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Selection and Target-Site Mapping of Peptides Inhibiting HCV NS5B Polymerase Using Phage Display

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A series of peptides binding to the HCV NS5B polymerase was selected from phage display peptide libraries. A conserved motif of Ser-Arg-X-Arg/Leu was identified among the selected peptides, and Pep2 (Trp-Ser-Arg-Pro-Arg-Ser-Leu) was chosen for further characterization. The binding of Pep2 to HCV NS5B *in vivo* was shown by a yeast two-hybrid assay and by subcellular colocalization analysis using immunofluorescence confocal microscopy. The *in vitro* interaction was also confirmed by GST pulldown assay. The replication of the HCV 1b subgenomic replicon was efficiently inhibited by the presence of the peptide. By using a subtractive biopanning against Pep2, the binding site of the peptide was mapped at the pocket of Pro388 to Pro391 in the thumb subdomain of the polymerase. A yeast two-hybrid analysis using Pro388Ala and Pro391Ala mutants of NS5B confirmed the binding.

Keywords: Hepatitis C virus, NS5B polymerase, phage display, peptide inhibitor, binding pocket

The hepatitis C virus (HCV) causes viral hepatitis in humans, leading to liver cirrhosis and hepatocellular carcinoma [22, 25, 37]. Worldwide, over 170 million people are infected with this virus, and there is no effective therapy or vaccine currently available. PEGylated interferon and ribavirin are the two available treatments for HCV, yet both have a limited efficacy [8, 21]. The HCV genome is a plus-sense single-strand RNA of 9.6 kilobases [10], encoding a polyprotein that is cleaved into 10 separate proteins. The 5' and 3' untranslated regions (UTRs) of the genomic RNA are necessary for replication, packaging, and translation [32, 35]. The NS5B RNA-dependent RNA polymerase is responsible for its RNA replication [13, 20]. NS5B polymerase is a 65 kDa protein and has a typical polymerase structure of fingers, palm, and thumb subdomains [1]. A GDD motif, which is present in all RNA polymerases, is found in the palm subdomain. The fingers and thumb subdomains interact with the RNA template. When the two loops extruding from the fingers subdomain make contact with the thumb subdomain, the protein becomes an active form of RNA polymerase [5, 18]. Over the past few years, various nucleoside and nonnucleoside chemicals were reported to inhibit the enzymatic activity of this polymerase [4, 9, 12, 29, 34, 36]. Some of the inhibitor binding sites have also been revealed by X-ray crystallographic analysis. Nonnucleoside inhibitors are believed to suppress RNA synthesis by inhibiting the conformational transition of the enzyme.

As a combinatorial biological method, a phage display is a powerful technique for protein-protein interaction analysis [31]. In addition, a phage library (usually $>10^{10}$) can be effectively and rapidly screened using a biopanning method. Peptides, proteins, and various forms of antibody fragments can be displayed on the surface of bacteriophages [2, 14, 26–28, 38], including filamentous bacteriophages (M13, f1), phage lambda, and phage T7. The ease of engineering involved in phage DNA, its rapid growth, and the availability of a high titer also make the system an attractive tool. Furthermore, the peptides and proteins displayed on the surface can easily be identified by a nucleotide sequence analysis of the phage DNA inside the capsid.

Amin *et al.* [3] previously reported on a selection of disulfide-constrained peptides inhibiting the enzymatic activity of HCV NS5B. The main focus was modulation by the peptides on the enzymatic activity of purified polymerase, meaning the experimental results were mainly biochemical. We report the effect of a novel NS5B-binding peptide on HCV replication in the subgenomic replicon level. In addition, the putative novel binding site of the inhibitory peptide is revealed.

MATERIALS AND METHODS

Purification of Recombinant NS5B

The gene encoding HCV NS5B, except for the hydrophobic C terminus 21 amino acids, was cloned into a pET21-a(+) vector and

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named pET-NS5BD21. *E. coli* BL21(DE3) transformed with pET-NS5BD21 was then grown and the overexpressed NS5B was purified using Ni-NTA affinity chromatography. PolyU sepharose chromatography was also performed to further purify the protein.

Phage Library and Biopanning

Ph.D 7mer and 12mer phage display peptide libraries were purchased from New England Biolabs (U.S.A.), and a C-9-C mer constrained peptide library was constructed using the pCANTAB5E vector (Pharmacia, U.S.A.). Two oligomers were annealed to encode a $C(NNK)_9C$ peptide and extended with a Klenow fragment and dNTPs. The resulting short double-stranded DNA was then ligated between the SfiI and NotI sites. *E. coli* JM109 was transformed with the ligated DNAs and further infected with helper M13 phages. Ten µg of purified NS5B was used to coat a well of a 96-well plate and blocked with 1% BSA. One hundred µl of the phage solution was then added and allowed to bind for 1 h. Thereafter, the well was washed three times with a TE buffer and the binding phages were eluted by lowering the pH to 3. The eluted phages were pooled and amplified by infecting ER2738 or JM109. These amplified phages were then used for a new round of biopanning and the process was repeated 3 to 5 times until certain peptide sequences were shown to be enriched.

Yeast Two-Hybrid Assay

Matchmaker two-hybrid 3 (BD Bioscience, U.S.A.) was used, where the NS5B was cloned in a pGAD vector and expressed, whereas Pep1 was cloned in a pGBK vector and expressed. The yeast AH109 strain was then transformed with the two plasmids, and the growth and enzymatic reaction were analyzed according to the manufacturer's manual.

Immunostaining and Subcellular Colocalization

The oligomers used to clone Pep2 into pEGFP were as follows: forward, 5'-GATCTTGGTCGCGCCCGCGGTCACTTTAAG-3'; reverse, 5'-5'-GATCCTTAAAGTGACCGCGGGCGCGACCAA-3'. Ten pmol of each oligomer was annealed and ligated to pEGFP-C1 (Clontech, U.S.A.) cleaved with BglII and BamHI. Meanwhile, pcDNA4V5 was used to clone the HCV NS5B. BHK-21 cells were co-transfected with pcDNA4V5-NS5B and pEGFP-Pep2. The cells were fixed and washed. Next, an anti-V5 antibody (Invitrogen, U.S.A.) and a TRITC-conjugated secondary antibody were used for detection under a laser confocal microscope (BioRad, U.S.A.).

Cell Culture and Transfection

BHK-21 and Huh-7 cells harboring an HCV subgenomic replicon were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin G, and 100 µg/ml streptomycin. One hundred mg/ml of G418 was also added to the replicon cell line for maintenance purposes. The BHK-21 cells were then seeded in a 6-well plate at a concentration of 5×10^4 cells/well and incubated for 24 h. One µg of pcDNA4-V5 and 1 µg of pECF-Pep2 were mixed with 5 µg of Lipofectamine 2000 (Invitrogen, U.S.A.) and 100 µl of serum-free DMEM and incubated for 20 min at room temperature. The mixture was then added to the culture with 2 ml of complete media and immunostaining was performed after 24 h. The Huh-7 replicon cells were seeded in a 6-well plate at a concentration of 1×10^5 cells/well. After 24 h, 1 µg of pcDNA-GST or pcDNA-GST-Pep2 was added with 0.5 µg of pCH110 (Pharmacia,

U.S.A., to measure the transfection efficiency) using Lipofectamins 2000.

GST Pulldown Assay

pcDNA-NS5B (FLAG tagged) was used for transfection of BHK-21 cells using lipofectamine. Cells were harvested in 24 h and lysed with RIPA buffer at 4°C for 1 h. Five µg of purified GST or GST-pep2 was mixed with 40 µl of glutathione sepharose 4B (Amersham Biosciences) beads and the volume adjusted with HNGT buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) to 200 µl. After an overnight incubation at 4°C, the mixture was washed with 1 ml of HNGT buffer four times, and then boiled for 5 min. The mixture was subjected to an SDS-PAGE followed by Western blot analysis using anti- FLAG and anti-GST antibodies.

Biochemical RdRp Assay

A 50-ml reaction mixture containing 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5 µCi of $[^{32}P]$-UTP, 50 µM UTP, 5 µg/ml poly(A)-oligo(U)$_{14}$ (Amersham, U.S.A.), and 2 µg recombinant NS5BΔ21 was incubated at 30°C for 90 min. The reaction was then stopped by the addition of EDTA to a final concentration of 100 mM. The unincorporated $[^{32}P]$-UTPs were removed by centrifugation of the mixture in a QuickSpin column (General Bio, Korea) at 10,000 ×g for 1 min. The remaining radioactivity was measured using a scintillation counter.

Real-Time RT-PCR

The total RNA was isolated from the replicon cell-line using a total RNA isolation kit (Qiagen, U.S.A.). An iScript cDNA synthesis kit (BioRad) was used with a random hexamer primer for the reverse transcription. The real-time PCR of the HCV replicon RNA was performed using iQ Supermix (BioRad) and the following primers: probe-5' 6FAM-CCACATTACGGCGGTGTCGGCT-BHQ1, forward-5'TTCCATGCTCACCGACCC3', reverse-5'CGCCCATCTCCTG-CCG3'. As the internal control, GAPDH mRNA was detected using the following primers: probe-5' 6FAM-CCGACGCCTGCTTCACC-ACCTT-BHQ1 3', forward-5' AAACCTGCCAAATATGATGACAT 3', reverse-5' GCCCAGGATGCCCTTGA 3'. The reaction conditions were as follows: denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 30 sec and 59.3°C for 30 sec.

Epitope Mapping

A subtractive biopanning method was used, where a library of random 12-mer display phages was allowed to bind in a well of a 96-well plate coated with 10 µg of purified GST for 1 h. While leaving the bound phages in the well, the supernatant was removed and added to a well coated with 10 µg of purified GST-Pep2. The standard biopanning procedure described above was performed.

Site-directed Mutagenesis

The pGAD-NS5B vector used in the yeast two-hybrid analysis was also subjected to site-directed mutagenesis. The primers used for the Pro388Ala mutagenesis were mutant1 primer (F 5'-CTACCTCAC-CCGTGATGCCACCACCCCCCTCGCAC, R 5'-GCGAGGGGGG-TGGTGGCATCACGGGTGAGGTAG) and mutant3 primer (F 5'-GGACCAAGC TCAAACTCACTGCAATCCCGGCTGCG, R 5'-GGACGCAGCCGGGATTGCAGTGAGTTT GAGCTTGG). After 12 cycles of a PCR reaction, DpnI was added and the mixture was incubated for 1 h at 37°C. The primers used for Pro391Ala

mutagenesis were; P391A mutant2 primer (F 5'-CGTGATCCCAC-CACCGCCGTGGCACGGGCTGC, R 5'-CGCAGCCCGTGCGAG-GGCGGTGGTGGGATCACGGG) and mutant3 primer (F 5'-GGA-CCAAGCTCAAACTCACTGCAATCCCGGCTGCG, R 5'-GGAC-GCAGCCGGGATTGCAGT GAGTTTGAGCTTGG). After 12 cycles of a PCR reaction, DpnI was added and the mixture was incubated for 1 h at 37°C.

RESULTS AND DISCUSSION

Selection of Peptides

The amino acid sequences for the selected peptides are shown in Table 1. This study used three different peptide libraries, 7mer, 12mer, and C-9-Cmer. A conserved motif of **Ser-Arg-X-Arg/Leu** was found in 4 peptides (Pep1, Pep2, Pep4, and Pep5), suggesting that they could bind to the same site in the target NS5B protein. Since Pep2 was the shortest peptide harboring the conserved motif and showing a high number of hits, it was selected for further characterization.

NS5B Interaction

First, the *in vivo* interaction between the selected peptide and the HCV NS5B protein was investigated using a yeast two-hybrid analysis (Fig. 1A). As such, Pep2 was cloned in a pGBK vector, whereas NS5B was cloned in a pGAD vector. From a reporter assay measuring α-galactosidase activity, the interaction between the peptide and NS5B was confirmed.

Next, the subcellular localization of the selected peptide and NS5B protein was observed. Pep2 was expressed as a fusion protein in an Enhanced Green Fluorescent Protein (EGFP), whereas NS5B was expressed with a V5 tag. Pep2 was expressed as a fusion protein, since a peptide composed of only seven amino acids would not be very stable in a cell. NS5B is known to localize predominantly in the perinuclear region because of its endoplasmic reticulum membrane-anchoring domain composed of C-terminal hydrophobic 21 amino acids, whereas EGFP is in both the nucleus and the cytoplasm. When the two constructs were coexpressed in a cell, the peptide and NS5B were shown to colocalize in the cytoplasm (Fig. 1B), providing further evidence of *in vivo* interaction. Interaction was also

Table 1. Selected peptides binding to HCV NS5B protein

Peptide name	Peptide sequence	Number of hits[a]
Pep1	CATRLGRSSRC	18/26
Pep2	WSRPRSL	16/26
Pep3	TGPLPKE	8/23
Pep4	CMAFFISRWRC	5/23
Pep5	NYNLSRNLTWFY	5/10

Bold characters show the conserved amino acid residues.

[a]Number of hits over number of sequenced clones.

observed in the nucleus, possibly due to overexpression and diffusion of Pep2 fused to EGFP.

In vitro interaction was also checked with GST-pulldown assay (Fig. 1C). NS5B was not seen in the sample pulled down with GST, but it was seen in the sample pulled down with GST-pep2, suggesting that HCV NS5B specifically interacts with pep2.

Inhibition of HCV Replication

As the interaction was confirmed both *in vivo* and *in vitro*, a test was conducted to determine whether the binding of
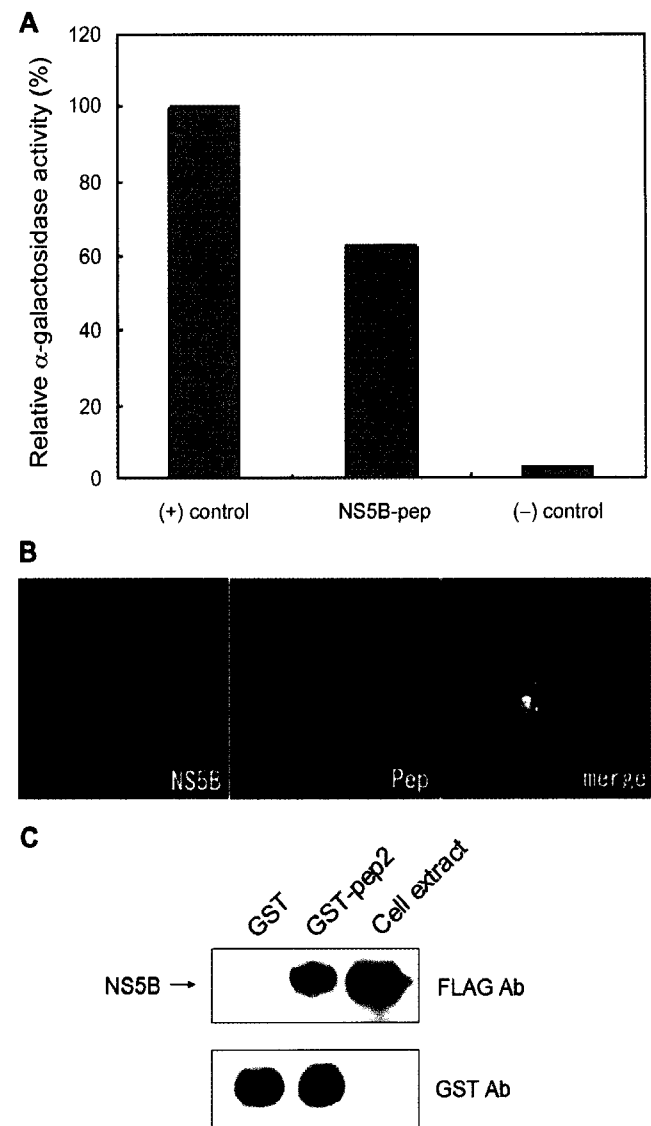

Fig. 1. A. Yeast two-hybrid analysis between Pep2 and HCV NS5B. SV40 large T antigen and murine p53 were used for a positive control. Empty vectors were used for a negative control. Relative α-galactosidase activities are shown. B. Confocal laser microscopy analysis of the subcellular localization of Pep2-EGFP and NS5B in a Huh-7 cell. Pep2-EGFP is shown in green, and NS5B is shown in red. C. GST pulldown assay of pep2 and HCV NS5B. HCV NS5B was expressed in a BHK-21 cell and pulldown was performed with purified GST-pep2. NS5B was detected with FLAG antibody.

the peptide affected the RNA-dependent RNA polymerase activity of NS5B. Thus, Pep2 was expressed as a GST-fused form in a Huh-7 cell line containing an HCV

A

B

C

Fig. 2. Inhibition of subgenomic HCV RNA replicon by Pep2-GST. A. Real-time RT-PCR analysis of the replicon RNA level. The amount of HCV subgenomic replicon RNA was measured in the presence of GST or GST-pep2. The average of three independent experiments is shown with error bars. B. Western blot analysis of HCV NS5A protein with anti-NS5A antibody. GST was detected with anti-GST antibody to confirm the expression of GST-pep2. Actin was detected as the internal control. C. Isotopic incorporation assay using synthetic RNA template and recombinant NS5B. Purified GST or GST-pep2 was added to the polymerase reaction mixture and the incorporation of UTP was measured.

subgenomic replicon [16]. A real-time RT-PCR analysis showed an approximate 50% reduction of the HCV replicon RNA in the presence of GST-pep2 compared with the presence of GST alone (Fig. 2A). A Western blot analysis of the HCV NS5A showed an approximate 90% reduction of the protein (Fig. 2B). Pep2 synthesized by a solid phase method using standard fluorenylmethoxycarbonyl (Fmoc) chemistry was also used for the biochemical RNA-dependent RNA polymerase assay. Consisting of just seven amino acids, Pep2 was added to an isotopic incorporation assay to measure the enzymatic activity of NS5B. Yet, the enzymatic activity of the purified protein was not shown to be inhibited by the presence of the 7mer peptide (data not shown). It is possible that the 7mer peptide was unable to retain the same structure as the form fused to phage coat protein, EGFP, or GST. Then, pep2 was expressed and purified as a fusion to GST. The fusion protein was shown to inhibit NS5B activity by 20% in an *in vitro* isotope incorporation assay (Fig. 2C). The inhibition was lower than that observed in a replicon assay, suggesting that some cellular factors were needed to exert its inhibitory effect for NS5B, or yet other factors were needed to stabilize the proper structural conformation needed for the peptide.

Target Site Mapping

Since Pep2 was shown to bind with NS5B and inhibit the RNA replication, the binding site was further investigated by a new round of biopanning. This time, Pep2 was the target and peptides binding to Pep2 were isolated. Since Pep2 was only shown to retain a binding conformation when expressed as a fusion peptide, GST-fused Pep2 was used for this purpose. Thus, a subtractive biopanning was required, to allow the the peptides binding to the GST portion of the fusion protein to be discarded first. Among the many peptides selected this way, a group of peptides was gathered harboring a conserved amino acid sequence of Pro-(Thr)-(Thr)-Pro (Fig. 3A). A CLUSTALW analysis with NS5B showed the motif was in the thumb subdomain containing Pro388 and Pro391. To confirm the binding between this region and Pep2, a yeast two-hybrid analysis using wild-type NS5B, Pro388Ala mutant, and Pro391Ala mutant was performed. As a control, Pro538Ala was constructed, in which the mutation was far from the suggested binding site. The result showed that the Pro388Ala mutation abolished the interaction with Pep2 (Fig. 3B), whereas Pro391 Ala and Pro538Ala mutations did not alter the binding, suggesting that Pro388 was critical for Pep2-binding. When considering the amino acid sequence of Pep2, the terminal amino acids, tryptophan and leucine, are hydrophobic and likely to bind to the hydrophobic pocket in the region (Fig. 4). The two arginines in the middle have long side chains and are positively charged, suggesting they may bind to negatively charged amino
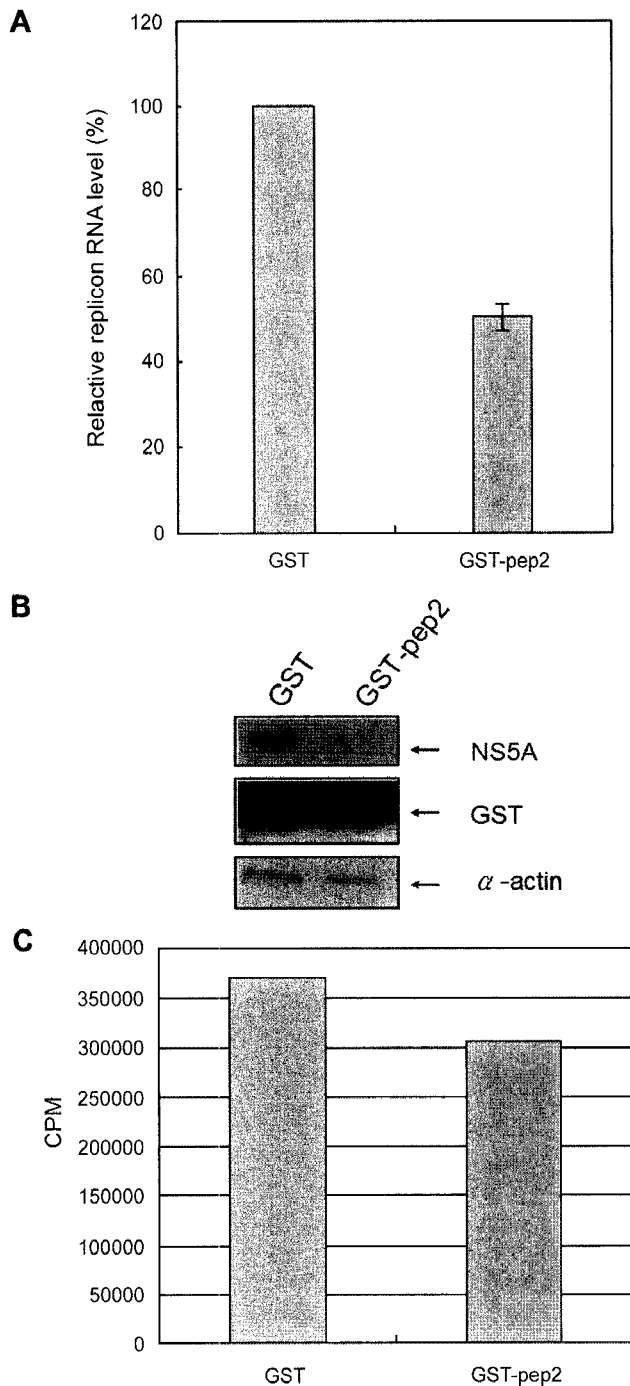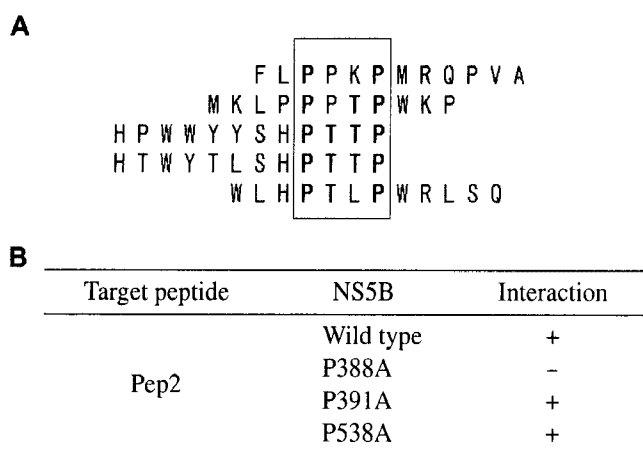
A

```
            F L  P P K P  M R Q P V A
        M K L P  P P T P  W K P
    H P W W Y Y S H  P T T P
    H T W Y T L S H  P T T P
        W L H  P T L P  W R L S Q
```

B

Target peptide	NS5B	Interaction
	Wild type	+
Pep2	P388A	–
	P391A	+
	P538A	+

Fig. 3. Target site mapping of Pep2.
A. Selected 12mer peptides binding to Pep2. An overlapping amino acid sequence is shown in bold in a box. B. Yeast two-hybrid analysis of wild-type or mutant NS5B with Pep2. P388A and P391A mutants were used for interaction analysis. The P538A mutant was used as a negative control.

acids or be exposed to a solvent. Finally, the two serines in the middle can mediate hydrogen bonds or be exposed to a solvent.

The current inhibitors of NS5B are grouped into 3 categories: nucleoside analogs, nonnucleoside inhibitors (NNIs), and pyrophosphate mimics. There are also three classes of NNIs [9], where one includes benzimidazoles and indoles that target Pro495 in the thumb subdomain. For NS5B to become active, GTP binding acts as an allosteric regulator and induces interaction between the fingers and thumb subdomains. As a result, the enzyme assumes a closed form and initiates RNA polymerization [6, 19]. These allosteric inhibitors target the binding site

Fig. 4. Predicted binding site of Pep2 in HCV NS5B protein.
The structural coordinates are from Adachi *et al.* [1]. The Pep2-binding site in the thumb subdomain is shown in pink. The thumb subdomain is shown in blue, the palm in green, and the fingers in red.

between the thumb and fingers subdomains, rendering the protein in an open form [33]. The second class contains thiophene-2-carboxylic acid and targets Met423 and Leu419 in the thumb subdomains [7]. Since the mode of action is similar to that of the first class, they are also allosteric inhibitors [9]. The third class includes benzothiodiazine and targets Met414 in the thumb subdomain. Here, the action is also focused on elongation complex formation like the other two groups [23, 24]. However, this group of inhibitors binds to the inside of the thumb subdomain, which is close to the active site of the enzyme. In our experiments, the Pep2 binding site is also mapped at a narrow cleft in the thumb subdomain. Based on the other small molecule inhibitor mechanisms, this is an allosteric site and may also affect conformational change of the enzyme. Accordingly, the identified site could be a novel target site on HCV NS5B polymerase for antiviral drug development.

Many successful epitope mappings by using a phage displayed peptide library were reported recently [11, 15, 17, 30]. The current work also exemplifies the usefulness of this methodology and provides a possibility of novel target site development in drug discovery.

Acknowledgments

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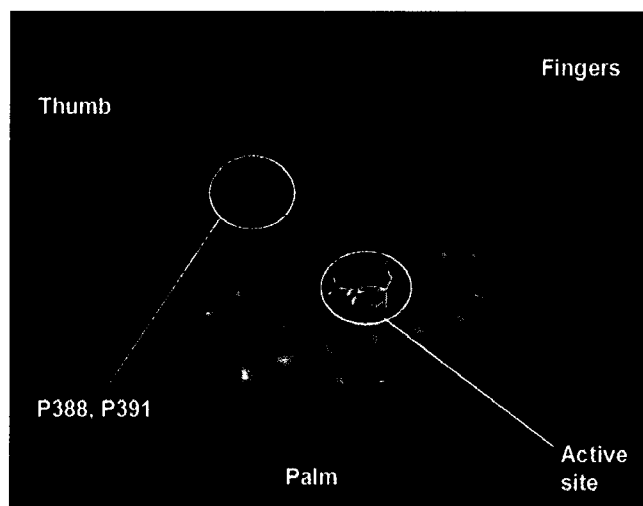
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