Antiviral Research 88 (2010) 197–206

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Ligand-induced changes in hepatitis C virus NS5B polymerase structure

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ARTICLE INFO

Article history:
Received 24 June 2010
Received in revised form 20 August 2010
Accepted 26 August 2010

Keywords:
Hepatitis C virus
NS5B polymerase
Trypsin digest

ABSTRACT

Hepatitis C virus (HCV) RNA-dependent RNA polymerase (NS5B) is required for viral replication. Crystal structures of the NS5B apoprotein show that the finger and thumb domains interact to encircle the active site, and that inhibitors defined by P495 resistance that bind to the thumb-finger interface displace the Δ1 finger loop and disrupt this structure. Since crystal structures may not reveal all of the conformations of a protein in solution we have developed an alternative method, using limited trypsin protease digestion, to investigate the impact of inhibitors as well as substrates on the movement of the Δ1 loop. This assay can be used to study NS5B under conditions that support enzymatic activity. In the absence of inhibitors, no specific region of NS5B was hypersensitive to trypsin, and no specific intermediate cleavage products were formed. Binding of P495-site inhibitors to NS5B induced specific trypsin hypersensitivity at lysine residues 50 and 51. Previously characterized inhibitors and mutant polymerases were used to link this specific trypsin hypersensitivity to movement of the Δ1 loop. Trypsin hypersensitivity identical to the inhibitor pattern was also induced by the binding of the RNA template. The addition of primer to the NS5B-template complex eliminated the hypersensitivity. The data are consistent with displacement of the Δ1 finger loop from the thumb by the binding of template, and reversal by the addition of primer or NTP. Our results complement inhibitor-enzyme co-crystal studies, and the assay provides a rapid and sensitive method to study dynamic changes in HCV NS5B polymerase conformation under conditions that support functional activity.

1. Introduction

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B viral hepatitis (Choo et al., 1989; Houghton, 1996). Although acute HCV infection is often asymptomatic, nearly 80% of cases become chronic. Chronic HCV may lead to progressive liver diseases such as cirrhosis or hepatocellular carcinoma and eventually cause liver failure. Currently, it is estimated that 170 million people are infected with HCV worldwide, with 3.9 million in the United States (Armstrong et al., 2006; Tanaka et al., 2002). The current standard of care (SOC) for HCV infection is a combination of pegylated interferon and ribavirin. SOC produces serious side effects in many treated patients. In addition, this treatment shows limited efficacy against genotype 1, the most prevalent type of HCV infection in the US (Cornberg et al., 2002).

This serious unmet medical need has driven pharmaceutical companies to develop small molecule inhibitors targeting HCV proteins that are essential for viral replication. One of these targets is the non-structural protein NS5B, an RNA-dependent RNA polymerase that catalyzes the polymerization of ribonucleoside triphosphates (rNTP) during viral RNA replication. The HCV polymerase NS5B is a very attractive target, since better selectivity and less toxicity are possible with inhibitors targeting the viral polymerase compared to inhibitors that target cellular pathways. Both nucleoside and nonnucleoside inhibitors have demonstrated antiviral activity against HCV in the clinic (Beaulieu, 2009; Feldstein et al., 2009; Wang et al., 2009). In addition to the therapeutic potential of these small molecule inhibitors, they provide powerful tools to probe the HCV RNA-dependent RNA polymerase mechanism.

The X-ray crystal structure of HCV NS5B has been solved (Lesburg et al., 1999; Ago et al., 1999; Bressanelli et al., 1999). Like other known polymerases, the NS5B structure resembles a right hand with fingers, palm, and thumb domains. In contrast to the well-characterized U-shaped structures of DNA polymerases, which exist in a 'partially open' conformation (Doublié et al., 1999), NS5B apoprotein was crystallized as a globular structure in a 'closed' conformation with a finger loop (Δ1) tucked into a hydrophobic pocket on the thumb. Other RNA-dependent RNA polymerases also have 'closed' structures (Ferrer-Orta et al., 2006).

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doi:10.1016/j.antiviral.2010.08.014

There is a single exception to the 'closed' apoenzyme structure; both 'open' and 'closed' structures were observed for genotype 2a (Biswal et al., 2005). It has been suggested that the closed structure endows NS5B with a 'preformed active site' in the absence of template (Bressanelli et al., 2002). Experiments showing that a circular template can be utilized by NS5B suggest that the 'closed' conformation opens during the formation of active polymerase complexes (Ranjith-Kumar and Kao, 2006).

Soaking the 'closed' apoenzyme crystals of NS5B with inhibitors and substrates yielded co-crystal structures with both 'closed' and 'open' conformations (Biswal et al., 2005; O'Farrell et al., 2003). Co-crystal structures with allosteric inhibitors binding in the thumb pocket defined by proline 495 resistance showed NS5B in an 'open' conformation, with complete displacement of the Δ1 finger loop. The co-crystal structures of HCV NS5B genotype 2a complexed with an allosteric thumb-binding inhibitor defined by M423T resistance also revealed NS5B in an 'open' conformation (Biswal et al., 2005); whereas, a co-crystal structure of genotype 1b (BK) with similar inhibitors did not show displacement of the Δ1 finger loop (Wang et al., 2003; Love et al., 2003; Biswal et al., 2006). A co-crystal structure of NS5B genotype 1b (J4) with an oligonucleotide (rU5) showed the small RNA bound at the active site of NS5B in a 'closed' conformation (O'Farrell et al., 2003). Co-crystal structures of HCV NS5B genotype 1b (BK) with high concentrations of ribonucleotides revealed GTP bound at the junction between the thumb and fingers domain, with NS5B in a 'closed' conformation. Binding of GTP at the site distal to the active site was hypothesized to be related to *de novo* initiation (Bressanelli et al., 2002).

Co-crystal studies also have revealed multiple allosteric inhibitor binding sites on HCV NS5B polymerase. In addition to two distinct sites located on the thumb, several overlapping sites have been observed in the palm domain (Beaulieu, 2009). We have used allosteric inhibitors of HCV NS5B as well as mutant NS5B proteins to correlate displacement of the Δ1 finger loop, previously observed in co-crystal structures, with specific trypsin hypersensitivity. The trypsin cleavage product provided an end-point for the development of an assay which can detect movement of the loop in solution phase studies.

The inhibitors most useful for validation of the trypsin hypersensitivity assay bind to the thumb pocket defined by P495 resistance substitutions. A mechanism of action for benzimidazole-based inhibitors that bind to this site was proposed based on kinetic and binding experiments in conjunction with resistance selection, before the availability of co-crystal structures (Tomei et al., 2003). Resistance selection in the replicon system identified substitutions at proline 495, an amino acid 30 angstroms from the active site, as responsible for resistance. Based on these studies the authors proposed that the allosteric P495-site inhibitors interact with the enzyme–RNA complex and effect a slow conformational transition, preceding nucleotide binding, which is required for the formation of a productive initiation complex. In 2005, co-crystal structures of NS5B and two structurally similar indole-based inhibitors were published, confirming the site of binding in a pocket of the thumb domain proximal to P495 (Di Marco et al., 2005). In the apoenzyme, the inhibitor binding site is occupied by a small alpha-helix which is an extension of the finger loops that connect fingers and thumb subdomains. Based on differences between the co-crystal structures and the structure of the apoenzyme, the authors hypothesized that P495 inhibitors interfere with enzyme activity by preventing formation of intramolecular contacts between fingers and thumb, precluding the coordinated movements required for RNA synthesis.

Although published co-crystal structures reveal the specific binding pocket of P495-site inhibitors, the NS5B conformation observed may represent one of many conformational changes that occur during inhibitor binding. We report the development of an assay that detects conformational changes in NS5B in solution phase studies. The assay was used to characterize changes in NS5B polymerase exposed to inhibitors as well as substrates; inhibitor-enzyme co-crystal structures in the presence of polymerase substrates (RNA template and primer or RNA template and NTP) have not been reported. The pattern of trypsin hypersensitivity observed defines a relationship between the binding of RNA template, RNA template-primer, rNTP and P495-site inhibitors, and the displacement of the Δ1 loop. Data from these solution phase studies complement inhibitor-enzyme co-crystal studies, expanding our understanding of the mechanism of inhibition of compounds that bind NS5B. The assay provides a rapid and sensitive method to detect conformational changes in HCV NS5B under conditions that support polymerase activity.

2. Materials and methods

2.1. Compound synthesis

Compound I was synthesized as described (Hirashima et al., 2006). Compound II was synthesized as described (Hudyma et al., 2006). Compound III was synthesized as described (Beaulieu et al., 2008). Compound IV was synthesized as described (Chan et al., 2005). Compound V was synthesized as described (Shaw et al., 2009). Compound VI was synthesized as described (Burns et al., 2007). All compounds had purity of ≥95%, as determined by LC–MS.

2.2. HCV NS5B protein expression and purification

The cDNA encoding the open reading frame for HCV NS5B Con 1 wild type, P495L, or L30S, with a C-terminal 18 amino acid truncation, was cloned into a pet21b vector for expression (Wang et al., 2006). The plasmids were used to transform competent BL21(DE3) *Escherichia coli* cells (Novagen) according to the Manufacturer's protocol. Proteins were expressed and purified using heparin sepharose and polyU sepharose chromatography (Wang et al., 2006). Enzymes were stored in buffer consisting of 20 mM Tris–HCL, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, 2 mM DTT, 0.5% Triton X-100, 50% glycerol.

2.3. Polymerase assay

NS5B enzymes (1.25, 2.5, 5 and 10 nM) were tested for activity in a polyC:pGpG assay at a final concentration of 20 mM Tris–HCl, pH 7.4, 5 nM polyC template, 8.6 μM pGpG primer (Oligo's Etc., Wilsonville, OR), 5 mM $MgCl_2$, 1 mM DTT, 2.5 mM KCl, 1 μM GTP, 0.5 μCi ^{33}P-GTP, 0.2 mM EGTA. The 30 μL reactions were incubated at 30 °C for 30 min. The reactions were precipitated by adding an equal volume of 20% TCA and incubating on ice for 20 min. Reactions were harvested onto GF/B filter plates (6005177, Perkin Elmer, Shelton, CT), and 30 μL Microscint scintillation fluid (6013611, Perkin Elmer, Shelton, CT) was added to each well of the filter plate. Plates were read in a TOPCOUNT (Perkin Elmer).

2.4. Tryptic digestion under high salt conditions

For tryptic digestion performed under high salt conditions (350 mM), 20 μg sequencing grade, modified trypsin (V511A, Promega, Madison, WI) was solubilized in 50 mM Tris–HCL, pH 7.6 (buffer A) to a concentration of 100 μg/mL. The trypsin stock was diluted to 25.6 μg/mL in buffer A and serial dilutions were performed down to 0.1 μg/mL. Tryptic digest reactions (30 μL) consisted of 5 μL inhibitor (540 μM in 6% DMSO, 90 μM final concentration) or 5 μL DMSO (6%), 5 μL purified NS5B (9 μM final concentration), 10 μL 3× reaction buffer consisting of 20 mM Tris–HCL, pH 7.4, 900 mM NaCl and 10 μL diluted trypsin. Enzyme with inhibitor or DMSO was preincubated at 30 °C for 30 min before trypsin was added, and the reactions were incubated at 30 °C for

an additional 30 min. The reactions were quenched with 30 μL 2× Tris–glycine SDS sample buffer (LC2676, Invitrogen, Carlsbad, CA) containing 700 mM 2-mercaptoethanol (61-0710, Biorad, Hercules, CA). The samples were heated at 95 °C for 5 min, and 10 μL of each sample was fractionated on a 4–20% Tris–glycine polyacrylamide gel (EC60255BOX, Invitrogen, Carlsbad, CA). The gels were stained with filtered Coomassie blue stain (161-0436, Biorad, Hercules, CA) and destained with 50% methanol, 10% acetic acid. For the P495L enzyme, the reactions were set-up as described, with 5 μg purified enzyme added to each reaction. The final concentration of enzyme (3 μM) was lower due to a lower concentration of the enzyme stock. In this case, the inhibitor stock that was added to the assay was 180 μM making the final concentration of inhibitor in the assay 30 μM.

To perform time course experiments, the reactions were assembled as described for wild type enzyme, but a fixed amount of trypsin was added (0.13 μg/mL final concentration) to each reaction. In order to observe the effect of the inhibitor on NS5B over time, the pre-incubation step was omitted. Reactions were assembled for each condition evaluated. Once the trypsin was added, the samples were placed at 30 °C, and an aliquot from each reaction was removed and quenched with 2× Tris–glycine SDS sample buffer (Invitrogen) every 6 min over 42 min. The samples were heated at 95 °C for 5 min, fractionated by SDS-PAGE, and stained as described above.

2.5. Tryptic digestion under low salt conditions

For tryptic digestion performed under low salt conditions (20 mM NaCl), trypsin was resuspended and diluted as described above. For reactions with no addition of RNA, 5 μL (30 μg, 8 μM final concentration in assay) wild type NS5B or L30S NS5B in storage buffer containing 200 mM NaCl was added to 5 μL 20 mM Tris–HCL, pH 7.4. The reaction mixtures were diluted 5-fold with 20 mM Tris–HCL, pH 7.4 and increasing concentrations (0–12.8 μg/mL) of diluted trypsin (10 μL) were added to the reaction mixtures. The samples were incubated at 30 °C for 20 min and quenched with an equal volume of 2× Tris–glycine SDS sample buffer (Invitrogen). The samples were heated at 95 °C for 5 min and fractionated on a 4–20% Tris–glycine polyacrylamide gel (Invitrogen). Gels were stained with filtered Coomassie blue (Biorad) and destained with 50% methanol, 10% acetic acid.

Reaction mixtures containing polyC template and pGpG primer or GTP consisted of 5 μL (12.5 μg, 4 μM final concentration in assay) wild type NS5B in storage buffer containing 200 mM NaCl, 5 μL polyC template (~1000 nt, ~1 μM final concentration), or 5 μL deionized water (as negative control), or 5 μL pGpG primer (0.1 mM final concentration), or 5 μL GTP (10 mM final concentration). The reaction mixtures were diluted 3-fold with 20 mM Tris–HCl, pH 7.4. 10 μL trypsin (12.8 μg/ml) was then added to each reaction mixture (final concentration 2.3 μg/ml). Reactions were set-up for each condition evaluated. Once the trypsin was added the samples were placed at 30 °C. A sample from each condition was quenched with 55 μL 2× Tris–glycine SDS sample buffer (Invitrogen) every 5 min for 25 min. The samples were heated and fractionated on the gel as above. All reaction components for these experiments were prepared in buffer made with RNase-free molecular biology grade water (W4502, Sigma–Aldrich, St. Louis, MO) with the exception of the NS5B enzyme which was purified with 18.2 Ω cm water from a MilliQ purification system.

2.6. Edman sequencing

Samples submitted for Edman sequencing were fractionated on a 4–20% Tris–glycine polyacrylamide gel (Invitrogen) so that each band on the gel represented at least 20 pmol of full-length or cleaved NS5B. The gel was transferred to sequencing grade PVDF membrane (162-0186 Biorad) using a standard protocol from Invitrogen. The PVDF was stained with filtered Coomassie blue stain (Biorad) and destained in 50% methanol, 10% acetic acid. The destaining step helps remove contaminating Tris–glycine that can interfere with Edman sequencing from PVDF. The stained bands were excised from the PVDF membrane and submitted for sequencing to the Keck Foundation, Biotechnology Resource Laboratory at Yale University (300 George St., Room 2104, New Haven, CT). Five cycles of sequencing were performed per sample.

3. Results

3.1. Displacement of the NS5B polymerase Δ1 loop detected by limited trypsin proteolysis

X-ray structures of the HCV NS5B apoprotein reveal the polymerase in a 'closed' conformation with a finger loop (Δ1) tucked into a hydrophobic pocket on the thumb; inhibitors that bind in the hydrophobic pocket displace the finger loop (Bressanelli et al., 1999). We used limited trypsin protease digestion of HCV NS5B in solution, with high salt buffer to mimic conditions used for co-crystal studies and low salt buffer to mimic conditions used for polymerase activity assays, to investigate movement of the finger loop. Based on the linear amino acid sequence of NS5B, trypsin was selected for protease digestion. There are 70 trypsin cleavage sites with >80% chance of trypsin cleavage recognition, as indicated by the Swiss Institute of Bioinformatics ExPASy Proteomics Server (http://us.expasy.org/tools/peptidecutter/). These potential cleavage sites are evenly distributed throughout the protein. We reasoned that even distribution of a large number of cleavage sites would increase the possibility of detecting a conformational change caused by movement of the finger loop.

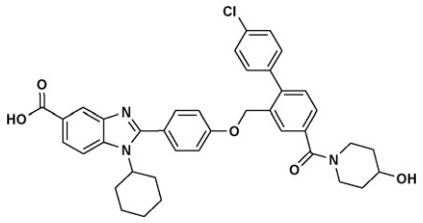
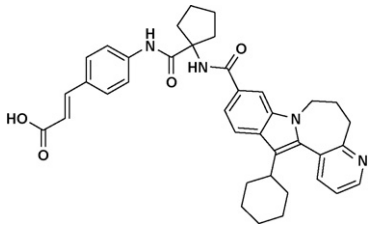
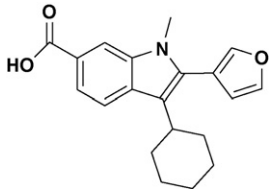
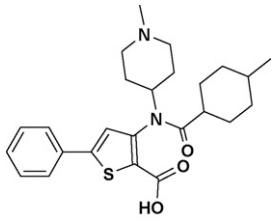
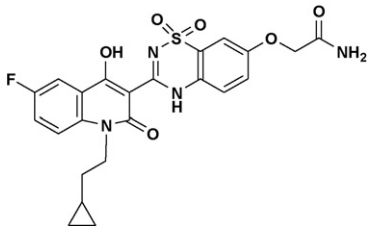
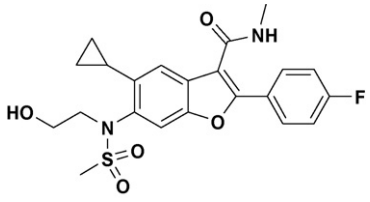
Inhibitors of NS5B that bind the thumb pocket and displace the Δ1 finger loop were used to determine if limited trypsin proteolysis of NS5B could detect displacement. Isolated NS5B protein with a wild type Con 1 sequence and an 18 amino acid deletion of the carboxyl terminus was incubated with a 10-fold molar excess of a previously characterized inhibitor (Hirashima et al., 2006; Tomei et al., 2003). Aliquots of NS5B protein alone or in complex with inhibitor (Table 1, Compound I) were treated with increasing concentrations of trypsin for 20 min and analyzed for cleavage (Fig. 1). The NS5B protein band from samples incubated without inhibitor disappears with increasing concentrations of trypsin, but no specific smaller band appears as the larger band fades (Fig. 1A). This pattern shows that the trypsin cleavage sites distributed over the surface of NS5B are equally sensitive to trypsin protease. This pattern contrasts sharply with the pattern of NS5B cleavage observed in the presence of Compound I, where a smaller band appears as the trypsin concentration increases (Fig. 1B). The cleaved band appearing in Fig. 1B lane 3, is equal in intensity to the uncleaved NS5B band in lane 4, and is the predominant band in lanes 5, 6 and 7.

In the presence of Compound I, a specific site close to the N-terminus of NS5B appears to be hypersensitive to cleavage by trypsin. These experiments were conducted under high salt conditions, similar to the conditions that are used to observe the displacement of the Δ1 loop in the co-crystal structures, as well as lower salt conditions that are compatible with enzyme activity and inhibition (Wang et al., 2006). The inhibitor-induced hypersensitivity was observed regardless of the salt conditions, suggesting that limited proteolytic digestion with trypsin could be a useful approach to correlate the changes observed in X-ray co-crystal studies with effects on NS5B polymerase activity.

3.2. Specific binding of P495-site inhibitors induces cleavage

Experiments were performed to determine if the trypsin digest pattern obtained in solution phase studies could be linked specifi-

Table 1
Compounds used to validate trypsin cleavage assay.

Compound	Structure	Binding site (resistance signature)	IC_{50} (μM) wild type/resistance
I		THUMB (P495)	0.1/1.5
II		THUMB (P495)	0.03/1.9
III		THUMB (P495)	0.43/>12.1
IV		THUMB (M423)	0.017/0.45
V		PALM (M414)	0.016/0.28
VI		PALM (C316)	0.008/2.16

Compounds I, II and III bind a pocket on the thumb domain that is occupied in the apoenzyme by a finger loop. Compound IV binds an alternative pocket on the thumb domain. Compounds V and VI bind to overlapping sites in the palm domain. Inhibitors shown with binding site identified by a signature resistance substitution and IC_{50} values for wild type and resistance.

cally to displacement of the Δ1 finger loop from the thumb-binding pocket, as shown by X-ray co-crystal structures (Di Marco et al., 2005). A P495-site inhibitor (Table 1, Compound II) and 3 inhibitors that bind to alternative allosteric sites on NS5B (Table 1, Compounds IV, V and VI) were tested for the ability to induce formation of the novel trypsin cleavage product (Fig. 2A). These compounds have similar EC_{50} values against wild type NS5B and show resistance when tested against NS5B enzymes carrying signature substitutions that define specific binding sites (Table 1), suggesting the inhibitors have similar binding affinities; however, the patterns of trypsin cleavage for the compounds were different. The pattern of NS5B cleavage generated in the presence of Compound II is similar to the pattern generated in the presence of Compound I (compare Fig. 2A, Compound II and Fig. 1B), but the pattern of NS5B cleavage in the presence of each of the alternative allosteric inhibitors (Compounds IV, V and VI) was the same as

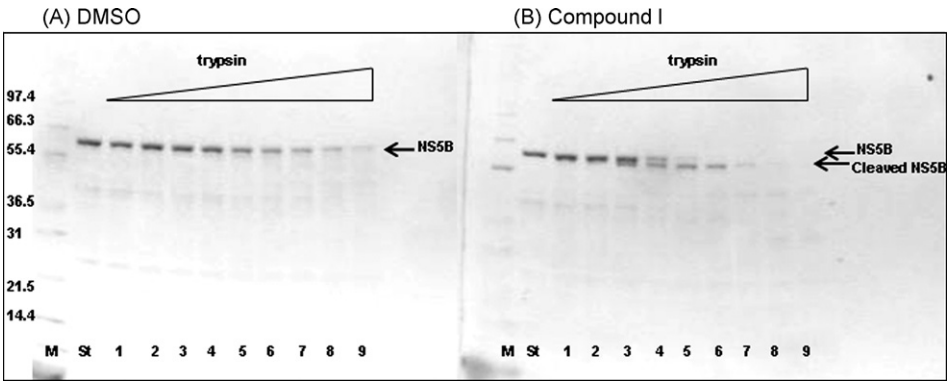


Fig. 1. Change in NS5B polymerase conformation detected as proteolytic cleavage. Isolated NS5B protein was incubated with increasing concentrations of trypsin (0, 0.03, 0.07, 0.13, 0.27, 0.53, 1.1, 2.1, 4.3, 8.5 μg/mL) under high salt conditions in the absence (A) or presence (B) of a 10-fold molar excess of Compound I. The final concentration of DMSO in each proteolytic reaction was 0.2%. Lane M contains protein markers with sizes (kDa) shown on the left. Lane St contains untreated NS5B protein. Samples in lanes marked with the same number were exposed to the same concentration of trypsin and can be compared directly.

control (DMSO). The data indicate that P495-site inhibitors induce a conformational change in NS5B, and that the same change is not induced by allosteric inhibitors that bind either to a different site on the thumb or to overlapping sites in the palm. Changes in conformation that occur with the binding of these alternative allosteric inhibitors are not revealed by partial proteolytic digestion with trypsin.

Resistance to Compounds I, II and III maps to proline 495 of NS5B (Tomei et al., 2003). The inhibitory activity of Compounds I, II, and III (EC_{50} values), assayed with NS5B protein carrying a resistance substitution (leucine substituted for proline 495), increase 10- to 50-fold compared to wild type (Table 1). NS5B protein carrying a P495L substitution was used to determine if mutant protein in the presence of Compound II is also resistant to lim-

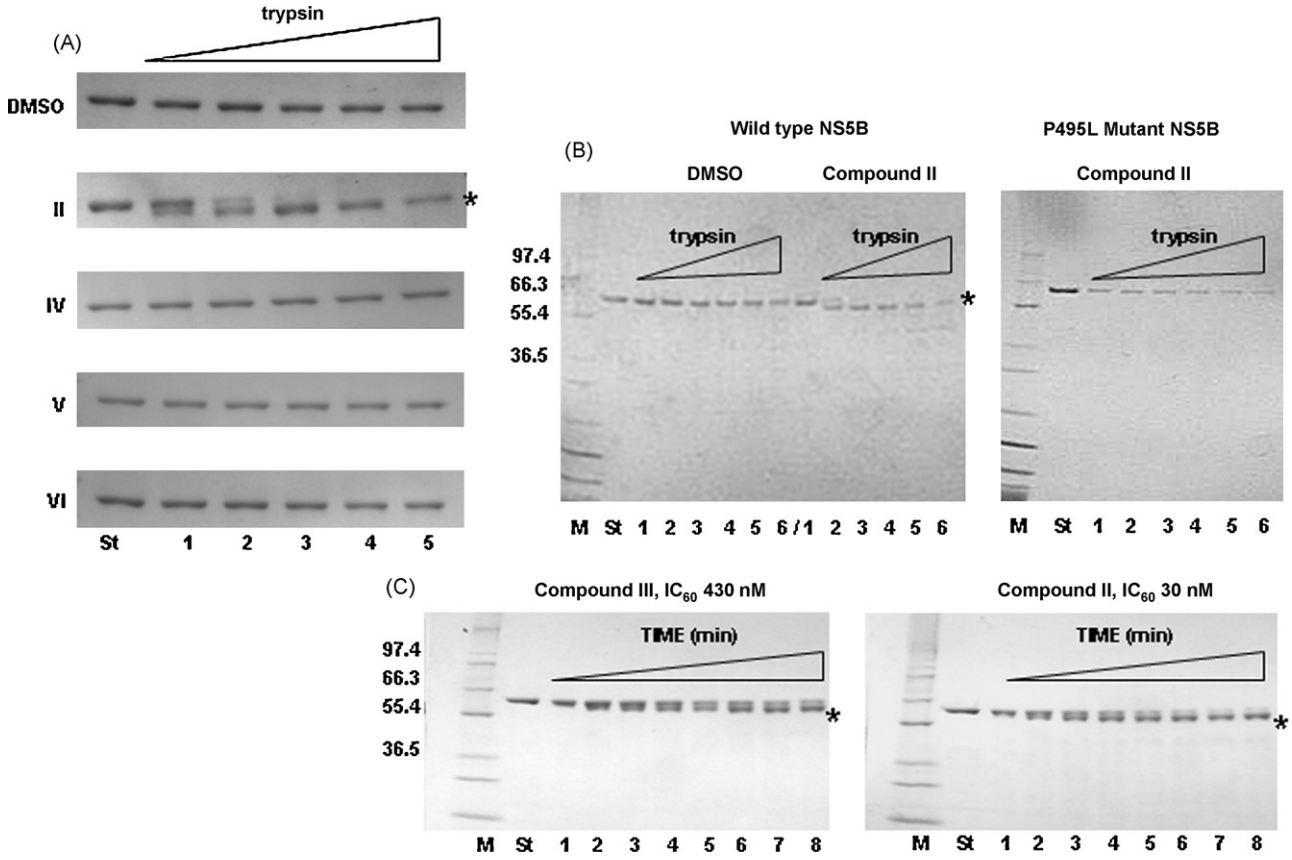


Fig. 2. Proteolytic cleavage induced by specific binding of a finger-thumb inhibitor. (A) Isolated NS5B protein was incubated with increasing concentrations of trypsin protease (0, 0.27, 0.53, 1.1, 2.1, 4.3 μg/mL) in the absence (1% DMSO) or presence (Compounds II, IV, V or VI) of a 10-fold molar excess of inhibitor. (B) Isolated wild type NS5B protein or NS5B protein with leucine substituted for proline 495 (P495L) was incubated with increasing concentrations of trypsin (0, 0.27, 0.53, 1.1, 2.1, 4.3 μg/mL) in the absence (1% DMSO) or presence of a 10-fold molar excess of inhibitor (Compound II). (C) Isolated wild type NS5B protein was incubated with a single concentration of trypsin (0.13 μg/mL) in the presence of a 10-fold molar excess of Compound II or III. Aliquots of each reaction were removed and quenched over time (0, 6, 12, 18, 24, 30, 36, 42 min). Proteolytic reactions were performed under high salt conditions. Lane M contains protein markers with sizes (kDa) shown on the left. Lane St contains untreated NS5B protein. In each individual experiment (A, B or C) lanes marked with the same number were exposed to the same concentration and time of treatment with trypsin and can be compared directly. The cleavage product generated is labeled with an asterisk.

Table 2
Sites of trypsin cleavage.

Sample	Salt condition	Residue at N-terminus	N-terminal sequence
NS5B	High	2	SM-X-YT
NS5B + Compound II	High	44	SASL-X-Q
NS5B + Compound II	Low	51, 52	KVTFD-X-LQ, VTFD-X-LQV
NS5B L30S mutant	Low	51, 52	KVTFD-X-LQ, VTFD-X-LQV
NS5B + RNA template	Low	52	VTFD-X-LQV

ited protease digestion. Isolated NS5B wild type protein or P495L was incubated with a 5-fold molar excess of Compound II and exposed to increasing concentrations of trypsin (Fig. 2B). Only wild type protein was specifically cleaved by trypsin. NS5B carrying the P495L substitution and treated with Compound II has the same pattern as untreated wild type NS5B. The limit of solubility prevented an increase in the concentration of Compound II to a level that is expected to inhibit activity and induce hypersensitivity of the mutant. Resistance of the mutant NS5B indicates that specific binding of inhibitor is required to induce the novel cleavage pattern.

Titration of inhibitors in the presence of NS5B can be used to generate dose-response curves that reveal the relative inhibitory potency of compounds. If hypersensitivity to trypsin is related to inhibition, then cleavage should also be sensitive to inhibitor potency. To test this potential linkage, isolated wild type NS5B was exposed to P495-site inhibitors that differ in potency by 10-fold (Table 1, Compounds II and III). NS5B was incubated with either Compound II or III (10-fold molar excess), and each reaction was treated with a single concentration of trypsin. Aliquots were removed over time and analyzed for the relative extent of cleavage (Fig. 2C). The more potent P495 inhibitor (II) induced greater trypsin hypersensitivity than the less potent inhibitor (III), indicated by the greater reduction of the upper band. The data shown in Fig. 2 demonstrate that the NS5B conformation that is more susceptible to limited proteolytic digestion with trypsin is induced by specific binding of P495-site inhibitors.

3.3. Site of cleavage exposes conformational change at the active site

Edman sequencing of the tryptic digest products generated under high and low salt conditions from wild type NS5B bound to a P495-site inhibitor revealed the sites of cleavage (Table 2). The cleavage site detected under salt conditions sufficient to stabilize the enzyme for crystallization was in the fingers domain, between arginine 43 and serine 44. The cleavage site detected under salt conditions compatible with the detection of enzymatic activity, between lysine 50 and lysine 51 and lysine 51 and valine 52, is directly adjacent to the active site of NS5B. Specifically, these residues are among a cluster of highly conserved basic residues thought to surround the triphosphate moiety of the incoming rNTP (Bressanelli et al., 1999). The shift in cleavage under high salt conditions shows that the conditions used to crystallize NS5B impact the conformational change. Sequence of the upper band demonstrated that the N-terminus of 'uncleaved' NS5B is intact (Table 2). The sequence of the cleavage site under salt conditions compatible with enzymatic activity shows that movement of the fingertip from the thumb-binding pocket exposes residues 50 and 51 to trypsin.

3.4. Cleavage induced by a finger tip mutant

The fingertip residues that interact with the thumb-binding pocket include amino acids 29–31. We confirmed a report that a serine substitution at leucine 30 (L30S) reduces NS5B activity >50-fold (Fig. 3A). Even though no co-crystal structure is available to demonstrate the impact of the L30S mutation on the NS5B structure, heparin sepharose column chromatography and analytical ultracentrifugation strongly suggest that only local alterations, rather than a general unfolding, are induced by the L30S mutant (Labonté et al., 2002).

If the L30S substitution in NS5B destabilizes the finger-thumb interaction, similar to the binding of P495-site inhibitors, the L30S mutant of NS5B should generate a trypsin cleavage pattern similar to the binding of Compounds I, II and III. To test this possibility, NS5B carrying an L30S substitution was exposed to trypsin (Fig. 3B). The pattern of trypsin hypersensitivity of the L30S mutant was similar to the pattern observed for wild type NS5B in the presence of Compounds I, II and III (Figs. 1B and 2A–C); but, the L30S mutant appeared to be more susceptible to cleavage, showing equal amounts of cleaved and uncleaved NS5B at the lowest concentration of trypsin tested. Sequencing of the cleaved fragment revealed the same site of trypsin cleavage (K50, K51) observed in the presence of P495-site inhibitors. These results demonstrate that trypsin cleavage is not dependent on the presence of an inhibitor, but rather on the displacement of the Δ1 loop.

Table 3
Impact on Compound II potency of pre-incubating NS5B with template and template/primer.

Pre-incubation condition	IC_{50} (nM)
NS5B + template + primer + Compound II[a]	260–377
NS5B + template[b]	31
NS5B + template + primer[c]	>2500

[a] Template/primer, inhibitor and enzyme were combined, and reaction was initiated by adding rNTP.
[b] Template and NS5B were preincubated for 60 min, and reaction was initiated with rNTP and primer.
[c] Template/primer/NS5B were preincubated for 60 min before the addition of inhibitor, and reaction was initiated with rNTP.

3.5. Polymerase substrates also impact the finger–loop interaction

Kinetic experiments have clearly shown that the formation of active polymerase complexes in the presence of template and primer is a time-dependent process (Liu et al., 2006). Presumably, correct positioning of the RNA template/primer is required before the enzyme can incorporate ribonucleotides. In addition, the formation of active polymerase complexes precludes inhibition by all allosteric inhibitors reported to date; the compounds inhibit initiation and not elongation of RNA synthesis (Howe et al., 2006; Liu et al., 2006; Tomei et al., 2003). Although pre-incubation of NS5B with template/primer prevents inhibition by P495-site inhibitors, pre-incubation with RNA template alone enhances inhibitor potency (Table 3). These observations suggest a possible connection between the conformational changes associated with active complex formation and the conformational change observed with wild type NS5B bound to P495-site inhibitors and with the L30S mutant. The ability to detect a conformational change by limited protease digestion of NS5B, in buffer conditions similar to the conditions used to measure polymerase activity, encouraged us to test NS5B substrates for the ability to stimulate a conformational change similar to P495-site inhibitors.

The pattern of trypsin hypersensitivity in the presence of RNA template (Fig. 4A, poly C) was similar to the pattern observed for NS5B bound to P495-site inhibitors (Fig. 1B and Fig. 2) or unbound L30S NS5B (Fig. 3B). Sequencing of the NS5B cleavage fragment generated in the presence of RNA template demonstrated that the cleavage is between lysine 51 and valine 52, the same site observed with P495-site inhibitors and the L30S mutant (Table 2). In contrast,

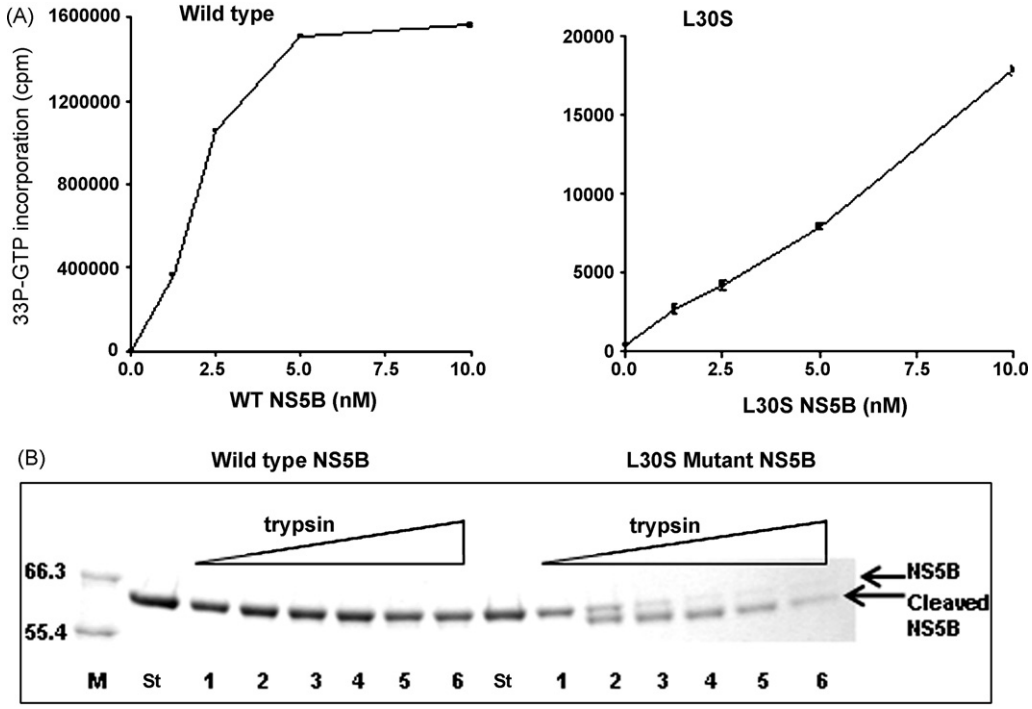


Fig. 3. Phenotype of the finger-thumb mutant L30S. (A) The activity of isolated wild type NS5B genotype 1b (Con 1) protein and NS5B protein with serine substituted for leucine 30 (L30S) were plotted in relation to the final concentration of NS5B in the assay (1.25, 2.5 5 and 10 nM). (B) Isolated wild type or L30S NS5B was incubated under low salt conditions with increasing concentrations of trypsin (0, 0.27, 0.53, 1.1, 2.1, 4.3 μg/mL). Lane M contains protein markers with sizes (kDa) shown on the left. Lane St contains untreated NS5B protein. Lanes marked with the same number can be compared directly. The uncleaved protein (NS5B) and cleaved product (Cleaved NS5B) are labeled.

NS5B in the presence of template/primer (Fig. 4A, poly C + pGpG) was more resistant to trypsin cleavage than NS5B bound to the RNA template. The combination of template and Compound II, on the other hand, generated more cleavage than either template or compound alone (Fig. 4A and B). NS5B in the presence of polyC/GTP (10 mM) (Fig. 4C), conditions required for *de novo* initiation, actually generated NS5B that was more resistant to trypsin than NS5B in the presence of polyC/pGpG.

The specific cleavage product generated by trypsin hypersensitivity is not detected in wild type NS5B in the absence of either P495 inhibitors or template; therefore, UTP, CTP and ATP were also tested for the ability to suppress trypsin hypersensitivity induced by the polyC template. The presence of 10 mM UTP, CTP or ATP suppressed the template induced cleavage (Fig. 4D). There was no effect of nucleotides alone (10 mM) on NS5B (data not shown).

These results demonstrate that displacement of the Δ1 loop ('opening') of NS5B is associated with binding of template alone. The 'closed' conformation is associated with conditions that support active complex formation, with NS5B bound to template and nucleotide or primer. Active complex formation is a prerequisite for polymerase initiation.

4. Discussion

The HCV NS5B apoprotein has a globular structure in a 'closed' conformation, with a finger loop (Δ1) tucked into a hydrophobic pocket on the thumb (Bressanelli et al., 1999; Di Marco et al., 2005). X-ray crystallography has revealed that HCV NS5B inhibitors bound at the finger-thumb interface displace the Δ1 finger loop to yield an 'open' conformation (Di Marco et al., 2005). Specifically, in the apoenzyme crystal structures of NS5B the Δ1 loop is clearly visible and tucked into a hydrophobic pocket on the thumb of the polymerase (Lesburg et al., 1999; Ago et al., 1999; Bressanelli et al., 1999). Co-crystal structures with P495 inhibitors soaked into apoenzyme crystals show no electron density for residues 22–35 of the Δ1 loop, indicating that the loop is no longer tucked into the thumb and that it is moving freely (Di Marco et al., 2005). In the co-crystal structure a slight opening of the polymerase with rigid body rotation of the fingers 2–4° in a clockwise direction from the palm and differences in the Cα positions in the fingers domain compared to that of the apoenzyme structure were observed. These results correlate well with solution studies under similar salt conditions where trypsin cleaves between residues 43 and 44 in the Δ1 loop. The authors concede that the rearrangement of the polymerase domains may have been limited in the crystals by packing interactions and thus could be even larger in solution. They found that the crystals could only be soaked in the presence of inhibitor for a very short period of time before the crystals disintegrated (Di Marco et al., 2005).

P495-site inhibitors provided tools to characterize a solution phase assay that was used to investigate dynamic change in the loop in the presence of substrates, under conditions that support polymerase activity. The assay revealed that movement of the fingertip translates to a change in conformation adjacent to the active site. Since the conformational change was observed under conditions (low salt) that support polymerase activity, the results complement inhibitor-enzyme co-crystal studies and show why P495 inhibitors are not effective after active complex formation (in the presence of RNA template and primer or RNA template and millimolar concentrations of NTP). Under these conditions the loop appears to be closed, blocking the binding of P495-site inhibitors.

Linkage between the movement of the finger loop and trypsin hypersensitivity is direct. In the presence of inhibitors that bind NS5B at allosteric sites other than P495, no cleavage was detected (Fig. 2A), consistent with published X-ray co-crystal structures of similar inhibitors and NS5B genotype 1 proteins that do not show displacement of the Δ1 loop (Biswal et al., 2006; Li et al., 2008; Hang et al., 2009). The 'open' conformation observed in the X-ray struc-

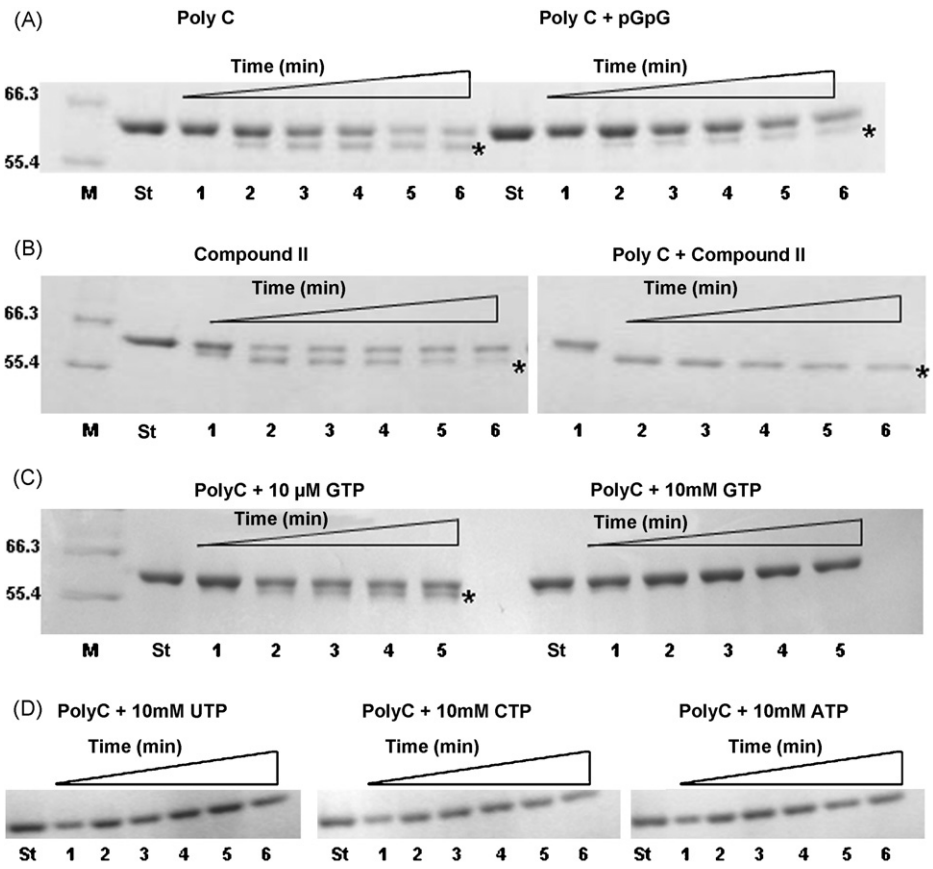


Fig. 4. Polymerase substrates induce and inhibit cleavage. (A) Isolated NS5B protein (4 μM) was incubated with trypsin in the presence of polyC RNA template (1 μM) or polyC RNA template (1 μM) + pGpG dinucleotide primer (0.1 mM). (B) Isolated NS5B protein (4 μM) was incubated with trypsin in the presence of Compound II and the absence or presence of polyC RNA template (1 μM). (C) Isolated NS5B protein (4 μM) was incubated with trypsin in the presence of polyC RNA template (1 μM) + GTP (10 μM and 10 mM). (D) Isolated NS5B protein (4 μM) was incubated with trypsin in the presence of polyC RNA template (1 μM) and 10 mM UTP, CTP, or ATP. Experiments were performed under low salt conditions with a single concentration of trypsin (2.3 μg/mL). Aliquots of each reaction were removed and quenched over time (0, 5, 10, 15, 20, 25 min). Lane M contains protein markers with sizes (kDa) shown on the left. Lane St contains untreated NS5B protein. Lanes marked with the same number were exposed to trypsin for the same period of time and can be compared directly. The cleavage product generated is labeled with an asterisk.

ture of genotype 2a NS5B bound to an M423T inhibitor was, most likely, contributed by inherent instability of the construct (demonstrated by the apoenzyme in both the open and closed states) and not a difference in inhibition mechanism between genotypes 1b and 2a (Biswal et al., 2005, 2006). Induction of the hypersensitivity required specific binding of a P495-site inhibitor, as demonstrated by (i) stability in the presence of Compound II of NS5B carrying the P495L resistance substitution (Fig. 2B), and (ii) greater trypsin hypersensitivity induced by a more potent P495 inhibitor. This was indicated by a greater reduction of the upper band stimulated by Compound II compared to III (Fig. 2C). Trypsin hypersensitivity was, however, not dependent on the inhibitor. Displacement of the Δ1 finger loop using genetic manipulation (L30S) generated trypsin hypersensitivity at the same site (Fig. 3B, Table 2).

To determine if polymerase substrates stimulate loop displacement, RNA template and RNA template/primer were tested. Under low salt conditions trypsin cleaves the NS5B-inhibitor complex, the L30S NS5B protein, and the NS5B-polyC template complex at exactly the same site between lysine 50 and lysine 51, lysine 51 and valine 52, or both (Table 2). This pattern suggests that the trypsin cleavage sites at lysines 50 and 51 are protected in the wild type apoprotein, and that P495 inhibitors and template cause a conformational change that exposes this region to trypsin.

The location of lysines 50 and 51 (K50 and K51), at the base of a finger loop, suggests that this could be a hinge region in NS5B (Fig. 5). Other polymerases have been shown to possess a hinge region near the rNTP binding site (Li et al., 1998). Superposition of the palm domain of HCV NS5B with a ternary complex of HIV RT (bound to DNA and dNTP) generated the hypothesis that the highly conserved, positively charged amino acids K50 and K51 line a tunnel through which NTP molecules access the NS5B active site

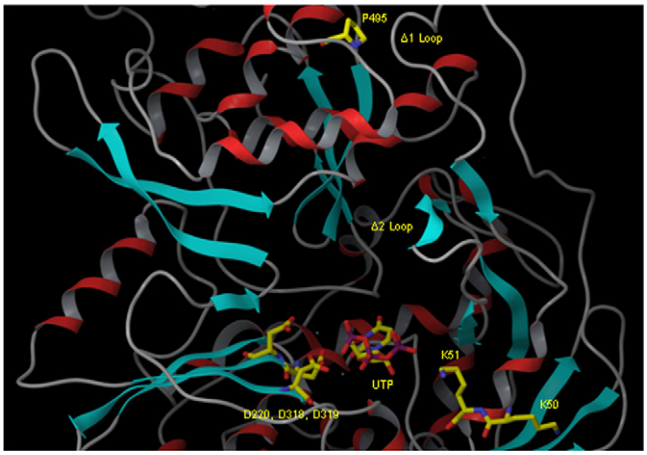


Fig. 5. Model based on the X-ray co-crystal structure coordinates of NS5B with UTP. The model shows the proximity of the trypsin cleavage sites at lysines 50 and 51 and bound UTP. Mn^{++} ions are shown as cyan spheres. Figure created with Maestro (Maestro v 8.5. Schrödinger, LLC, New York, NY, 2009) (Berman et al., 2000; Bressanelli et al., 2002; O'Farrell et al., 2003).

(Bressanelli et al., 1999). A co-crystal structure of NS5B and UTP shows that K50 and K51 are adjacent to the triphosphate moiety of UTP in the interrogation 'I site', and K51 makes an electrostatic interaction with UTP (Bressanelli et al., 2002; O'Farrell et al., 2003). Susceptibility of K50 and K51 in the presence of inhibitor or RNA and not in the free enzyme or in complex with template/primer suggests displacement of the Δ1 loop translates to movement in a region that could directly impact polymerase activity. Susceptibility of K50 and K51 in the presence of RNA template suggests that the dynamic change in conformation that can, in the presence of inhibitor, lead to inactivation of the enzyme is reversible and necessary for the positioning of template.

The data show how an allosteric inhibitor that binds 30 Å from the active site can inhibit polymerase activity (Fig. 5). These results are consistent with mechanism of action experiments that indicate P495-site inhibitors block initiation; this conformational change could block incorporation of the first rNTP (Liu et al., 2006; Tomei et al., 2003). Absence of the K50, K51 cleavage in the presence of NS5B inhibitors of equal potency that bind to alternative allosteric sites shows that these inhibitors have a distinct inhibition mechanism, even though they also inhibit initiation (Liu et al., 2006; Ma et al., 2005). It is conceivable that conformational changes in NS5B induced by these allosteric inhibitors, which also inhibit the initiation of RNA replication, could be detected by using a different protease cocktail or by altering the assay conditions.

These results clarify the relationship between the enhancement of inhibitor potency and RNA template binding (Table 3) (Tomei et al., 2003). High trypsin concentrations were required to detect cleavage in the presence of polyC template alone. However, enhanced cleavage was observed in the presence of RNA template plus inhibitor, indicating the combination generated more NS5B molecules with loop displacement than either ligand alone (Fig. 4A and B). This data suggests that the presence of RNA template enhances the binding of P495-site inhibitors by shifting the number of NS5B molecules that have a displaced Δ1 loop and are more accessible to inhibitor. Co-crystal structures with a U5 RNA revealed no displacement of the Δ1 finger loop or opening of the NS5B structure (O'Farrell et al., 2003). This is consistent with U5 RNA behavior as a primer such as pGpG and not the RNA template (Fig. 4A).

To investigate the conformation of the Δ1 loop with RNA primer and template bound, assays were done in the absence of Mg^{++} or Mn^{++} to mimic either primer-dependent or *de novo* initiation conditions without rNTP incorporation. The pGpG primer (0.1 mM) and GTP (10 mM) in the presence of polyC template elicit similar effects, suppressing the trypsin hypersensitivity stimulated by template alone. The GTP (10 mM)/RNA template combination showed greater stabilization of NS5B than pGpG primer/RNA template (Fig. 4A and C). GTP alone at 10 mM, but not 10 μM, stabilized the enzyme in the presence of RNA template (Fig. 4C). A stabilizing effect by high concentrations of dNTP on members of the DNA polymerase I family has also been observed (Li et al., 1998).

Stabilization of template bound NS5B was not restricted to high concentrations of GTP. ATP, CTP or UTP (10 mM) in the presence of polyC template produced the same stabilizing effects (Fig. 4C and D). A fluorescence-tagged trypsin substrate was used to establish that neither the rNTP reagents nor any of the other ligands used in these experiments inhibited trypsin directly (data not shown) (Bolger and Checovich, 1994). We are also confident that stabilization is not due to a physical blockade by NTP. If bound NTP, in the presence of the RNA template, were physically blocking the cleavage at Lys 51 without inducing loop closure, we could still detect hypersensitivity at Arg 43. Co-crystal structures indicate no interaction between Arg 43 of NS5B and bound NTP. Based on the co-crystal structure of NS5B and UTP, Lys 50 and 51 are located in the nucleotide interrogation site ("I site") not at the catalytic site. The "I site" is thought to transiently hold an NTP while the enzyme interrogates the template strand; therefore, no preference for one NTP over another is expected. This is consistent with the trypsin digest results that show all four NTPs have the same affect on the Δ1 loop in the presence of the polyC template (Fig. 4D).

Our results provide evidence to support the hypothesis that movement of the Δ1 loop occurs with RNA template binding (Chinnaswamy et al., 2008). It is possible that inhibitor binding interferes with the proper positioning of template-primer. It has been proposed that opening of the Δ1 loop during elongation allows the double stranded RNA product to exit the enzyme (Chinnaswamy et al., 2008). Data presented here demonstrate that opening of the Δ1 loop causes solvent exposure of K50 and K51 and correlates with inactivation of NS5B. This observation supports an alternative hypothesis that movement of the thumb, as proposed by Bressanelli, accommodates the double stranded product, rather than movement of the finger loop (Bressanelli et al., 2002).

Our data support a model with the finger loop of NS5B displaced to accommodate the positioning of template and remaining closed once the initiation complex forms. This model is consistent with published data showing that P495-site inhibitors have no effect on the polymerase once elongation begins (Liu et al., 2006; Tomei et al., 2003). Co-crystal structures of NS5B and P495-site inhibitors have clearly shown displacement by inhibitor of the Δ1 finger loop from a thumb-binding pocket. Our work demonstrates that inhibitor binding, which is 30 angstroms from the active site, induces a conformational change that exposes K50 and K51, amino acids that interact with incoming nucleotides. Inhibitor-induced conformational changes in this region could block nucleotide incorporation and inactivate NS5B.

Acknowledgements

The authors thank their Virology colleagues Jin-Hua Sun, Dr. Ira Dicker and Dr. Brian Terry for critical reading and useful discussions of this manuscript; and Dr. Robert Fridell and Dike Qui for constructs used to prepare isolated proteins. Authors also thank their Chemistry colleagues Dr. Piyasena Hewawasam and Henry Wong for the preparation of reference compounds.

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