaScreen) to follow core dimerization using GST-fused and Flag-fused core

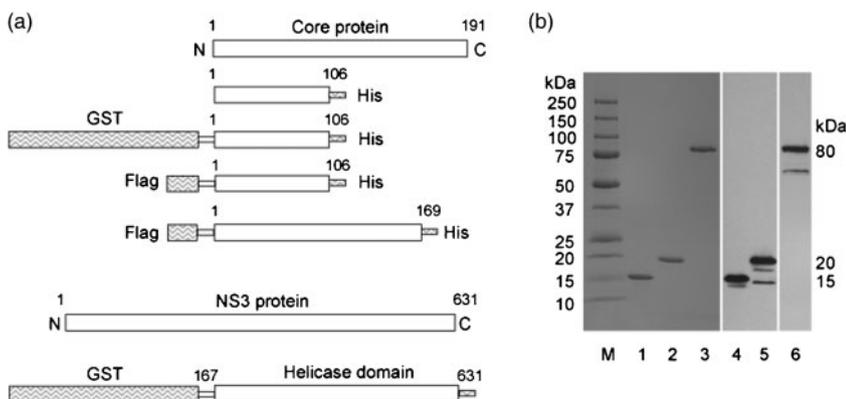


Fig. 1. (a) HCV core protein and NS3 protein constructs. (b) Analysis of purified HCV core and NS3h protein. Lanes 1 and 4: core106 (15 kDa); lanes 2 and 5: core169 (20 kDa); and lanes 3 and 6: GST-NS3h (80 kDa) were resolved by SDS-PAGE and stained with Coomassie blue (lanes M and 1–3) or detected by immunoblotting using mouse monoclonal anti-core or anti-helicase antibodies (lanes 4–6). The immunoblot revealed also minor degradation products of core106 (13 kDa), core169 (14 and 17 kDa) and GST-NS3h (60 kDa).

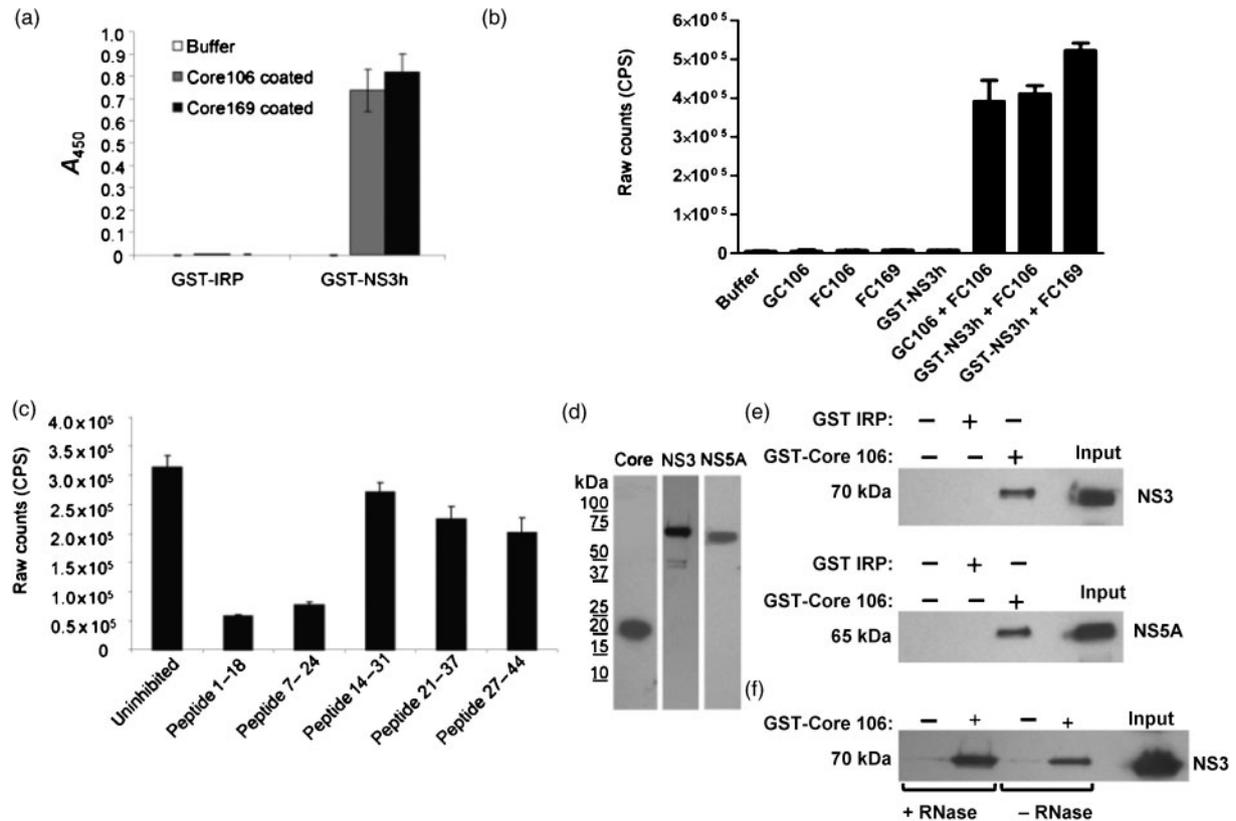


Fig. 2. Direct interaction of core with NS3h. (a) Sandwich ELISA revealing the interaction between either core106 or core169 coated on the plate with GST-NS3h. The background controls in the assay contained buffer only, core106 alone and core169 alone (mean absorption of 0.06 mAU) and were used to normalize the signal. An irrelevant GST-fusion protein (GST-IRP) was used as a negative control. The data shown were representative of three independent experiments with triplicate data points in each experiment. (b) AlphaScreen showing interaction of either Flag-core106 (FC106) or Flag-core169 (FC169) with GST-NS3h. The GST-core106 (GC106)-FC106 interaction was included as a positive control. The data shown were one out of three representative experiments with duplicate assay points. (c) Core-derived peptides spanning the first 1–44 residues on core-NS3h interaction tested by AlphaScreen. A Student's *t*-test confirms that the difference between the uninhibited control compared with the inhibition by the peptides (1–18 and 7–24) was statistically significant. The data shown are representative of three independent experiments with duplicate data points in each experiment. (d) Cell lysates infected with HCV 2a J6/JFH-1 virus were analysed by immunoblotting and revealed the presence of HCV core, NS3 and NS5A proteins. (e) GST pull-down of NS3 by GST-core106 from the lysate. NS5A was also pulled down as a control for the assay. The resulting precipitates were examined by immunoblotting using anti-NS3 antibody (top panel) or NS5A (bottom panel). One-tenth of the cell lysate used in the GST pull-down assay was immunoblotted for NS3 and NS5A as the input control. (f) GST pull-down of NS3 by GST-core106 in the presence of RNase.

proteins, and core106 as a specific competitor (Kota *et al.*, 2009). This assay, displaying a signal-to-background ratio greater than 50-fold, confirmed both the Flag-core106-GST-NS3h and the Flag-core169-GST-NS3h interactions (Fig. 2b). Several groups have previously reported that the cellular DEAD-box protein DDX3, a human helicase homologous to HCV NS3h, interacts with viral core (Angus *et al.*, 2010; Mamiya & Worman, 1999; Owsianka & Patel, 1999; You *et al.*, 1999): the first 40 aa residues of the N-terminal part of HCV core are important for this interaction. We evaluated the effects of five peptides spanning the 44 first residues of the N-terminal part of core (Fig. 2c) by AlphaScreen. The two most potent mostly

hydrophilic peptides were able to inhibit core-NS3h interaction by about 80 %, indicating that residues localized between 1 and 24 are important for the interaction but other residues of core are also required.

Full-length NS3 from HCV-infected cells interacts with the N-terminal domain of core

First, the expression of core, NS3 and NS5A in HCV-infected Huh-7.5 cells was verified by immunoblotting revealing the expected molecular mass of the full size proteins: 20, 70 and 65 kDa, respectively (Fig. 2d). A GST pull-down assay was then used to verify that NS3 and

NS5A (as a control) produced by the virus in cells could interact with core (Fig. 2e). After incubation of GST-core106 with a lysate of HCV-infected Huh-7.5 cells, the protein complexes were pulled down by glutathione-Sepharose beads and analysed by immunoblotting using mouse anti-NS3 and anti-NS5A antibodies. As a negative control, the cell lysate was incubated with beads alone to exclude non-specific interactions of NS3 with the glutathione-Sepharose beads. Moreover, GST-IRP was analysed in parallel to exclude the eventual participation of the GST moiety in the interaction. The presence of protein bands of apparent molecular mass of 70 and 65 kDa, respectively, seen only with beads coated with GST-core106, confirmed specific pull-down of the full-length NS3 and NS5A produced in cells (Fig. 2e). A 2 h RNase treatment of GST-core106 and of the cell lysate did not decrease the amount of pulled-down proteins, suggesting that interaction between core and NS3 is not mediated by RNA (Fig. 2f).

Core oligomerization is required for binding of NS3h

Core is known to oligomerize to form the viral particle (Klein *et al.*, 2004; Kunkel *et al.*, 2001). We investigated the size of the complex formed between core and NS3h by performing cross-linking experiments using dimethyl suberimidate (DMS), which reacts with amino groups, and which we previously used to characterize core oligomerization (Kota *et al.*, 2009). While no evidence was found for cross-linking of a core106 monomer to NS3h monomer (expected molecular mass 95 kDa), trimer formation was readily apparent between core106 dimer (30 kDa) and GST-NS3h (80 kDa): the identity of the band of the expected total size of 110 kDa (Fig. 3a) was confirmed by immunoblotting with both anti-core and anti-GST antibodies (Fig. 3b). With core169, GST-NS3h formed not only trimers (120 kDa) but also tetramers (140 kDa) (Fig. 3c, d). The immunoblots confirm the presence of both core169 and NS3h in these two bands. Regarding apparent molecular masses, the trimers and tetramers may correspond to the interaction of GST-NS3h (80 kDa) with two (2×20 kDa) or three molecules of core169 (3×20 kDa), respectively. Higher molecular-mass oligomers, associated with NS3h are also visible for core106 (Fig. 3a, b) and core169 (Fig. 3c, d).

Interestingly, in the AlphaScreen experiments, the interaction between GST-NS3h and core yielded a signal superior to that seen for the core–core interaction alone, reflecting a better transfer of energy between GST-NS3h and the two molecules of dimerized Flag-core (Fig. 2b).

Disruption of HCV core–NS3h interaction by inhibitors of core dimerization confirms role of oligomerization

To verify the role of dimerization of core in the binding of NS3h, we evaluated the effects of an inhibitor (SL201) and

of a competitor (core106) by AlphaScreen (Kota *et al.*, 2010; Wei *et al.*, 2009). Buffer only and individual protein partners in the presence of donor/acceptor beads alone provided the background value for the assay (Fig. 4a). Addition of 1% DMSO without SL201 did not reveal any effect of the solvent on the interaction. As positive control, we used the Flag-core106–GST-core106 interaction (Fig. 4a). The AlphaScreen showed the Flag-core106– and Flag-core169–GST-NS3h interactions were inhibited by core106 (10 μ M) and SL201 (15 μ M) (Fig. 4b). Core106 inhibited the Flag-core106–GST-NS3h interaction with an IC_{50} of 0.344 μ M, i.e. four times higher than the IC_{50} of 0.089 μ M for the core106 homodimer (Fig. 4c and Table 1). The higher IC_{50} could be explained by the need for two molecules of core106 to abrogate completely the interaction between Flag-core106 dimer and GST-NS3. The inhibition by one molecule of core106 only weakened the signal partially. Similarly, the IC_{50} for the Flag-core169–GST-NS3h was 0.873 μ M (Fig. 4e and Table 1). SL201, a 513 Da inhibitor of core106 dimerization, was discovered when screening with a time-resolved fluorescence resonance energy transfer (TR-FRET)-based screen assay, the Center for Chemical Methodology and Library Development at Boston University library of 2240 compounds (Kota *et al.*, 2010; Wei *et al.*, 2009). When evaluated on the Flag-core106– or Flag-core169–GST-NS3h pairs of proteins, SL201 strongly inhibited ($\sim 90\%$) these interactions (Fig. 4b). In a dose–response format, SL201 displayed strikingly consistent IC_{50} values ranging from 9.3 μ M for the homologous core106 self-association to 9.0 μ M and 10.5 μ M for Flag-core106–GST-NS3h and Flag-core169–GST-NS3h interactions, respectively (Fig. 4d, f and Table 1). These results suggest that it is most likely that SL201 acts on the same single interaction site on core, regardless of the protein interaction partner, in contrast to what is observed when the much larger core106 molecule is used as the disruptor of the interaction.

Treatment by SL201 abolishes infectious virus production

Recently developed cell-culture-based systems for production of infectious HCV from genotype 2a JFH-1 have facilitated the study of the virus life cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). SL201 was previously shown to substantially decrease HCV production by HCV-infected Huh-7.5 cells, as revealed by quantitative RT-PCR (Kota *et al.*, 2010; Wei *et al.*, 2009). The compound was added here at various dilutions with infectious supernatant from electroporation of 2a J6/JFH-1 RNA into naïve cells and incubated for 3 days (early stage – T1). This stage corresponds to the entry and replication of the virus. T1 culture supernatant was used to infect naïve cells to study infectivity (late stage – T2) (Fig. 5a). The compound was shown to be active with an effective concentration at 50% (EC_{50}) of 8.8 μ M for T1 and 8.1 μ M for T2 (Fig. 5b). The compound SL201 had no effect on production of a subgenomic HCV replicon which

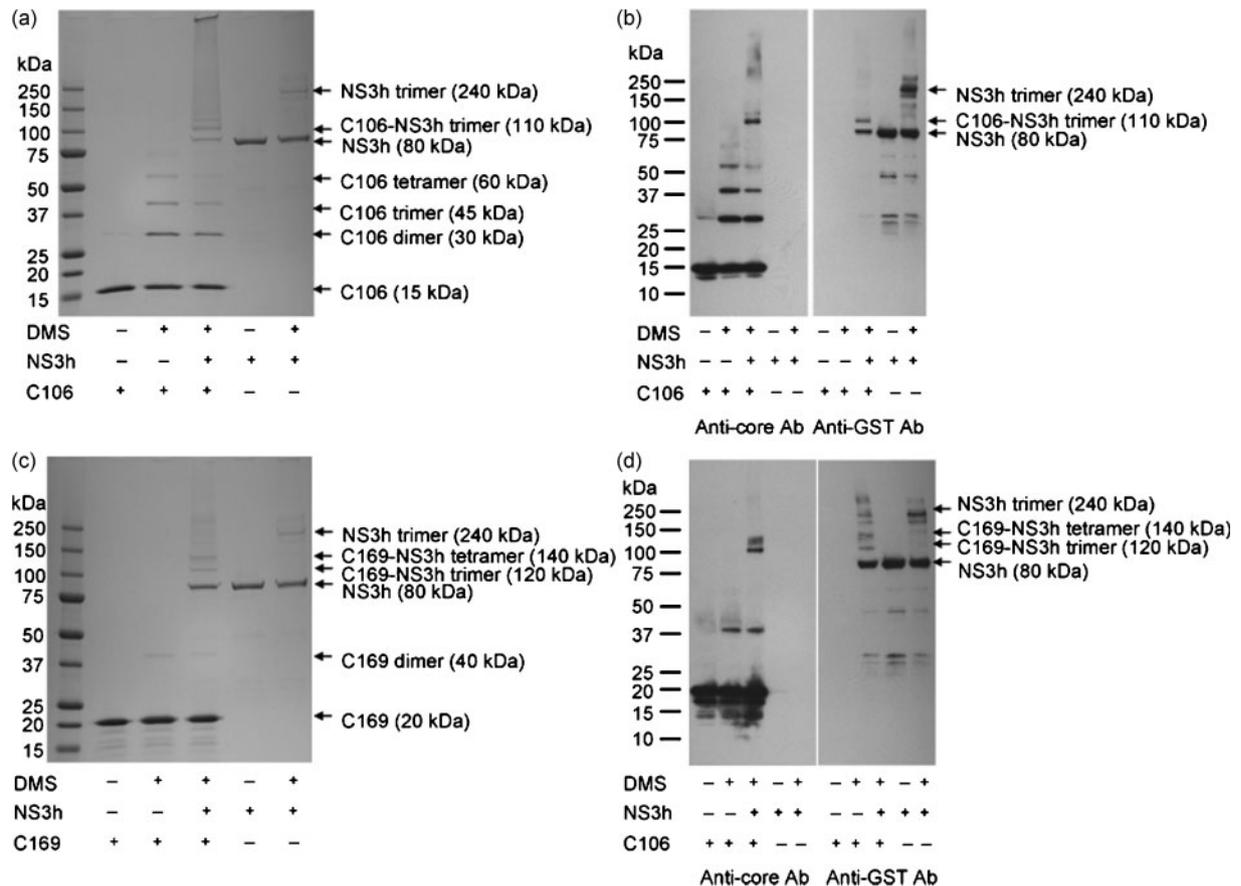


Fig. 3. Cross-linking of HCV core protein oligomers with HCV NS3h. (a, c) SDS-PAGE stained with Coomassie blue of core106 (C106) and core169 (C169), respectively, with or without GST-NS3h cross-linked in the presence of DMS. (b, d) Immunoblotting with an anti-core antibody (left) or anti-GST antibody (right).

contains only the NS proteins, confirming that the compound does not affect replication and supports the hypothesis that it blocks HCV particle assembly by acting on core or core's interactions with other HCV proteins, including NS3 (Fig. 5c).

DISCUSSION

Until recently, the only known role for HCV NS3 in the viral life cycle was thought to be linked to its protease activity in HCV polyprotein processing and its helicase activity in viral replication. There is, however, growing evidence that NS3 may participate in the assembly/production of infectious viral particles (Ma *et al.*, 2008; Murray *et al.*, 2008; Patkar & Kuhn, 2008), as do other NS proteins such as NS5A (Appel *et al.*, 2008; Miyanari *et al.*, 2007; Tellinghuisen *et al.*, 2008) and NS2 (Jirasko *et al.*, 2008; Jones *et al.*, 2007; Murray *et al.*, 2007; Yi *et al.*, 2007, 2009). With regard to NS3h, two successive spontaneous mutations were identified that restored the production of infectious virus by a strain that was otherwise quite unproductive (Ma *et al.*, 2008). Core and NS3h were also

found to be colocalized on LDs of cells transfected with HCV RNA (Ma *et al.*, 2008; Miyanari *et al.*, 2007).

In the present work, we demonstrate for the first time that NS3h directly interacts with core as shown by several biochemical methods. After showing the physical interaction between recombinant affinity-purified core and NS3h proteins by ELISA and AlphaScreen, we confirmed the interaction of GST-core106 with the full-length NS3 protein produced in HCV-infected hepatoma cells using a GST pull-down assay. We verified that prior treatment with RNase did not reduce the interaction, thus discarding an indirect effect due to the presence of RNA. Taken together, these experiments showed that NS3h binds to HCV core and that the N-terminal fragment core106 and the helicase domain of NS3 (167–631) are sufficient for the interaction. Using an AlphaScreen, the ability of core106 to block association of GST-NS3h with Flag-core106 confirmed the involvement of N-terminal residues in the interaction. By screening the ability of peptides spanning the N-terminal part of core known to interact with DDX3, a human homologue of HCV NS3h (You *et al.*, 1999), two peptides, 1–18 and 7–24, were found to block binding by

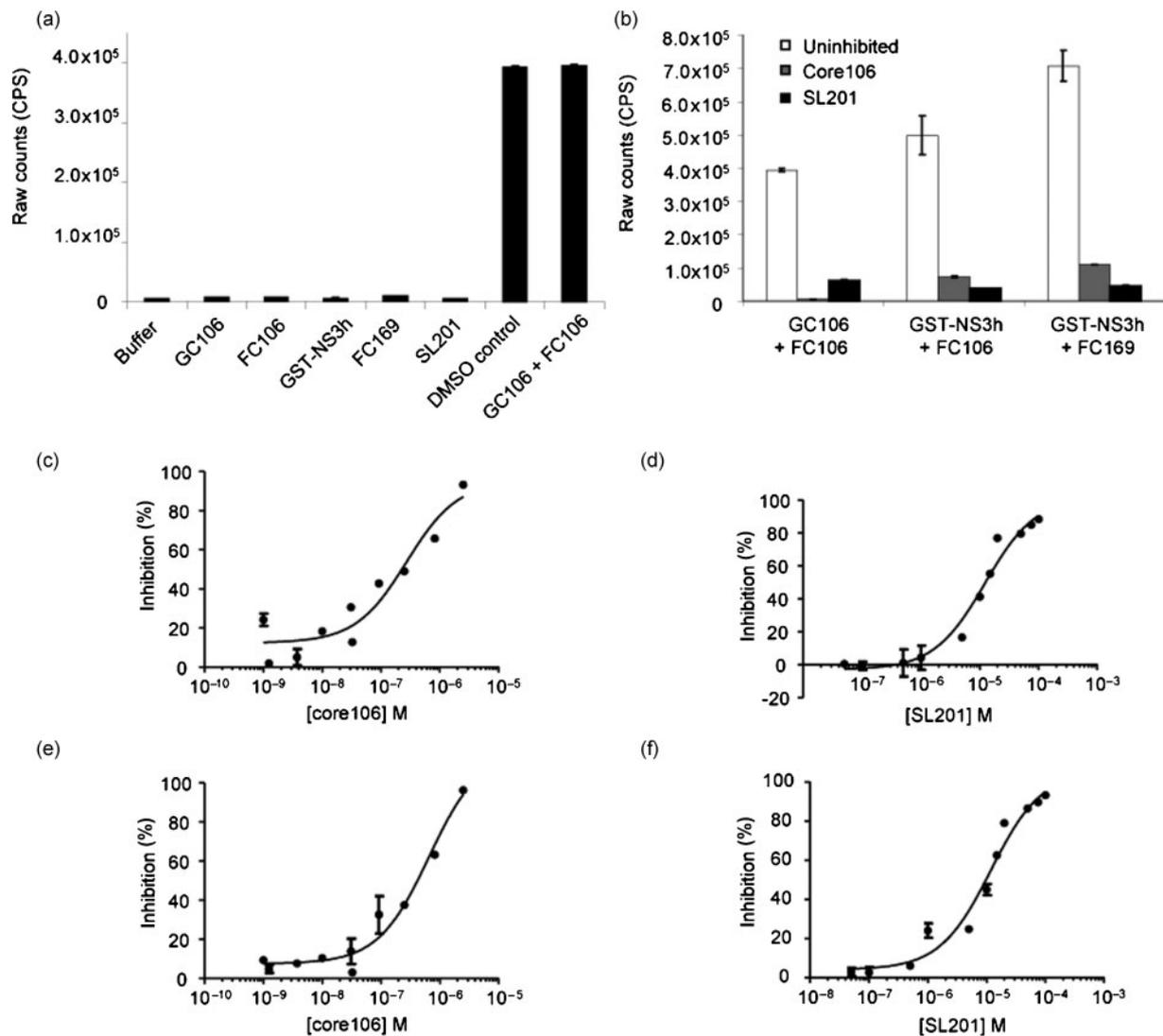


Fig. 4. Inhibition of core-NS3h interaction by core dimerization inhibitors. (a) Positive and negative controls of the AlphaScreen experiments performed in Fig. 4(b). Buffer, GST-core106 (GC106), Flag-core106 (FC106), GST-NS3h, Flag-core169 (FC169) and SL201 with beads only were included in the assay as negative controls. All these controls displayed a signal close to background. GC106 and FC106 at 150 nM each were included in the assay as a positive control for interaction in the presence or absence of DMSO. (b) AlphaScreen showing inhibitions of FC106- and FC169-GST-NS3h interactions by core106 (10 μ M) and SL201 (15 μ M). The data shown were representative of three independent experiments with duplicate data points in each experiment. Student's *t*-test confirmed that the difference between the uninhibited control compared with the inhibition by core106 or SL201 was statistically significant. (c, d, e and f) Representative graphs showing dose-response analysis of (c) core106 or (d) SL201 as inhibitors of FC106-GST-NS3h; and (e) core106 or (f) SL201 as inhibitors of FC169-GST-NS3h. Dose range for core106 was from 125 pM to 2.5 μ M and for SL201 was from 0.05 μ M to 100 μ M. Data points are presented in terms of percentage inhibition, which was calculated using the uninhibited control as 0% reference. The data shown are representative of four independent experiments with duplicate data points in each experiment.

80%, indicating the importance of one or several residue(s) of those N-terminal peptides in the interaction. Moreover, the C-terminal region of core may contain additional residues important for the binding since interaction between NS3h and core169 displays a higher signal than that between core106 and NS3h as shown by AlphaScreen.

We previously developed an ELISA and an AlphaScreen using core106, the region essential for self-association, and identified SL201, a small molecule that inhibits HCV core dimerization (Kota *et al.*, 2010; Wei *et al.*, 2009). Core106 and SL201 potently inhibit the binding with the helicase. Using a molecular beacon-based helicase assay (Belon & Frick, 2008), no effect of SL201 was observed on DNA

Table 1. IC₅₀s of core106 and SL201 to block GST-NS3h and core interactions

IC₅₀s were calculated from Fig. 4 using Graphpad Prism software. IC₅₀s were calculated using an 'n of 2' with duplicate data points. '+' Indicates that core106 or core169 is actually present as a dimer or trimer in the complex formed with NS3. '*' Indicates data previously published (Kota *et al.*, 2009; Wei *et al.*, 2009) included here as a control.

Partners		Disruptors	
GST-fusion protein	Flag-fusion protein	IC ₅₀ [μM]	
		Core106	SL201
Core106 ⁺	Core106 ⁺	0.09 ± 0.10*	9.30 ± 0.90*
Core106 ⁺	NS3h	0.22 ± 0.15	9.00 ± 1.00
Core169 ⁺	NS3h	0.87 ± 0.53	10.50 ± 2.00

unwinding (D.N. Frick, unpublished observations). The strong inhibition by core106 and SL201 suggests that NS3h recognizes and interacts preferentially with dimers or oligomers of core. This observation is strengthened by the cross-linking experiments, which show that NS3h interacts with core106 dimers and core169 dimers and trimers, but not monomers. The presence of cross-linked tetramers containing core169 trimers and NS3h but not core106 trimers suggests that NS3h better stabilizes oligomeric forms of core169 than of core106.

The NS3 protein of yellow fever virus (YFV), the prototype flavivirus, which like HCV is a member of the family *Flaviridae*, also plays an essential role in virus assembly as shown by *trans*-complementation of a mutation (Trp349Ala) by expression of the wild-type NS3 protein (Patkar & Kuhn, 2008). Since domains 1 and 2 of the HCV helicase show a high degree of similarity with the corresponding domains in YFV helicase, and the sequence alignment reveals 21% similarity including the presence of a conserved aromatic residue (Tyr350) in HCV at the position corresponding to 349 in YFV (Wu *et al.*, 2005), they may also share mechanistic features in the process of virus assembly and release. The interaction observed between core and NS3h might be equally important for HCV assembly. We propose the following sequence of events: initially, HCV core is processed by signal peptide peptidase, oligomerizes, and then progressively coats LDs and recruits NS5A and NS3 to the LDs, a prerequisite for particle assembly (Appel *et al.*, 2008; Ma *et al.*, 2008; McLauchlan, 2009a; Miyanari *et al.*, 2007; Strosberg *et al.*, 2010; Tellinghuisen *et al.*, 2008). SL201 potently blocks HCV production by infected hepatoma cells (Kota *et al.*, 2010; Wei *et al.*, 2009), but had no effect on multiplication of human immunodeficiency virus in CD4-transfected HeLa cells (S. Valente, personal communication). The compound did not modify HCV replicon propagation, indicating lack of effect on viral replication. These results suggest that the inhibitor disrupts viral particle assembly.

To differentiate between an effect on assembly and entry, time-of-addition intracellular and extracellular infectivity assays were performed. To analyse the results, we used both real-time RT-PCR and HCV-limiting-dilution (TCID₅₀) assays. These data confirm that SL201 appears at least 10-fold less effective when added before viral infection than during or after infection (see Supplementary Figs S1 and S2 and Supplementary Table S1, available in JGV Online).

The colocalization and interaction of core with NS proteins on LDs confirms core as a key organizer of virus assembly and suggests that it acts by recruiting the replication complex near the site of assembly to facilitate the packaging and integration of the newly synthesized RNA. Whether core actually modulates the HCV RNA unwinding activity of the viral helicase, and thus actually affects replication and translation, remains an open question.

In conclusion, the present studies establish that HCV helicase physically interacts with the oligomerized HCV core protein, and that this interaction involves the first 106 residues from core and residues 166–631 from NS3. Binding is stronger when domain D2 of core, up to residue 169, is present, and is inhibited by small molecule SL201, a disruptor of core dimerization and of infectious HCV production.

METHODS

Materials. Anti-GST (Sigma-Aldrich), anti-core (MA1-080) (Thermo Scientific), anti-NS3 [H23] (Abcam), anti-NS5A [9E10] (gift from Dr T. Tellinghuisen, Scripps-Florida, USA) were purchased. HRP-conjugated secondary antibodies were obtained from Jackson ImmunoResearch. Infectious HCV 2a strains J6/JFH-1 and Jc1 were produced using previously published protocols (Lindenbach *et al.*, 2005; Pietschmann *et al.*, 2006; Wakita *et al.*, 2005; Zhong *et al.*, 2005). Cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Akron Biotech), 1X Pen-Strep-Gln and 1× non-essential amino acids (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO₂.

Cloning, expression and purification of proteins. A vector encoding GST-NS3h (from HCV HC-J6CH genotype 2a; AF177036) was generated by excising the HCV helicase-encoding region from p24Hel-2a (Lam *et al.*, 2003) and inserting it into vector pET41a. Recombinant protein was expressed in transformed *E. coli* BL21(DE3) cells and purified by Hi-Trap nickel-nitrilotriacetic acid (Ni-NTA) column and affinity-capture of GST-NS3h with glutathione-Sepharose beads as described previously (Lam *et al.*, 2003).

The cloning, expression and purification of the recombinant HCV core106, Flag-core106 and GST-core106 were done as described previously (Kota *et al.*, 2009). Core106 was further purified for the cross-linking assays by reverse-phase HPLC on a semi-preparative column Agilent Zorbax Extend C₁₈ using an isocratic (0–5 min, 22%) followed by linear gradients (5–55 min, 32%; 55–60 min, 100%) of buffer B (acetonitrile, 0.01% trifluoroacetic acid) at 2 ml min⁻¹ flow rate and at room temperature. Core106 fractions were dialysed overnight at 4 °C against 20 mM Tris (pH 8.0), 0.5 M NaCl and stored at –80 °C.

The sequences of core covering the first 169 residues were amplified by PCR from HCV H77 genotype 1a (NC-004102) plasmid pCV-H77C and inserted into pET42b. For expression, this plasmid or

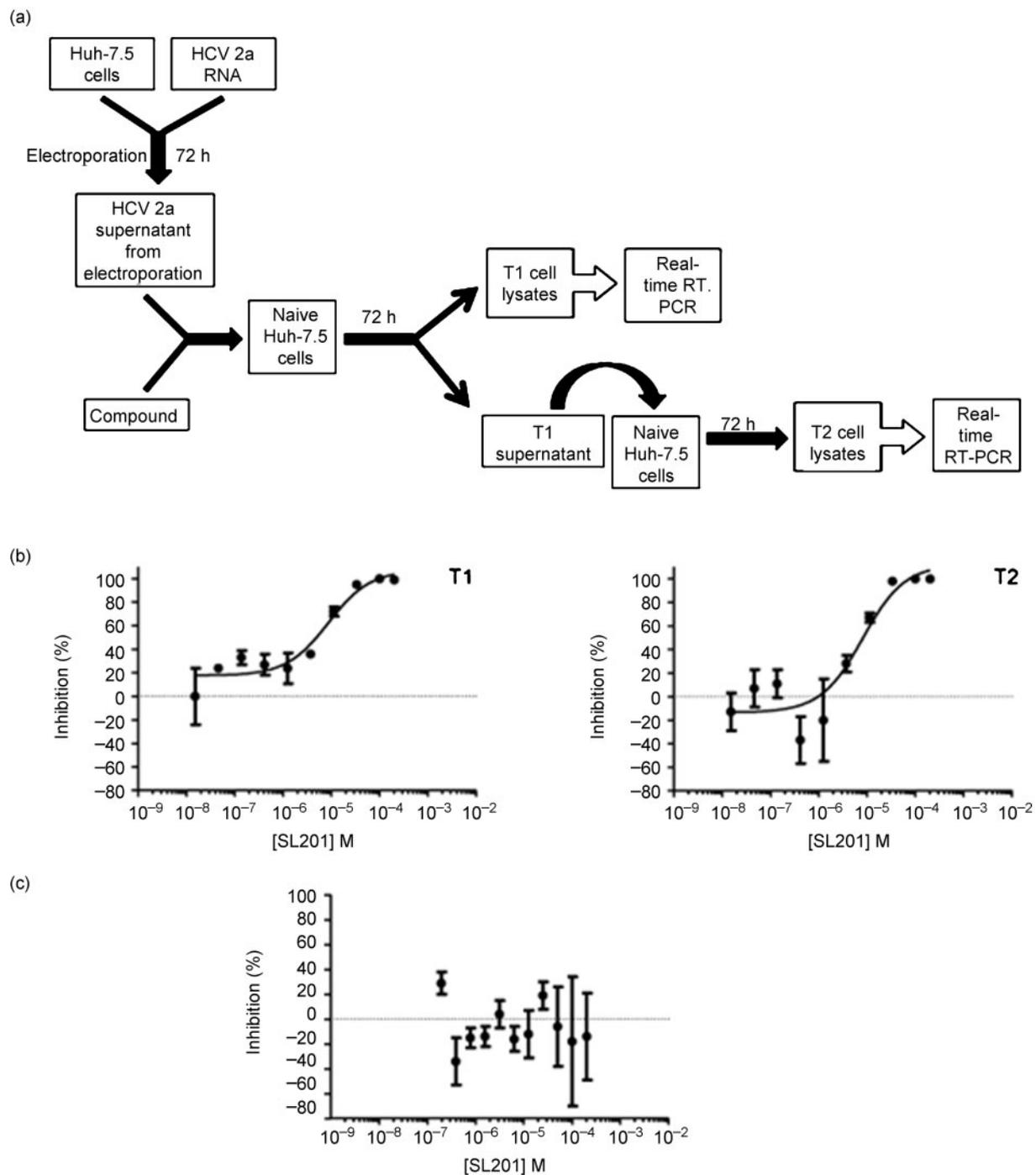


Fig. 5. Effect of SL201 on HCV 2a J6/JFH-1 virus and PSGR-JFH1 replicon. (a) Scheme of infection of naïve Huh-7.5 cells by HCV. Infectious supernatants were obtained from cells electroporated with RNA from HCV (Kota *et al.*, 2009). (b) Inhibition assay of SL201 was performed on HCV 2a J6/JFH-1 virus by adding serially diluted SL201 and virus onto naïve Huh-7.5 cells and then incubating for 3 days (T1). Culture supernatant from T1 was transferred onto naïve Huh-7.5 cells and the cells were incubated for an additional 3 days (T2). RNA was purified from lysed cells from both T1 and T2 and analysed by real-time RT-PCR. EC_{50} values for this new batch of SL201 were 8.8 μ M and 8.1 μ M, respectively. (c) Effect of SL201 on PSGR-JFH1 replicon cells. Inhibition assay of SL201 was performed on PSGR-JFH1 replicon cells by adding serially diluted SL201 onto cells and incubating for 3 days. RNA was purified from cell lysates and analysed by real-time RT-PCR. RNA production from cells in the presence of various concentrations of SL201 was compared with the cell-only control. Data points are presented in terms of percentage inhibition, which was calculated using the uninhibited control as 0% reference. These are representations of one experiment with triplicate data points.

pT7Flag plasmid containing Flag-core169 were transformed into *E. coli* BL21(DE3) cells using standard expression procedure (Boulant *et al.*, 2005). Core169 (containing only an 8-His tag) or Flag-core169-containing pellets were solubilized using lysis buffer containing 6 M urea (Thermo Fisher Scientific) and the clarified lysate was applied to Ni-NTA affinity columns pre-equilibrated with 20 mM Tris/HCl buffer (pH 8.0), 0.5 M NaCl, 6 M urea and 0.1% dodecyl β -maltoside (DM; Sigma-Aldrich). Recombinant proteins were eluted with 50, 100 and 150 mM imidazole (Thermo Fisher Scientific) and dialysed overnight at 4 °C against 20 mM Tris (pH 8.0), 0.1% DM and stored at -80 °C. The homogeneity of the purified proteins was determined by SDS-PAGE and confirmed by immunoblotting using mouse monoclonal anti-core antibodies.

Indirect sandwich ELISA. The assay was performed similarly as described previously (Kota *et al.*, 2009). Core106 and core169 (500 pmol) were coated to high-binding 96-well plates and GST-fusion proteins were tested at 1 pmol per well. Rabbit anti-GST antibodies were used at a dilution of 1:1000 and anti-rabbit IgG-HRP antibodies were added at a dilution of 1:10 000. Colour development was assessed by using the Ultra 3,3',5,5'-tetramethylbenzidine (Thermo Fisher Scientific). The colour reaction was stopped by adding 1 N H₂SO₄ and absorbance measured at 450 nm.

GST pull-down. Huh-7.5 cells were grown for 24 h before electroporation with infectious HCV 2a strain J6/JFH-1. At day 4 post-electroporation, the supernatant was removed and cells were lysed in 400 μ l lysis buffer (PBS with 1% Triton X-100, 1 mM PMSF, protease-inhibitor cocktail) and stored at -20 °C. The presence of HCV proteins core, NS3 and NS5A in cell lysate was verified by immunoblotting using anti-core, anti-NS3 and anti-NS5A antibodies. For GST pull-down, GST-core106 or GST-IRP (kindly provided by Dr N. Ayad, Scripps-Florida) immobilized on glutathione-Sepharose 4B beads (GE Healthcare Biosciences) was incubated with 80 μ l of cell lysate in incubation buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 0.05% Triton X-100] for 4 h at room temperature under end-over-end agitation. As control, GST-core106 and the cell lysate were alternatively treated with 10 U of RNase (New England Biolabs) at 37 °C for 2 h. The beads were washed three times and centrifuged at 5000 g for 1 min using a SigmaPrep spin column and boiled in the SDS loading buffer for 5 min, and the pulled-down proteins were detected by immunoblotting.

Cross-linking analysis. For cross-linking experiments, core106 and NS3h were dialysed against cross-linking buffer (200 mM phosphate buffer, pH 8.0), and core169 in cross-linking buffer containing 0.01% CHAPS, and stored at -80 °C. Cross-linking analysis was performed by incubating 8 μ M of core106 or core169 proteins with or without 2.5 μ M of NS3h in cross-linking buffer followed by the addition of 200 molar equivalents of DMS for 1 h at room temperature. The reaction was stopped by adding an equal volume of SDS loading buffer and sample was boiled for 10 min. The products were resolved by SDS-PAGE and analysed by Coomassie blue staining or by immunoblotting using anti-core and anti-GST antibodies to detect core and GST-NS3h, respectively.

AlphaScreen. This method was developed as described previously (Kota *et al.*, 2009). In brief, the GST-fusion proteins and Flag-fusion proteins at a concentration of 208 and 250 nM, respectively, were used. GST-core106 and Flag-core106 at 150 nM each were included as a positive control. Alternatively, in order to evaluate the possible role of RNA in the core-NS3h interaction, proteins were treated with 10 U of RNase A for 2 h at 37 °C. The untagged core106 domain (10 μ M) or compound SL201 (15 μ M) (previously published as compound #15) (Wei *et al.*, 2009) were added to the proteins as reference competitors. Core-derived peptides were evaluated as potential inhibitors on core-NS3h interaction and/or core-core

interaction at 40 μ M concentration. Anti-Flag acceptor beads and glutathione donor beads were added to the proteins at a final concentration of 20 μ g ml⁻¹. The data of the uninhibited control compared with the inhibition by either core106 or SL201 were analysed using unpaired Student's *t*-test.

Inhibition of HCV 2a J6/JFH-1 in Huh-7.5 cells. The addition of SL201 to infected cells was done as per previously published protocols (Kota *et al.*, 2009) for an initial 72 h period (T1) and an additional 72 h (T2). The compound was dosed from 200 μ M, 100 μ M, and then 1:3 serial dilutions down to 0.015 μ M. For time-of-addition experiments, naïve Huh-7.5 cells were treated with serial dilutions of compound from 1 to 100 μ M before (for 6 h), during and after (24 h) infection with HCV 2a J6/JFH-1. Supernatants from cells were removed and titrated at 10⁻⁶ dilution for HCVcc-limiting-dilution assay to determine TCID₅₀ values (Lindenbach *et al.*, 2005). Infected cells were lysed by three freeze-thaw cycles and titrated in medium to 10⁻⁶ dilution for HCVcc-limiting-dilution assay to obtain TCID₅₀ values. Cells in a duplicate plate were lysed for RNA analysis by real-time RT-PCR.

PSGR-JFH1 replicon cell assay. PSGR-JFH1 replicon cells (Tellinghuisen *et al.*, 2008) were maintained in G418 selection and taken out of selection for assay. Cells were plated into 24-well plates and incubated with 0.195–200 μ M final concentration of SL201 for 3 days, along with a cells-only control. Cells were then lysed for RNA purification with Qiagen RNeasy kit. Real-time RT-PCR was performed on RNA samples, using Roche's LightCycler RNA amplification kit HybProbe, for analysis.

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