VAPC, an Human Endogenous Inhibitor for Hepatitis C Virus (HCV) Infection, Is Intrinsically Unstructured but Forms a “Fuzzy Complex” with HCV NS5B

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Abstract

Nearly 200 million people are infected by hepatitis C virus (HCV) worldwide. For replicating the HCV genome, the membrane-associated machinery needs to be formed by both HCV non-structural proteins (including NS5B) and human host factors such as VAPB. Recently, the 99-residue VAPC, a splicing variant of VAPB, was demonstrated to inhibit HCV replication via binding to NS5B, thus acting as an endogenous inhibitor of HCV infection. So far, the structure of VAPC remains unknown, and its interaction with NS5B has not been biophysically characterized. In this study, we conducted extensive CD and NMR investigations on VAPC which led to several striking findings: 1) although the N-terminal 70 residues are identical in VAPC and VAPB, they constitute the characteristic β-barrel MSP fold in VAPB, while VAPC is entirely unstructured in solution, only with helical-like conformations weakly populated. 2) VAPC is indeed capable of binding to NS5B, with an average dissociation constant (Kd) of ~20 μM. Intriguingly, VAPC remains dynamic even in the complex, suggesting that the VAPC-NS5B is a “fuzzy complex”. 3) NMR mapping revealed that the major binding region for NS5B is located over the C-terminal half of VAPC, which is composed of three discrete clusters, of which only the first contains the region identical in VAPC and VAPB. The second region containing ~12 residues appears to play a key role in binding since mutation of 4 residues within this region leads to almost complete loss of the binding activity. 4) A 14-residue mimetic, VAPC-14 containing the second region, only has a ~3-fold reduction of the affinity. Our study not only provides critical insights into how a human factor mediates the formation of the HCV replication machinery, but also leads to design of VAPC-14 which may be further used to explore the function of VAPC and to develop anti-HCV molecules.

Introduction

Hepatitis C virus (HCV), first discovered in 1989, infects about 200 million people worldwide [1–3], and is a leading risk factor for the development of severe chronic liver diseases including cirrhosis and hepatocellular carcinoma [4]. HCV is a member of the Flaviviridae family of enveloped, positive-stranded RNA viruses, and has a genome of approximately 9.6 kb encoding a single polyprotein of ~3,000 amino acids, which is subsequently processed into 10 individual proteins by viral and cellular proteases [5–9]. Interestingly, replication of positive-stranded RNA viruses involves intracellular membrane structures, such as the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome [10]. The membrane-associated machinery copies the RNA genome into a negative-strand intermediate, which is then used to generate additional positive-stranded RNA copies for subsequent rounds of translation and packaging into virus particles. HCV replication is initiated immediately after translation and processing of the viral protein, and all HCV gene products remain associated with intracellular membranes [11–17]. Although the exact mechanisms, identities of the host factors and detailed interactions among them are poorly understood, HCV nonstructural proteins including NS3, NS4A, NS4B, NS5A, and NS5B have been characterized to be the key components of the replication machinery.

Thus far, no efficient HCV vaccine is available, and the most common therapy is based on a combination of interferon-alpha and ribavirin. However, this treatment has a success rate of only ~50%, with severe side effects [18,19]. As a consequence, identification of novel targets to design HCV antiviral drugs is urgently required [20–23]. At present, the most common targets are two viral enzymes, i.e. the NS3/4A serine protease and the NS5B RNA-dependent RNA polymerase [24], as they are amenable to the development of biochemical assays for inhibitor screening [25]. However, targeting enzymatic actions also appear to have a considerable disadvantage, i.e. rapid development of drug resistance [25,26]. Therefore, there is promising potential to target non-enzymatic processes such as those required for RNA
Conformation of VAPC and Its Binding to NS5B

hVAPB-MSP: 1MAKVEQVLSL EPQHELKFRG PFTDVTNTNL KLGNPTDRNV
hVAPC: 1MAKVEQVLSL EPQHELKFRG PFTDVTNTNL KLGNPTDRNV

CFKVKTAPR RXCVRPNSTGI IDAGASINVSLVMLQPFYIDP
CFKVKTAPR RXCVRPNSTGI IDAGASINVSGRRWTADEED

NEKSKHHFHVQ SMFAPTSTS DMEAVWKEAS PEDLMDSKLR CVFEL_{125}
SAEQQQPHFSI SPNWGRRP_{99}

(a)

(b) (c)

(d)
replication. Although how cyclophilins regulate HCV replication remains unknown, a molecular chaperone cyclophilin A that catalyzes the cis-trans isomerization of proline residues, has been demonstrated to be an important drug target for chronic hepatitis C therapy [27,28].

Recently, the formation of the HCV replication machinery has been shown to require interactions of viral NS5A/NS5B to host proteins including human vesicle-associated membrane protein-associated protein (VAP) subtypes A and B [15–17]. NS5A is a critical component of HCV replication [29], and is additionally involved in the modulation of cell signaling pathways, interferon response, pathogenesis, and apoptosis regulation [30]. Although NS5B functions as an RNA-dependent RNA polymerase, it appears also to engage in protein-protein interactions critical for forming the replication machinery. Indeed, VAPC, a splicing variant of VAPA/VAPB, was recently identified to block the HCV RNA replication as well as HCV propagation. In particular, VAPC expression has been detected in various tissues, but...
Figure 3. Binding between VAPC and NS5B. (a) Far-UV CD spectrum of HCV NS5B. (b) One-dimensional $^1$H NMR spectrum of HCV NS5B. Blue arrows are used to indicate very up-field NMR resonance peaks characteristic of a well-folded protein with tight tertiary packing. (c) Superimposition of $^{1}{H}^{15}{N}$ NMR HSQC spectra of VAPC in the absence of (blue) and in the presence of unlabeled NS5B (red) at a molar ratio of 1:1.5 (VAPC:NS5B). (d) Residue-specific changes of integrated $^1$H and $^{15}$N chemical shifts of VAPC in the presence of unlabeled NS5B at a molar ratio of 1:1.5 (VAPC:NS5B); doi:10.1371/journal.pone.0040341.g003

negligibly in the liver. As a consequence, VAPC has been proposed to be an endogenous inhibitor of HCV infection [31,32].

The human VAP family proteins were initially identified as homologs of vesicle-associated membrane protein (VAMP)-associated protein (VAP) with a size of 33 kDa in Aplysia californica, including VAPA, VAPB, and VAPC, and several newly-identified spliced variants [33–35]. VAPA and VAPB share ~60% sequence identity, and are composed of three conserved domains, i.e., an N-terminal immunoglobulin-like β sheet domain that has 22% sequence identity to the major sperm protein (MSP); a central coiled-coil domain; and a C-terminal transmembrane domain [36–38]. On the other hand, the 99-residue VAPC lacking the transmembrane segment possesses the N-terminal 70 residues completely identical to the 125-residue MSP domain of VAPB, and the C-terminal 29 residues unique in VAPC (Figure 1). VAP proteins are ubiquitously expressed type II integral membrane proteins that localize to the ER and pre-Golgi intermediates [39]. Moreover, VAP proteins target lipid-binding proteins carrying a short motif containing two phenylalanines in an acidic tract (FFAT motif) to the ER [36]. The FFAT-motif consists of the consensus amino acid sequence EFFDAXE, which is conserved in several lipid-binding protein families implicated in the transfer of lipids between the ER and other organelles, such as the Golgi, endosomes, and plasma membrane [40]. The VAP proteins also interact with intracellular proteins (including N1r, N1r2, and N1r3) via the FFAT motif which differentially affects the organization of the ER [41]. Recently, it was also shown that the VAPB-MSP domain also serves as a ligand for Eph receptors [37,38,42]. Strikingly, two point mutations (P56S and T46I) in the VAPB-MSP transformed into loop or helical-like conformations in the unfolded or partially folded proteins [46,47]. As judged from small but positive Cα conformational shifts over the majority of VAPC residues (Figure 2c), it appeared that loop/helical-like conformations were weakly populated over several segments of the sequence, even over the first 70 residues which are identical to the VAPB-MSP domain, and adopted β-sheet secondary structures as demonstrated by our previous crystallographic and NMR characterization [37]. More precisely, over the identical 70 residues, some residues adopting β-stands in the VAPB-MSP transformed into loop or helical-like conformations in VAPC (Figure 2c).

VAPC Forms a “Fuzzy Complex” with NS5B

Recently, it was discovered that by interacting with HCV NS5B, the human VAPC acts as an inhibitor of HCV replication. Therefore, in the present study, we cloned and expressed the HCV NS5B protein. As shown in Figure 3a, NS5B has a far-UV CD spectrum characteristic of a well-folded helix-dominant protein, with two negative signals at ~209 and 222 nm, as well as a very large positive signal at ~195 nm. However, due to the short transverse relaxation time (T2) resulting from its very large molecular size, the NMR resonance peaks are very broad (Figure 3b). Nevertheless, the manifestation of several highly up-field signals clearly demonstrate that NS5B is well-folded, with a tight tertiary packing in solution.

Subsequently, we titrated the $^{15}$N-labeled VAPC protein with the unlabeled NS5B. As shown in Figure 3c, addition of NS5B triggered the shifts of many HSQC peaks of VAPC, indicating that VAPC was indeed able to bind to NS5B. It is also noteworthy that the HSQC spectral dispersions of VAPC did not significantly increase even upon binding to NS5B, implying that VAPC remained largely flexible even in the complex. On the other hand, based on the sequential assignment, we were able to map the perturbation of the HSQC peaks to the VAPC sequence (Figure 3d). Interestingly, the most significantly-perturbed residues are located on the C-terminal half of the VAPC sequence, mostly composed of the residues unique to VAPC (Figure 3d).

Results

VAPC is Highly-unstructured

The recombinant VAPC protein was overexpressed as a His-tagged fusion protein in E. coli, and subsequently purified by Ni$^{2+}$-affinity chromatography under native conditions. Subsequently, VAPC was separated from the His-tag by on-gel cleavage with thrombin, and was further purified by HPLC on a reverse-phase (RP) C8 column. As shown in Figure 2a, VAPC has far-UV CD spectra typical of a highly-disordered protein with pH ranging from 6.5 to 3, with the maximal negative signal at ~198 nm, and lacking any positive signal at ~192 nm. However, there was a small negative signal at ~225 nm, implying that the helical conformation may be populated in VAPC to some degree. The $^{1}$H,$^{15}$N NMR HSQC spectrum (Figure 2b) also indicated that VAPC lacks any tight tertiary packing as evident from its very narrow $^{1}$H (~0.9 ppm) and $^{15}$N (~19 ppm) spectral dispersions. Consequently, it can be concluded that VAPC is predominantly disordered, lacking of well-formed secondary structures and tight tertiary packing.

By analyzing triple-resonance heteronuclear NMR spectra including HNCACB and CBCA(CO)NH acquired on a $^{15}$N–$^{13}$C-labeled VAPC sample at a protein concentration of 300 μM, we succeeded in achieving its sequential assignment and obtaining Cα conformational shifts of all residues except for several in the C-terminal portion whose HSQC peaks were not detected (Figure 2c). It is well-established that Cα chemical shift deviations from their corresponding random-coil values are very sensitive indicators of protein secondary structures, thus representing a powerful probe for detecting residual secondary structures in unfolded or partially folded proteins [46,47]. As judged from small but positive Cα conformational shifts over the majority of VAPC residues (Figure 2c), it appeared that loop/helical-like conformations were weakly populated over several segments of the sequence, even over the first 70 residues which are identical to the VAPB-MSP domain, and adopted β-sheet secondary structures as demonstrated by our previous crystallographic and NMR characterization [37]. More precisely, over the identical 70 residues, some residues adopting β-stands in the VAPB-MSP transformed into loop or helical-like conformations in VAPC (Figure 2c).
Conformation of VAPC and Its Binding to NS5B

(a) MAKEQVLSL EPOHELKFRG PFTDVVTNL KLNPTDRNV
    CFKVKTAPR RYCVRP57NSGI IDAGASINVSGRRWTADEED
    SAEQQPHFSL SPNWEGRRP99

(b) Ellipticity vs. Wavelength (nm)

(c) 

(d) Hα Conformational Shift (ppm)

(e) NSGI I DAGASINVSGRRWTADEEDSAEQQPHFSL SPNWEGRRP

\[ d_{NN}(i,i+1) \]
\[ d_{NN}(i,i+2) \]
\[ d_{NN}(i,i+3) \]
Conformational and Binding Properties of VAPC-43

As most significantly-perturbed residues triggered by adding NS5B are located on the C-terminal half of VAPC (Figure 3d), we then cloned into a GST-fused expression vector a truncated VAPC designated as VAPC-43 spanning residues 57–99 (Figure 4a). The recombinant VAPC-43 protein was over-expressed in E. coli, and subsequently purified by affinity chromatography with glutathione-Sepharose 4B beads. VAPC-43 was released from the GST-fusion protein by on-gel cleavage with thrombin, and was further purified by HPLC on a reverse-phase (RP) C18 column.

As shown in Figure 4b, the 43-residue VAPC-43 is also highly disordered in solution as evident from its far-UV CD spectra. Furthermore, the lack of tight tertiary packing in VAPC-43 is clearly evident from its narrow HSQC spectral dispersions (Figure 4c). To gain residue-specific insights into the solution conformation of VAPC-43, we collected a pair of three-dimensional heteronuclear NMR spectra, namely 15N-edited HSQC-TOCSY and HSQC-NOESY, and subsequently achieved its sequential assignment. As judged from the negative Hα conformational shifts for most residues (Figure 4d), VAPC-43 appears to have loop or helical-like conformations weakly-populated over four segments, i.e. the first centered at Ile61-Asp62; second at Asp77-Glu78, third at Pro86, and fourth at Glu95-Gly96, similar to the patterns observed for Cα conformational shifts in the context of the full-length VAPC (Figure 3d). Interestingly, the first helical segment is composed of residues identical in both VAPC and VAPB-MSP domain. The existence of the loop or helical-like segments was further supported by characteristic NOEs (Figure 4e) over the VAPC-43 sequence defining the helical conformation, including dNN(i, i+1), dNN(i, i+2), dNN(i, i+3), and dNN(i, i+4). The existence of the loop or helix over the VAPC-unique region is consistent with the prediction results of secondary structures (Figure 1d). However, since no dNN(i, i+4) NOEs were observed, the helical conformation in VAPC-43 appears to be mainly dynamic 310-helix/loop, rather than α-helix, consistent with the above CD result [56,57].

Although it is highly unstructured in solution, it is able to bind NS5B as demonstrated by HSQC titrations of 15N-labeled VAPC-43 with unlabeled NS5B (Figure 5a). However, even at a molar conformational shift...
The ratio of 1:2.5 (VAPC-43:NS5B) where the peak shifting of many residues were largely saturated (Figure 5b), the HSQC spectral dispersions still remained largely unchanged. This implies that like the full-length VAPC, VAPC-43 also remains largely flexible without any tight tertiary packing in the complex with NS5B. On the other hand, as judged from the chemical shift differences (Figure 5b), three discrete regions appears to be critical for binding with NS5B, i.e. the first one centered at Ile62, second over Arg73-Ala82, and third centered at Glu95. The first and third regions contain only ~2 residues while the second spans ~10 residues.

To explore the role of the second region in binding to NS5B, we thus mutated four residues (Arg73, Trp74, Glu78 and Asp80) into Ala. We successfully expressed and purified the recombinant mutant protein. The VAPC-43 mutant again appears to be highly unstructured without any well-formed secondary structures as judged from its far-UV spectrum (Figure 6a), and lacks any tight tertiary packing as demonstrated by the very narrow HSQC spectral dispersions (Figure 6b). On the other hand, the VAPC-43 mutant appears to undergo some dynamic aggregation, or/and conformational exchanges on µs to ms time-scale, and consequently the intensities of its HSQC peaks were not uniform, i.e. some were very strong but some very weak (Figure 6b). We also titrated 15N-labeled VAPC-43 mutant with unlabeled NS5B. Remarkably, even at a molar ratio of 1:2.5 (VAPC-43 mutant:NS5B), no significant shift of the HSQC peaks was observed (Figure 6b), clearing demonstrating that this second region is indeed critical for binding with NS5B.

Conformation and Binding Properties of VAPC-14

Subsequently we cloned, expressed and purified a 14-residue fragment designated as VAPC-14 containing residues Arg72-Gln85. As shown in Figure 7a, VAPC-14 is highly disordered in solution as evident from its far-UV CD spectra. Furthermore, the lack of any tight tertiary packing is also evident from its narrow HSQC spectral dispersions (Figure 7b). Nevertheless, VAPC-14 was able to bind NS5B as demonstrated by HSQC titrations of 15N-labeled VAPC-14 with unlabeled NS5B (Figures 7b, 7c). Again, even at a molar ratio of 1:2.5 (VAPC-14:NS5B), the HSQC spectral dispersions still remained largely unchanged (Figure 7b). This indicates that like the full-length VAPC and VAPC-43, even in the complex with NS5B, VAPC-14 remains largely flexible without any tight tertiary packing.

We attempted to perform the ITC measurements on the binding of NS5B to VAPC and its fragments, but failed to obtain high-quality data, probably due to the complex binding mode, and/or the fact that many VAPC residues still remain largely flexible even in the complex. Therefore, to quantitatively assess the binding between VAPC-43/VAPC-14 and NS5B, we fitted the shift tracings of the VAPC-43/VAPC-14 HSQC peaks to obtain dissociation constants (Kd) as we have previously conducted on other systems [48,49]. The fitting is exemplified in Figures 5c and d for VAPC-43 and 7d for VAPC-14, and the derived Kd values are summarized in Table 1. Strikingly, the Kd value of VAPC-14 is 49.13 µM, only ~3-fold larger than that for VAPC-43 (18.29 µM), suggesting that deletion of the first and third binding regions only resulted in a 3-fold reduction of the binding affinity to NS5B.

Discussion

The human VAPC is a 99-residue splicing variant of the 243-residue VAPB, with the N-terminal 70 residues being identical in
both proteins. As demonstrated in the crystal structure of the human VAPB-MSP domain that we previously determined [37], the 70 residues adopt β-strand or turn secondary structures (Figure 1). Prediction of the VAPC secondary structures by several computational programs, including GOR4 [50], Predator [51], and SHIMPA96 [52], also suggest that several fragments within the identical 70 residues possess high intrinsic propensity to form β-stranded conformation (Figure 1d). Strikingly, by use of CD and NMR spectroscopy, we provide here the first residue-specific evidence that VAPC is in fact highly unstructured in solution, lacking of any stable secondary structure and tight tertiary packing. More surprisingly, NMR parameters such as conformational shifts and NOE connectivity patterns reveal that unlike the MSP domain also characterized by NMR spectroscopy [37], in VAPC, no β-strand conformation is populated. Instead, loop or helical-like conformations are identified to be weakly populated over several regions of the VAPC sequence. This observation is consistent with the notion that the formation of β-sheet conformation requires the stabilization of complex long-range interaction networks, and thus is highly context-dependent [53,54]. Indeed, by NMR characterizations from our and other groups, the classic SH3 β-barrel fold adopted by >4,000 sequences has been previously unraveled to transform into helical conformations at certain solution conditions [55,56] or triggered by mutations [55,57]. In particular, we also demonstrated that one ALS-causing mutation (P56S) is sufficient to eliminate the β-barrel fold of the human VAPB-MSP domain, and to trigger the conversion into the highly-unstructured state only with weakly populated conformation [37]. Therefore, in VAPC, replacement of the remaining sequence of the 125-residue VAPB-MSP domain by 29 residues unique to VAPC is anticipated to disrupt the long-range interaction network critical for specifying or/and stabilizing the MSP fold. Consequently, VAPC would lose the ability to form β-stranded conformation, only with weakly-populated loop or helical conformations which are mostly stabilized by local interactions [53–57].

On the other hand, we have demonstrated that despite being highly disordered, VAPC is indeed active in binding to NS5B, with an average dissociation constant of ~20 μM by use of NMR spectroscopy. This renders VAPC as an intrinsically unstructured protein. Interestingly, since the first 70 residues assume a well-folded β-stranded conformation in the context of the MSP
and also represents a promising starting point to develop potent flexible (Figure 5b). The separate site is relatively low [68], and also many residues are not multi-binding site scenario for which the binding affinity of each the VAPC-NS5B complex is most likely to result from its discrete intrinsically unstructured proteins, and thus being designated as phenomenon is starting to be recognized to actually exist in a large could be obtained for the ApLLP-DNA interaction. Currently, this intriguingly remains largely unstructured even upon forming a transcriptional activator ApLLP for long-term memory forma-

Table 1. Residue-specific dissociation constants (Kd) for the binding of NS5B to VAPC-43 and VAPC-14.

| Residues | VAPC43 Kd (μM) ± SD | VAPC14 Kd (μM) ± SD |
|----------|---------------------|---------------------|
| I61      | 1.26 ± 19.53        |                     |
| R72      |                     | 161.32 ± 63.86      |
| R73      | 41.39 ± 19.53       |                     |
| W74      | 19.93 ± 5.37        |                     |
| D77      | 17.18 ± 5.76        |                     |
| E78      | 0.01 ± 0.07         |                     |
| E79      | 5.52 ± 2.06         | 1.41 ± 1.34         |
| D80      | 4.35 ± 2.15         | 68.86 ± 18.93       |
| A82      | 0.01 ± 0.22         | 27.34 ± 12.63       |
| E83      | 18.62 ± 3.32        |                     |
| Q85      | 0.11 ± 0.2          |                     |
| E95      | 20.27 ± 7.63        |                     |
| Average  | 18.29 ± 7.35        | 49.13 ± 17.64       |

Note: underlined Kd values have large errors, and thus were not included for calculating the average Kd values.

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NMR characterization allows the identification of VAPC residues critical for binding to NS5B. Interestingly, it appears that some discrete VAPC regions are involved in binding with NS5B. The first centered at Ile62 and the third centered at Glu95 are very short, while the second region is much longer, spanning Arg73-Ala82 (Figure 6b). Interestingly, only the first is located within the identical sequence of VAPC and VAPB, while the second and third are both within the VAPC unique sequence. The key role of the second region was further confirmed by NMR assignments. The concentration of all protein samples was checked by SDS-PAGE, and their molecular masses were verified by a Voyager STR matrix-assisted laser desorption ionization time-of-flight-mass spectrometer (Applied Biosystems).

The production of the isotope-labeled VAPC and its fragment or mutant proteins for NMR studies followed a similar procedure except that the bacteria were grown in M9 medium with the addition of [15N]glucose for 15N labeling and [13C]-glucose for 13C double labeling [37,38,48,49]. The identities of VAPC and its fragment or mutant proteins were further confirmed by NMR assignments. The concentration of protein samples was determined by the spectroscopic method in the presence of deuterium [37,38,48,49,72].

Circular Dichroism (CD) and NMR Experiments

All CD experiments were carried out in a Jasco J-810 spectropolarimeter (Jasco Corporation) at 25°C as previously described [37,38,48,49]. The protein concentration was 20 μM in 2 mM phosphate buffer (pH 6.5) for all far-UV CD experiments.

NMR samples were prepared in 10 mM phosphate buffer in the presence of 10 mM DTT (pH 6.5). All NMR data were collected at 25°C on an 800-MHz Bruker Avance spectrometer equipped with a shielded cryoprobe as described before [37,38,48,49]. For HSQC characterization, samples were prepared at a protein concentration of 100 μM. For sequential assignments of VAPC, triple-resonance NMR experiments including HNCA and CBCA/CO/NH were acquired on a double-labeled sample at a protein concentration of ~300 μM. For sequential assignments of peptide inhibitors for interfering with the VAPC-NS5B interface in the treatment of HCV infection by use of NMR-guided approaches [60–70].

Materials and Methods

Cloning, Expression and Purification

The VAPC gene was constructed by first amplifying the DNA fragment encoding the N-terminal 70 residues identical to VAPB from our previous MSP construct [37], followed by linking to the de novo synthesized DNA fragment for the 29 residues unique to VAPC [48]. The genes for truncated and mutated VAPC (including VAPC-43 and its mutant VAPC-14) were generated by PCR using different pairs of primers. The full-length VAPC was cloned into pET32a expression vector, while VAPC-43 and its mutant VAPC-14 were cloned in pGEX-4T-1. The NS5B gene was PCR-amplified from the pXJ40flag-NS5B plasmid, which contains NS5B of HCV genotype 1b [71], and then cloned into pET32a.

All the expression vectors were transformed into E. coli BL21 (DE3) Star cells (Invitrogen). For expression of recombinant proteins, cells were grown in Luria-Bertani (LB) medium in the presence of ampicillin (100 μg/ml) at 37°C to reach an absorbance of 0.6 at 600 nm, and subsequently induced with optimized IPTG concentrations. Harvested cells were resuspended and lysed by sonication in lysis buffer (50 mM Tris, 500 mM NaCl, 10% glycerol, 20 mM imidazole, 10 mM 2-mercaptoethanol, pH 7.5) containing protease inhibitor cocktail (Roche). His-tagged proteins were purified by Ni2+ affinity chromatography (Qiagen), while GST-fused proteins were purified by affinity chromatography with glutathione-Sepharose 4B beads (Pharmacia Biotech) under native conditions. The recombinant proteins were released from the fused tags by in-gel thrombin cleavage, and further purified either by FPLC on a Superdex 200 column for NS5B (Pharmacia Biotech), or by HPLC on RP (reverse phase) C8 and C18 columns (Vydey) for VAPC, VAPC-43 and its mutant VAPC-14, respectively. The purity of all protein samples was checked by SDS-PAGE, and their molecular masses were verified by a Voyager STR matrix-assisted laser desorption ionization time-of-flight-mass spectrometer (Applied Biosystems).

NMR view of VAPC-43, three-dimensional heteronuclear NMR experiments including HSQC-TOCSY and HSQC-NOESY were acquired on a 1H,15N-labeled sample at a protein concentration of ~500 μM. NMR data were processed with NMRpipe [73] and analyzed with NMRView [74].

NMR Characterization of Binding Interactions
For NMR HSQC characterization of the binding interactions of NS5B to VAPC fragments or mutant, two-dimensional 1H-15N HSQC spectra of the 15N-labeled VAPC proteins were acquired at a protein concentration of 100 μM in the absence or presence of the unlabeled NS5B at different molar ratios. By superimposing the HSQC spectra at different molar ratios, the shifted or disappeared HSQC peaks could be identified, and further assigned to the corresponding residues as previously described [37,38,48,49]. The degree of perturbation was reflected by an integrated chemical shift difference (CSD) calculated by the formula \( \Delta\delta = \left( \Delta\delta^1 + \Delta\delta^2 \right)/2 \) [48]. The CSD tracings were fitted by using the one binding site model [49] to obtain residue-specific dissociation constants (Kd) as summarized in Table 1.

Author Contributions
Conceived and designed the experiments: JS. Performed the experiments: SG. Analyzed the data: SG JS. Contributed reagents/materials/analysis tools: GG HQ MHU YJT VG. Wrote the paper: JS.

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