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West Nile Virus (WNV) protease and membrane interactions revealed by NMR spectroscopy

Shovanlal Gayen, Angela Shuyi Chen, Qiwei Huang, CongBao Kang *

*Experimental Therapeutics Centre, Agency for Science, Technology and Research (A*STAR), Singapore 138669, Singapore*

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ABSTRACT

West Nile Virus (WNV) protease is a two-component protease, important for the maturation of virus by cleaving the viral ploypeptide into functional proteins. WNV protease contains a Nonstructural (NS) protein 3 possessing the protease active sites and is regulated by a cofactor region containing approximately 40 amino acids from an integral membrane protein, NS2B. Although NS2B was demonstrated to be important for the location of the protease on the membrane, there was no direct evidence to show the interaction between protease (NS3) and membrane. Herein, we investigated the interaction between WNV protease and dodecylphosphocoline (DPC) micelles using NMR spectroscopy. The results showed that amino acids (31–33) from NS3 were important for the interaction with detergent micelles, which was similar to the finding in the study of protease from Dengue virus. This region may serve as an anchoring site to stabilized NS3 protease domain on the membrane.

1. Introduction

West Nile Virus (WNV) belongs to the family of Flaviviridae containing more than 70 members and is a mosquito-borne virus causing severe neurological disease and fatalities [1]. It has spread into many other countries since its introduction to New York in 1999 [1,2]. WNV affected many human beings and has killed many horses and birds [3]. No efficient clinical treatment or vaccine is currently available to prevent WNV infection. Like other Flavivirus, the genome of WNV encodes a polyprotein processed by host and viral proteases [3] to three structural proteins and seven nonstructural proteins including NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [4]. The NS3 is a potent drug target against WNV because of its important role for virus maturation by cleaving the junctions of NS2A/B, NS2B/3, NS3/4A and NS4B/5 [5,6].

Studies have demonstrated that the NS3 protein from dengue virus (DENV) and WNV contains multiple functional domains. The N-terminal approximately 180 residues (NS3pro) comprise the protease activity, while the C-terminus has helicase, nucleoside triphosphatase and RNA triphosphatase activities [1,7]. The protease of WNV or DENV is two-component protease because the NS3 protease is only active when it forms a heterodimer with NS2B- an integral membrane protein through approximately 40 residues from the hydrophilic domain between two potential transmembrane segments [1,8]. Structural studies of the NS3pro of WNV and DENV in the complex with its cofactor domain from NS2B (NS2B.G4SG4.NS3pro) have been investigated by X-ray crystallography and NMR spectroscopy [3,9,10]. It was demonstrated that the C-terminus of the cofactor domain of NS2B wraps around the NS3 pro to form a closed structure when an inhibitor is present, while in the absence of inhibitor, it localizes away from the active site to form an open conformation [10].

NS2B is an integral membrane protein and predicted to have three hydrophobic regions that are important for its localization on membrane [11]. NS2B and NS3 were demonstrated to localize with the convoluted membrane structure [11]. Although the structures of WNV protease have been studied using different methods, the interaction between NS3pro and membrane is not well understood. Previous surface plasmon resonance (SPR) assay on the NS3pro from DENV confirmed that the loop containing G29 to G32 was important for membrane association [12]. The loop was conserved in flavivirus family and may be important for affecting the accessibility of substrate by anchoring protease domain on the membrane surface. Due to the sequence and structural homology of proteins between DENV and WNV, this loop in WNV protease may also involve in interaction with membrane. In a functional study, it was demonstrated that different detergents have different effects on WNV protease activity [13]. To test the possible interaction between WNV protease and membrane, we conducted NMR study to identify residues of WNV protease that may be important for the molecular interaction with detergent micelles. In this study, we used dodecylphosphocoline (DPC) micelles that were commonly used as a membrane mimic. The region containing residues

* Corresponding author. Address: Experimental Therapeutics Centre, Agency for Science, Technology and Research (A*STAR), 31 Biopolis Way Nanos #03-01, Singapore 138669, Singapore. Fax: +65 64788768.
E-mail address: cbkang@etc.a-star.edu.sg (C. Kang).

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L31 to S33 of NS3 was shown to interact with DPC micelles, indicating that this region is the membrane anchoring site. Our results provided an evidence for the protease and membrane interactions, which will be important for understanding the NS3 functions and its interaction with substrates or inhibitors.

2. Materials and methods

2.1. Protein expression and purification

The WNV protease containing 47 residues from NS2B and 184 residues from NS3 linked through a G4SG4 linker was cloned into pET21d vector. The resulting plasmid was chemically transformed into *E. coli* BL21 (DE3) codon plus RILP competent cells. Two to three colonies were incubated in 20 ml of M9 medium with 30 μg/ml kanamycin. The overnight culture was further transferred into 1 L of M9 medium. Protein was induced for 3 h at 25 °C by adding IPTG (β-D-1-thiogalactopyranoside) to 1 mM final concentration when OD_{600} reached 0.8. *E. coli* cells were harvested by centrifugation at 8,000*g* for 10 min at 4 °C. The cell pellet was resuspended in a lysis buffer containing 20 mM Tris–HCl, pH 7.8, 300 mM NaCl, 2 mM -mercaptoethanol. Cells were broken by sonication in an ice bath and cell lysate was cleared by centrifugation at 40,000*g* for 20 min. The supernatant was passed though a gravity column containing Ni^{2+}-NTA resin. Resin with protein was washed with at least 10 resin volumes of washing buffer containing 20 mM Tris, pH 7.8, 1 M NaCl, 20 mM imidazole and 2 mM -mercaptoethanol to remove unspecific proteins binding to the resin. Protein was eluted with elution buffer containing 500 mM imidazole, pH 6.5, 300 mM NaCl and 2 mM -mercaptoethanol. Purified protein was changed to a buffer containing 20 mM Tris–HCl, pH 7.8, 10 mM NaCl, 1 mM DTT (dithiothreitol) using a PD10 column. Protein was further purified using an ion-exchange column. Protein was concentrated to 0.2–0.5 mM in an NMR buffer containing 20 mM HEPES, pH 7.1, 1 mM DTT and 10% D_2O.

2.2. Protein concentration determination

The protein concentration was determined by measuring the absorbance at 280 nm (A_{280}) on a NanoDrop Spectrophotometer. The extinction coefficient was estimated to be 49000 M^{-1} cm^{-1} corresponding to 1.83 mg ml^{-1} cm^{-1} using the protein calculator server (http://www.scripps.edu/~cdputnam/protcalc.html). The spectrophotometer was first referenced with water and buffer, and then 2 μl of sample was used for absorbance measurement. The protein concentration was determined with the above calculated extinction coefficient.

2.3. NMR spectroscopy

Uniformly ^{15}N- or ^{13}C, ^{15}N-labeled protein samples were concentrated. Backbone assignment was conducted using 3D-HNCA, CBCACOHN experiments. Triple labeled (^{15}N, $^{13}C/^{2}H$) with 0.8–1.0 mM concentration in the NMR buffer was used for data acquisition. The $^{1}H^{N}$, ^{15}N assignment for WNV protease was obtained by transferring partial published assignment and confirmed using standard triple resonance NMR experiments. All the NMR experiments were performed at 25 °C on a Bruker 700 MHz spectrometer equipped with a cryogenic probehead. The NMR data were processed with NMRPipe [14] and Sparky (http://www.cgl.ucsf.edu/home/sparky/). The transverse R_2 rate and $\{^{1}H\}$-^{15}N steady state NOE were performed as previously described [15].

2.4. Titration experiment with DPC detergent

For the titration experiments, ^{15}N-labeled WNV protease with a concentration of 0.2 mM was prepared in the NMR buffer. Aliquots of a 280 mM DPC in NMR buffer were added to protease solution. 2D ^{1}H-^{15}N-HSQC spectra were collected at each DPC concentration. The ^{15}N chemical shift changes were measured. The dissociation constant (K_D) between protease and DPC was determined using the following equation [16]. $\delta = \delta_{max}*([M_0] - cmc)/(K_D*n + [M_0] - cmc)$. Where, δ is the chemical shift difference calculated from the following equation $\Delta = ((0.154*\Delta(^{15}N)^2 + \Delta(^{1}H)^2)^{0.5}$. $\Delta(^{15}N)$ and $\Delta(^{1}H)$ are chemical shift difference between the backbone amide nitrogen and amide proton resonances in the absence and presence of a given DPC concentration. $[M_0]$ is the detergent concentration in the sample, cmc is the critical micelle concentration and n is the aggregation number of DPC. We used value suggested by manufacture and cmc was 1.5 mM and n was 54, individually. The fractional saturation value (Y) as a function of ligand concentration was plotted. The K_D is the value of ligand concentration that gives Y = 0.5.

2.5. Model of protease and membrane interactions

The model of WNV protease on the membrane was made in PyMol (www.pymol.org) using X-ray structure of the protease and inhibitor complex (PDBID: 3e90) and predicted topology of full length NS2B. The helical structures of potential transmembrane domains of NS2B were generated in PyMol to indicate its possible structure without any simulation and energy minimization. The position of NS3 protease domain on the membrane was docked manually to membrane surface.

3. Results

3.1. Assignments for protease

The three-dimensional (3D) structures of WNV protease in the presence and absence of inhibitors were studied using both X-ray crystallography and NMR spectroscopy. From previous NMR studies, the assignment of the free protease is challenging due to missing of cross peaks arisen from the intermediate exchanges [10,17]. The complete backbone assignment of WNV protease in the presence of an inhibitor was achieved, while only partial assignment for free WNV protease was obtained [10,17]. In this study, we are investigating the interaction between protease and membrane. Partial assignment of the HSQC spectrum will still be useful to understand their interactions. We assigned the HSQC spectrum of free WNV protease based upon published data [10,17] as well as from our 3D HNCA and CBCACONH experiments (Fig. 1A). Although we obtained more than 56% assignment for WNV protease due to the signal overlap and unfavorable protease dynamics, the obtained set of assignments would still be informative for the definition of the binding sites [10,17].

3.2. WNV protease interacts with DPC micelles

To elucidate the interaction between WNV protease and membrane, we added different amounts of DPC micelles to the protease (Fig. 1). DPC micelles were widely used as a model membrane because the phosphocholine head groups were expected to simulate a membrane interface [16,18]. The assignment of the free protease was used as a starting point. The position of the cross peaks in the HSQC spectrum was traced when DPC was added gradually. Using this way, the HSQC assignment could be traced up to a concentration of 60 mM because higher DPC concentration will cause line-

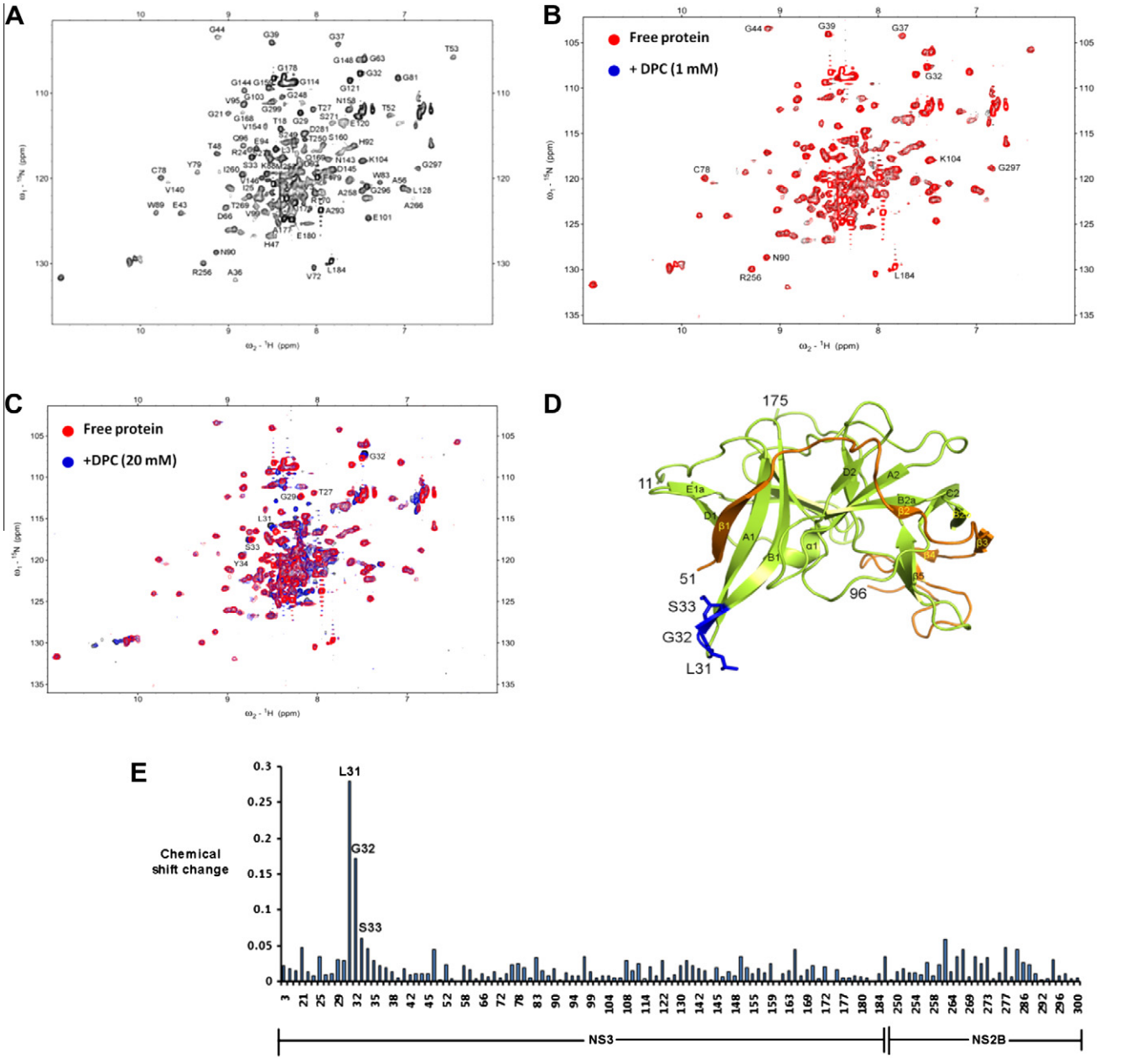

Fig. 1. Interactions of WNV protease and the DPC micelles. (A) Assignment of the ^{1}H-^{15}N-HSQC of WNV protease. (B) Superimposed ^{1}H-^{15}N-HSQC spectra in the presence (blue) and absence (red) of 1.0 mM of DPC micelles. (C) Superimposed ^{1}H-^{15}N-HSQC spectra in the presence (blue) and absence (red) of 20.0 mM of DPC micelles. (D) Residues affected by DPC titration. The structure of WNV protease (PDBID: 3e90) was shown in cartoon representation. NSpro3 is shown in green and NS2B is shown in brown. Residues showed significant chemical shift perturbations are shown in stick mode and in blue, while residues with moderate chemical shift changes are highlighted in yellow. (E) Chemical shift changes for WNV protease in the presence and absence of DPC micelles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

broadening of the cross peaks. The interaction was monitored by comparing the chemical shift changes in the HSQC spectra caused by addition of increasing concentration of DPC. There was no clear interaction between protease and detergent when the DPC concentration was below its cmc that is 1.5 mM (Fig. 1B). Chemical shift perturbation of backbone amino proton and nitrogen was observed when the DPC concentration was higher than cmc, indicating that protease interacts with micelles (Fig. 1C).

3.3. Identification of DPC binding sites

By comparing the HSQC spectra of WNV protease in the absence and presence of DPC micelles, residues showed chemical shift variations were mapped to the 3D structure of WNV protease solved by X-ray crystallography (Fig. 1D and E). The binding site was mainly mapped to a loop region between β sheets A1 and B1 containing L31 to S33. These three residues showed DPC concentration dependent chemical shift changes (Fig. 1C). The assignment of L30 was not followed due to resonance overlap, while this residue was also predicted to be interacting with micelle because of its hydrophobic side chain. The G29 showed no chemical shift perturbation, indicating it was not interacting with micelles (Fig. 1C). One indole side chain of a Trp without assignment also showed moderate chemical shift change, indicating an interaction with micelles. Overall, the largest chemical shift changes between free and DPC bound protease was identified in the loop region, demonstrating that this is the possible binding site to membrane.

3.4. Binding affinity between protease and DPC micelles

To obtain the dissociation constant (Kd) between protease and micelles, titration of different amounts of DPC to ^{15}N-labeled protease solution was analyzed with focus on these three residues (L31 to S33) (Fig. 2A–C) which are conserved among DENV and WNV (Fig 3A and B). DPC dependent chemical shift changes of the amide proton and nitrogen of the HSQC spectra were shown in Fig. 2. These three residues showed similar binding affinity to DPC micelles (Fig. 2E and F) with an average dissociation constant (Kd) of ~0.30 mM. Relaxation study showed that in the presence of DPC micelles, ^{15}N transverse relaxation rates and {^{1}H}-^{15}N-NOE values for residues (L31-S33) were increased, which may arise from the interaction with DPC micelles (Fig. 3C). The presence of DPC micelles affected the flexibility of this loop due to their direct interaction (Fig. 3D).

3.5. Interaction between WNV protease and CHAPS

High throughput screening (HTS) is a frequently used approach in identification of lead inhibitors [13]. To avoid false positives due to undesirable properties of compounds libraries such as aggregation, detergents are usually added in the assay buffer to eliminate the false positive hits. The *in vitro* assay for WNV protease has been set up using different substrates in screening campaigns against WNV and DENV proteases [13]. Study have shown that some non-ionic detergents such as Triton X-100 could increase protease activity while Detergent CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) was a suitable choice for HTS [13]. We then tested if CHAPS used in the assay buffer interacted with protease. Unlike DPC micelles, CHAPS interacted with protease even at a concentration below cmc (~8 mM) (Fig. 4A). Chemical shift perturbations were observed for some residues such as C78 and G121 (Fig. 4A and B). In addition, residues such as T52 and T53 that are close the protease active site were also affected by addition of CHAPS. This result indicated that the detergent-induced enhancement of protease activity was not through stabilizing the orientation of protease from the detergent because residues L31 to S33 showed no interaction with CHAPS (Fig. 4A).

4. Discussion

The NS2B of WNV or DNV is an integral part of the viral membrane protease, playing the role as a cofactor of NS3 [19]. The hydrophilic region between two potential transmembrane domains was shown to be crucial for the stability and activity of NS3pro [11]. NS3 and NS2B were shown to co-localize on cell membrane. In the DENV protease, mutation analysis and SPR showed there was a membrane binding motif that is the loop between two β sheets (A1 and B1) of NS3pro [12]. It was proposed that this membrane-interacting loop has a similar role to the N-terminal amphipathic helix of the hepatitis C viral protease (NS3-NS4A) [12]. Our results from titration study revealed that residues L31-S33 from the loop of WNV NS3pro were also involved in membrane interaction. The only difference was that G29 was not involved in the binding to DPC micelles (Fig. 3). Although several cross peaks showed modest chemical shift perturbations and some new peaks appeared after DPC was added (Fig. 1), this loop was still the major binding region. In the presence of DPC micelles, tumbling time of some residues might be changed, which made their cross peaks observed in the HSQC spectrum. We also demonstrated that adding DPC micelles did not denature the protease structure, which was also confirmed by a near-UV circular dichroism (CD) spectroscopy (supplementary Fig. 1).

From the structural study on WNV protease, the membrane-binding region is close to the N-terminal part of NS2B co-factor region. A model of full-length NS2B and NS3 on the ER membrane was generated using the crystal structure of protease and peptide

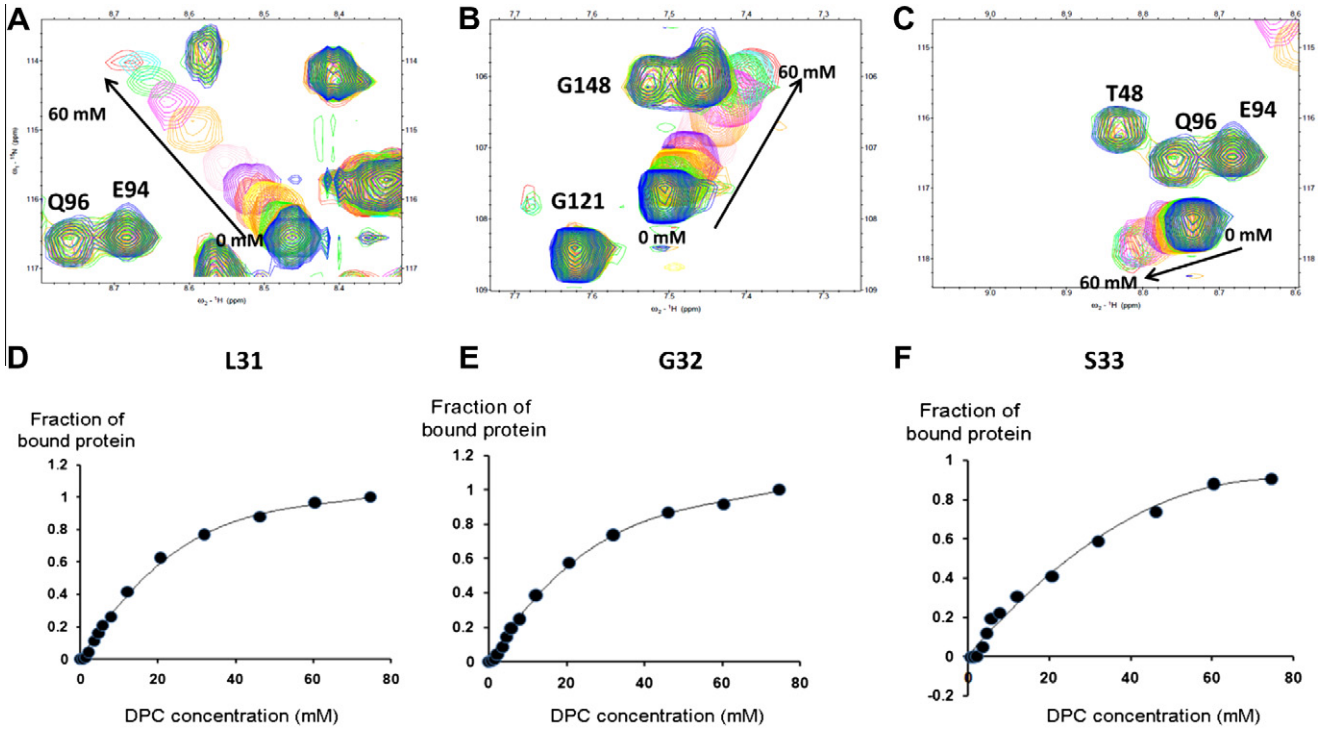

Fig. 2. Dependence of chemical shift changes on DPC concentration. The enlarged regions of superimposed ^{1}H-^{15}N-HSQC spectra in the presence of DPC micelles for residues L31 (A), G32 (B) and S33 (C) are shown. The differences between the chemical shifts in the absence of DPC micelles against DPC concentration are plotted for of residues L31 (D), G32 (E) and S33 (F). The DPC concentration is the total detergent concentration. The micelle concentration was determined as described in Materials and Methods.

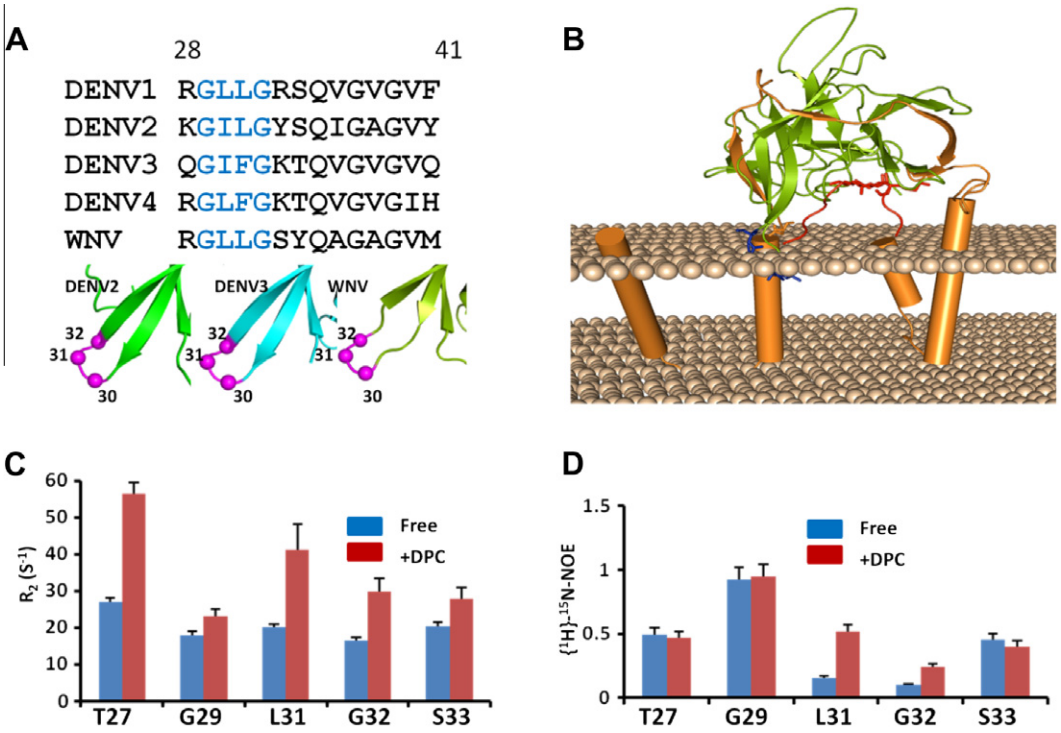

Fig. 3. Model of membrane and NS3pro interaction. (A) Sequence alignment for the potential membrane binding region of DENV (1-4) and WNV. The sequence was from previous publication [23]. The coordinate for the structures are from PDB with accession numbers of 2fom, 3uli and 3e90. (B) Model of NS2B.NS3pro of WNV on the ER membrane. The NS3pro is in green and cofactor domain of NS2B is shown in brown. This was from the structure of WNV-inhibitor complex (PDBID: 3e90). The L31-S33 region is shown in stick representation and in blue. The region shown in red is the modeled substrate. The three possible transmembrane domains of NS2B are shown as cylinder helix mode. (C) The R_2 of WNV protease in the presence (red) and absence (blue) of DPC micelles (30 mM). (D) The {^{1}H}-^{15}N-NOE of WNV protease in the presence (red) and absence (blue) of DPC micelles (30 mM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

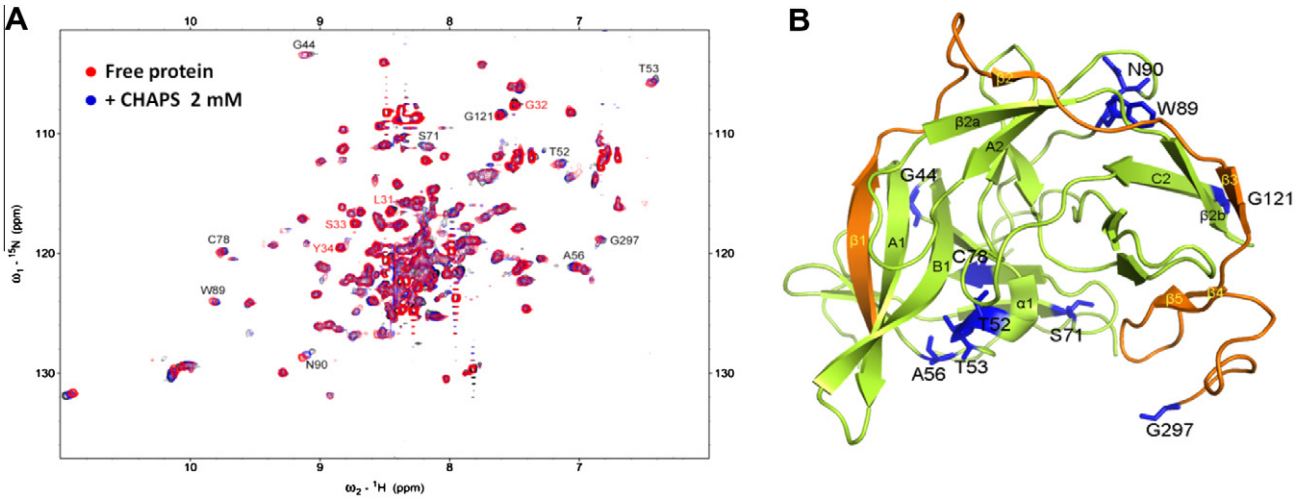

Fig. 4. WNV protease and CHAPS micelles interactions. (A) Superimposed ^{1}H-^{15}N-HSQC spectra in the presence (blue) and absence of 1.0 mM of CHAPS. The residues interacting with DPC micelles are shown in red. B. Residues affected by CHAPS titration. The structure of WNV protease (PDBID: 3e90) was shown in cartoon representation. NS3pro is shown in green and NS2B is shown in brown. Residues showed chemical shift perturbations are shown in stick mode and in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

inhibitor complex and shown in Fig. 3B. This membrane-binding loop identified by our titration experiment may be functional as a site anchoring on the membrane, which stabilizes the complex on the membrane. We found, from titration experiments, that the active site has no interaction with membrane (Fig. 1). A peptide inhibitor showed similar interaction with WNV protein to that in the presence of DPC micelles (Supplementary Fig. 2), indicating that protease-membrane binding did not affect ligand binding. The interaction between protease and membrane exposed the protease active sites to the solvent and close to the membrane, facilitating substrate cleavage (Fig. 3). NS3 contains several functional domains. Studies demonstrated that there is a flexible linker between protease domain (NS3pro) and the helicase domain. It was also shown that there were reorientations between protease and helicase domains from X-ray studies [12,20]. From our study, NS3pro interacted and may be subsequently stabilized by the

membrane. We postulate that this helicase domain reorientation may provide a structural basis for the helicase to have different regulatory roles for viral replication, which was observed in DENV NS3 protein. Indeed, study has shown that NS3 helicase domain with different orientation possessed different activity such as ATP binding [12,20].

Efforts have been made to develop potent DENV or WNV protease inhibitors, as there is currently no drug available so far [21,22]. Different approaches such as HTS or *in silico* based drug design have been used [21], the inhibitors have IC_{50}s in nanomolar range have still not been used in therapy. A possible reason may be that the construct used for the assay does not contain the transmembrane domains of NS2B. The molecular interaction between protease and membrane may affect the efficacy of the inhibitors designed. Our result showed that NS3pro had a membrane interacting site, which may serve to restrict the orientation of the protease on the ER membrane. How the cell membrane affects the interaction between enzyme containing full-length NS2B and NS3 and an inhibitor should also be investigated in the future.

In summary, we observed direct interaction between WNV protease and DPC micelles. A loop region between βA1 and βB1 from NS3pro that is conserved among flavivirus family was shown to be important for the interaction with micelles. This region may exist as a membrane-binding site, which makes protease has rigid structure on the cell membrane. In addition, we demonstrated that detergent CHAPS used in the enzyme assay buffer does not interact with the loop region. Our results indicate that membrane may affect the function of NS3, which will be important for drug discovery against WNV and DENV.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.043.

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