Dengue protease activity: the structural integrity and interaction of NS2B with NS3 protease and its potential as a drug target

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Synopsis
Flaviviral NS3 serine proteases require the NS2B cofactor region (cNS2B) to be active. Recent crystal structures of WNv (West Nile virus) protease in complex with inhibitors revealed that cNS2B participates in the formation of the protease active site. No crystal structures of ternary complexes are currently available for DENv (dengue virus) to validate the role of cNS2B in active site formation. In the present study, a GST (glutathione transferase) fusion protein of DENv-2 cNS2B49–95 was used as a bait to pull down DENv-2 protease domain (NS3pro). The affinity of NS3pro for cNS2B was strong (equilibrium-binding constant \(< 200 \text{ nM}\)) and the heterodimeric complex displayed a catalytic efficiency similar to that of single-chain DENv-2 cNS2B/NS3pro. Various truncations and mutations in the cNS2B sequence showed that conformational integrity of the entire 47 amino acids is critical for protease activity. Furthermore, DENv-2 NS3 protease can be pulled down and transactivated by cNS2B cofactors from DENv-1, -3, -4 and WNv, suggesting that mechanisms for activation are conserved across the flavivirus genus. To validate NS2B as a potential target in allosteric inhibitor development, a cNS2B-specific human monoclonal antibody (3F10) was utilized. 3F10 disrupted the interaction between cNS2B and NS3 in vitro and reduced DENv viral replication in HEK (human embryonic kidney)-293 cells. This provides proof-of-concept for developing assays to find inhibitors that block the interaction between NS2B and NS3 during viral translation.

Key words: allosteric inhibitor, dengue, flavivirus, NS2B, NS3, protease

INTRODUCTION

DENv (dengue virus) belongs to the family of Flaviviridae and is the most prevalent arthropod transmitted virus in humans. It causes a range of diseases from self-limiting dengue fever to the sometimes-fatal DHF (dengue hemorrhagic fever) and DSS (dengue shock syndrome) [1,2]. There are four closely related, but antigenically distinct, viral serotypes (DENv-1–4) that are grouped together with other human disease causing viruses such as WNv (West Nile virus), yellow fever virus and Japanese encephalitis virus within the genus flavivirus [1,2].

As with other members of the Flaviviridae family, the genomes of DENVs consist of a positive single-stranded RNA of approx. 10 700 bases in length [3]. Co- and post-translational processing of the polyprotein gives rise to three structural proteins and seven non-structural proteins [3]. Correct processing of these proteins is essential for virus replication and requires host proteases such as signalase and furin [4] and a two-component viral protease NS2B/NS3 [3]. The N-terminal 172 amino acids of DENv NS3 contains a trypsin-like protease domain [5] and the activity of this domain is dependent on at least 40 amino acids of NS2B [5,6]. NS2B/NS3 cleaves at the junctions between NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4B/NS5 [5,7,8], within NS2A [9], NS3 [8,10,11], NS4A [12,13] and also at the junction of C-prM [14–16].

NS3 protease in the absence of NS2B is inactive and is only able to poorly hydrolyse the small chromogenic substrate N-α-Bz-1-Arg p-nitroanilide (Bz is benzoyl) [17]. Substrate profiling studies with single-chain NS3 protease containing the NS2B...
cofactor region (cNS2B) suggest the substrate-binding pocket of NS3 protease is extended by NS2B [18], a hypothesis supported by recent crystallographic and NMR studies (Figures 1B and 1C) [19–22]. Crystal structures of WNV single-chain cNS2B-NS3pro in the substrate-free form demonstrate that the N-terminal region of cNS2B stabilizes NS3pro, while its C-terminal sequence is highly flexible [19,20]. In the presence of inhibitors, the C-terminal region becomes ordered in the WNV structure and contributes directly to the catalytic pocket of the protease [19–22]. Crystal structures for DENV single-chain cNS2B-NS3 have been obtained for the substrate-free form and similarly demonstrate a role for the N-terminal region in stabilization and a highly flexible C-terminal region [19,23]. However, despite much effort, there are currently no co-complexed DENV protease structures available to ascertain whether the dual role of WNV NS2B in protease activation is also relevant for dengue.

Dengue NS3pro by itself is insoluble [17], while fusion to cNS2B gives rise to a highly soluble protein [18,24]. Furthermore, refolded DENV NS3 protease can bind and be trans-activated \textit{in vitro} by a GST (glutathione transferase)–cNS2B fusion protein [25]. In the absence of a co-complexed DENV cNS2B-NS3 protease structure, we employed this strategy to decipher DENV cNS2B residues that are important for protease binding from those required for protease activation. Mutation and truncation analyses of cNS2B confirmed that the C-terminal region of cNS2B is responsible for protease activation and a loss of structural mobility in cNS2B abolishes activity. Transactivation studies with DENV-2 NS3 protease and cNS2B from other members of the flavivirus genus (DENV-1, -3 and -4 and WNV) suggest that cNS2B activation mechanisms are conserved across the genus. Finally, we examined the effect of blocking the NS2B-NS3 interaction on DENV replication with a cNS2B-specific antibody. Together, our findings strongly support observations from co-complexed WNV NS2B-NS3 protease structures [19–21] that flavivirus NS2B cofactor is needed to stabilize DENV NS3 protease as well as to form part of the catalytic site. These findings provide important insights for the development of flavivirus protease inhibitors.
MATERIALS AND METHODS

Materials
Fluorogenic peptide substrate Bz-Nle-Lys-Arg-Arg-AMC (Nle is norleucine and AMC is 7-amino-4-methylcoumarin) was purchased from LSU Health Sciences Center, New Orleans, LA, U.S.A., and Bz-L-Arg-AMC was from Fisher. PCR was carried out using Turbo Pfu polymerase from Stratagene. Restriction enzymes and modifying enzymes were purchased from New England Biolabs. Oligonucleotides were synthesized from Research Biolabs. Rabbit polyclonal anti-NS3 antibodies and mouse monoclonal anti-NS3 antibodies were a gift from Dr Prida Malasit, Siriraj Hospital, Bangkok, Thailand. Anti-His monoclonal antibodies were purchased from Qiagen.

Preparation of expression constructs
The DENV-2 TSV01 NS3pro (amino acids 1–185) construct was obtained by PCR using the primer pair NS3pro185XhoI-F and NS3pro185BamHI-R (Supplementary Table S1 at http://www.bioscirep.org/bsr/031/bsr0310399add.htm). The plasmid DENV-2 pET15b-CF40-gly-NS3pro185 [18] was used as a template. PCR conditions were: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min and 72°C for 5 min. The PCR product was digested with XhoI and BamHI, and ligated into the same sites in pET15b. DENV-2 pET15b-CF40-gly-NS3pro185 [18] was used as a template for the forward primer, NS2BcfXho-F [18] and the reverse primer NS2BdelC7-REV , NS2BdelC7-REV or NS2BdelC7-REV (Supplementary Table S1) respectively. To obtain the sequence of NS3pro185 [NS3 protease domain (amino acids 1–185 in NS3 protease; amino acids 1476–1660 in DENV polyprotein)] with glycine linker, PCR was performed with the primer pair NS3pro185LK-F and NS3pro185BamHI-R using DENV-2 pET15b-CF40-gly-NS3pro185 [18] as a template. Each of the truncated cNS2B sequences and NS3pro were joined by overlap PCR with primers NS2BcfXho-F and NS3pro185BamHI-R to generate CF40(ΔC7)-gly-NS3pro185, CF40(ΔC9)-gly-NS3pro185 and CF40(ΔC11)-gly-NS3pro185.

To prepare the GST–cNS2B expression constructs cNS2B PCR fragments from DENV-1–4 and WNV were amplified with the following primer pairs: DV1FOR and REV, DV2(NGC)FOR and REV, DV2(TSV01)FOR and REV, DV3FOR and REV, DV4FOR and REV, WNVFOR and REV (Supplementary Table S1). Their respective PCR templates were: DENV-1–4 pET15b-CF40-gly-NS3pro185 [18], DENV-2 NGC FL genome pDVWS601 (a gift from Dr Andrew Davidson, Cellular and Molecular Medicine, University of Bristol, Bristol, U.K.) and WNV pET15b-CF40-gly-NS3pro185 [19]. PCR conditions were as described above. The PCR fragments were digested with BamHI and EcoRI and cloned into the same sites in pGEX-4T-1.

Plasmid DENV-2 TSV01 pGEX-4T-1-cNS2B was used as a template for preparation of the truncated and mutagenised GST–cNS2B constructs. To generate pGEX-4T-1-cNS2B(C29), the primer pairs CF29-FOR and -REV were used. Alanine or proline substitutions were introduced into the cNS2B sequence with the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The following primer pairs were used for the site-directed mutagenesis: W61A-FOR and REV, G69P-FOR and REV, D81A-FOR and REV, G82P-FOR and REV, I86A-FOR and REV, K87A-FOR and REV (Supplementary Table S1). In addition, a K74A/I78A double mutant was generated using overlapping PCR. In the first round of PCR, two separate reactions were performed with the following primer pairs: cNS2BFOR and NS2BDMREV and NS2BDMFOR and cNS2BREV. The two products were joined in the second round of PCR using the primers cNS2BFOR and cNS2BREV. The resulting PCR product was digested with BamHI and EcoRI and cloned into the same sites in pGEX-4T-1.

Expression and purification of protein constructs
Expression and purification of DENV-2 CF40-gly-NS3pro185 have been previously described [18]. For refolding and purification of DENV-2 NS3pro, competent Escherichia coli BL21-CodonPlus-(DE3) were transformed with pET15b-DEN2-TSV01 NS3pro185 and grown in LB (Luria–Bertani) broth as described [18]. Cell pellets were resuspended with binding buffer (6 M guanidine hydrochloride, 20 mM Tris/HCl, pH 8.5, 0.5 M NaCl and 5 mM imidazole) and solubilized by stirring for 60 min at 4°C. The suspension was clarified by centrifugation and on-column refolding was performed using published protocols [26]. Fractions containing NS3pro were further purified by gel filtration (HiLoad 26/60 Superdex 75, Amersham Biosciences). Compositions of the buffers used in refolding are shown in Supplementary Table S2 (http://www.bioscirep.org/bsr/031/bsr0310399add.htm).

For pGEX-4T-1-cNS2B constructs, transformed E. coli BL21-CodonPlus(DE3)-RIL cells were grown in LB broth at 37°C until a D₅₅₀ of 0.5, was reached. Protein expression was induced with 0.4 mM IPTG (isopropyl β-D-thiogalactoside) at 16°C for 20 h. Cell pellets were re-suspended in 4 ml of PBT (PBS containing 1% Triton X-100) and lysed by sonication. Clarified cell lysate was incubated with 200 μl of 50% slurry of glutathione–Sepharose 4B (Amersham Biosciences) for 2 h at 4°C. Beads were extensively washed with PBT and finally stored in binding buffer (50 mM Tris/HCl, pH 8.5, 2.5 mM CHAPS and 20% glycerol) at −80°C.

Pull-down assay for NS3 protease
Glutathione–Sepharose 4B beads (20 μl of a 50% slurry; Amersham Bioscience) bound with 160 μg of either GST–cNS2B or GST only, were incubated with 25 μg of refolded NS3pro at room temperature (25°C) for 1.5 h in 2 ml of binding buffer (50 mM Tris/HCl, pH 8.5, 2.5 mM CHAPS and 20% glycerol). Beads were washed three times with 1 ml of binding buffer and the GST–cNS2B/NS3pro complex was eluted from the beads with two 40 μl incubations with elution buffer (10 mM reduced glutathione, 50 mM Tris/HCl, pH 8.5, 1 mM CHAPS and 20% glycerol) for 10 min at 25°C. Pull-down complexes were then resolved by SDS-PAGE and western blotted with anti-NS3 antibodies.
glycerol) for 1 h at 4°C. Eluted samples were buffer-exchanged to protease assay buffer (50 mM Tris/HCl, pH 8.5, 1 mM CHAPS and 20% glycerol) with Zeba Desalt Spin Columns (Pierce) and 49 μl was used directly for in vitro protease assays. To determine the amount of NS3pro pulled-down from each binding experiment, 5–8 μl of the eluted complex was separated by SDS/PAGE (12% gel), and Western blotted with anti-His antibodies. The intensity of the NS3pro bands obtained was measured using ImageQuant TL v2003.02 software (Amersham Biosciences). Band intensities from 0 to 40 ng of refolded NS3pro were similarly obtained, plotted as a standard graph and used to quantify the amount of pulled-down NS3pro from each experiment. To normalize the amount of GST–cNS2B bound on the beads in each experiment, 0.5–1 μl of the eluted complex was separated by SDS/12% PAGE and stained with Coomassie Blue. All pull-down experiments were performed at least thrice.

Affinity measurements of NS3 protease for cNS2B
GST–cNS2B or GST alone (32 pmol) was bound to glutathione–Sepharose 4B beads and incubated with 0–500 nM refolded NS3pro at room temperature for 1.5 h in 1.5 ml of binding buffer. Beads were washed three times with 1 ml of binding buffer, after which 25 μl of SDS/PAGE gel-loading buffer was added to the beads and 5 or 10 μl of the protein samples were separated by SDS/PAGE (12% gel), and Western blotted with anti-NS3 antibodies to determine the amount of NS3pro pulled down from each binding experiment. The intensity of the NS3pro bands obtained was quantified with ImageQuant TL v2003.02 software and compared with a standard curve as above. To normalize the amount of GST–cNS2B bound on the beads in each experiment, the membranes were stripped with 1 × Re-Blot Plus Strong Solution (Chemicon International) and re-probed with anti-GST antibodies. In experiments where the anti-NS2B antibody fragment 3F10 [27] was utilized, an equivocal amount of this was added to beads previously bound with GST–cNS2B. Refolded NS3pro was added to the beads in 1.5 ml of binding buffer following the 3F10 incubation in a sequential fashion. All affinity pull-down experiments were performed at least twice.

Protease assays
The assay with the simple fluorogenic substrate Bz-L-Arg-AMC was performed as previously described [17] except that the activity of refolded NS3pro was measured in 50 μl of 50 mM Tris/HCl, pH 8.5, 1 mM CHAPS, 20% glycerol, 300 mM NaCl and 4 mM of Bz-L-Arg-AMC. Reaction mixtures contained 500 nM of refolded NS3pro or 3 μM CF40-gly-NS3pro185 as control.

Assays with the tetrapeptide substrate Bz-Nle-Lys-Arg-AMC have been previously described [28]. Briefly, 50 μl reaction mixtures containing 50 mM Tris/HCl, pH 8.5, 1 mM CHAPS, 20% glycerol and 50 μM Bz-Nle-Lys-Arg-AMC were incubated at 37°C. Activity was measured in a Victor® plate reader (PerkinElmer) (λex: 390 nM; λem: 460 nM). Control reactions contained 50 nM CF40-gly-NS3pro185 [cNS2B fused to NS3 protease domain via nine amino acids (Gly4-Ser-Gly4)]. Fluorescence readings [RFU (relative fluorescence units)/min] were converted into M/s from a standard AMC calibration curve and further normalized to per nmol of eluted NS3pro185 (see above). Results were fitted into Michaelis–Menten equation by non-linear regression using GraphPad Prism. Steady-state kinetic constants of each substrate were determined from triplicate measurements and reported as means ± S.E.M.

Inhibition of viral replication with 3F10
HEK (human embryonic kidney)-293 cells were maintained in DMEM (Dulbecco’s modified eagle’s medium) containing 10% FCS (fetal calf serum) and 1% penicillin–streptomycin. BHK-21 cells (baby-hamster kidney-21 cells) were maintained in RPMI 1640 medium containing 10% FCS and 1% penicillin–streptomycin. DENV-2 (GenBank® EU081177.1) was propagated in C6/36 cells prior to infection. For Fab transfection, 5 × 104 HEK-293 cells were transfected with 1 μg Fab (3F10 or the non-binding control Fab (3F6)) using the TurboFect protein transfection reagent (Fermentas) and control cells were mock-transfected with TurboFect according to the manufacturer’s instructions. Cells were infected 4 h post-transfection with DENV-2 at an MOI (multiplicity of infection) of 1.0 in fresh medium. For plaque assays, media supernatants were collected 48 h post-infection and virus titres [PFU/ml (plaque-forming units per ml)] were determined with plaque assays on BHK-21 cells as previously described [29].

RESULTS
Characterization of complex of DENV-2 GST–cNS2B and NS3 protease
Expression of recombinant DENV-2 NS3pro (amino acids 1–185) as a N-terminal His-tag fusion protein in bacteria produced mainly insoluble protein. To recover NS3pro from the inclusion bodies, cell pellets were denatured, refolded on a Ni-affinity column and finally subject to gel filtration chromatography. Purified NS3pro eluted as a single peak and cleaved the simple fluorogenic substrate Bz-L-Arg-AMC (Supplementary Figure S1 at http://www.bioscirep.org/bsr/031/bsr0310399add.htm) indicating that it was correctly folded and active following refolding. Consistent with the results from Yusof et al. [17], NS3pro was significantly more active in turning over this substrate than the single-chain DENV-2 construct comprising cNS2B (amino acids 49–95) linked to NS3pro (CF40-gly-NS3pro185; Supplementary Figure S1). In contrast, NS3pro showed no activity with the tetrapeptide substrate Bz-Nle-Lys-Arg-AMC (results not shown), which was previously identified as a highly specific substrate for single-chain cNS2B/NS3 protease complexes (DENV-1–4) [18]. To test whether the NS2B cofactor can be provided in trans to restore activity against the tetrapeptide substrate, DENV-2 NS3pro was incubated with GST–cNS2B (amino acids 49–95 of DENV-2 NS2B linked to the C-terminal of GST). As shown by Western blot, NS3pro bound to beads with GST–cNS2B but not to beads with GST alone (Figure 2A). Following elution from the beads, the reconstituted GST–cNS2B/NS3pro complex readily cleaved...
Flavivirus NS2B-mediated NS3 protease activation

Figure 2  DENV-2 TSV01 NS3pro pull-down and transactivation
Protease activation was measured with substrate Bz-Nle-Lys-Arg-Arg-AMC and all assays were performed thrice. Activity is reported as means ± S.E.M. per nmol of NS3pro and activities were normalized to the amount of pulled-down NS3pro in each experiment. (A) A representative Western blot of NS3pro eluted from GST beads or GST–cNS2B (DENV-2 TSV01) beads probed with mouse anti-NS3 antibody. Lane 1, NS3pro was not detected in beads with GST control alone; lane 2, NS3pro was detected in beads with GST–cNS2B. Arrow indicates NS3pro. (B) Protease activity of eluted NS3pro bound to GST or GST–cNS2B (DENV-2 TSV01). (C) A representative Western blot of the pull-down of DENV-2 NS3pro by GST–cNS2B from different flaviviruses. The arrow indicates NS3pro. (D) Protease activity of DENV-2 NS3pro transactivated by GST–cNS2B from various flaviviruses.

Table 1  Steady-state kinetic parameters of DENV-2 TSV01 NS3 protease
Activities of GST–cNS2B/NS3pro and single-chain CF40-gly-NS3pro185 were assayed over a range of Bz-Nle-Lys-Arg-Arg-AMC substrate concentrations (15.6–250 μM). Results were obtained by non-linear regression analysis of initial velocities prior to 10% substrate depletion. Pull-down and protease activity assays were performed three times and results reported as means ± S.E.M.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Vmax (μmol·min⁻¹·μmol⁻¹ NS3)</th>
<th>kcat (s⁻¹)</th>
<th>Km (μM)</th>
<th>kcat/Km (M⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST–cNS2B/NS3pro</td>
<td>21.53 ± 1.87</td>
<td>0.36 ± 0.03</td>
<td>38.04 ± 4.10</td>
<td>9667 ± 1328</td>
</tr>
<tr>
<td>CF40-gly-NS3pro185</td>
<td>24.43 ± 0.60</td>
<td>0.41 ± 0.01</td>
<td>36.86 ± 1.72</td>
<td>11075 ± 320</td>
</tr>
</tbody>
</table>

the Bz-Nle-Lys-Arg-Arg-AMC substrate (Figure 2B) and its catalytic efficiency (kcat/Km) was comparable with that of a single-chain cNS2B–NS3pro complex, CF40-gly-NS3pro185 (Table 1).

Interaction of DENV-2 NS3pro with GST–cNS2B from other flaviviruses
The requirement for cNS2B as cofactor for optimal NS3 protease activity is a pre-requisite for all members of the flavivirus genus and within the family there is a high degree of sequence homology in the cNS2B sequence (Figure 1A). To study the mechanism of cNS2B-mediated activation of NS3, transactivation of DENV-2 NS3pro by cNS2B from other members of the flavivirus genus was assessed. Western-blot analyses of pull-down experiments showed that DENV-2 NS3pro bound to cNS2B from DENV-1–4 and WNV (Figure 2C). Higher levels of DENV-2 NS3 protease activity were induced by DENV-2 and -4 cNS2B compared with cNS2B from DENV-1 and -3 (Figure 2D). Interestingly, WNV cNS2B transactivated DENV-2 NS3pro to levels comparable with DENV-1 and -3 cNS2B suggesting mechanisms for
Effects of C-terminal residues in cNS2B on protease activity

Previous studies have mapped the minimal DENV cNS2B cofactor sequence to 40 amino acids starting at Leu53 up to and modelling efforts [31], in the absence of an inhibitor-bound dengue crystal structure, it remains unclear as to which residues are important for active site formation and/or for interaction with NS3pro residues.

To address the roles of specific amino acids in the DENV cNS2B C-terminal region, single alanine substitutions were made at positions Trp61, Asp81, Ile86 and Lys87 and a double alanine substitution was made at positions Leu74/Ile78 (shown in Figure 1A). The conserved amino acids Gly69 and Gly82 were separately mutated to proline to assess the effect of reducing the flexibility of cNS2B on cofactor activity. All mutants were expressed as NS2B–GST fusion proteins, incubated with refolded NS3pro and assessed for NS3pro interaction and protease activity as described above.

As shown in Figure 4(A), NS3pro bound all mutant GST–cNS2B proteins to a similar degree. However, the various mutants exhibited different abilities to transactivate NS3pro (Figure 4B and Table 2). W61A decreased NS3 protease activity by 40% compared with the complete absence of activity found with a single-chain cNS2B/NS3pro by Niyomrattanakit et al. [30]. Nevertheless, in agreement with the former study, the double alanine mutant Leu74/Ile78 had no protease activity. Alanine substitutions at residues Asp81 and Ile86 reduced NS3 protease activity by 85% and 76% respectively reaffirming the role of C-terminal NS2B residues in NS3 protease activation. Interestingly, mutating Lys87 to alanine increased protease activity by 64%. Mutants G69P and G82P exhibited dramatic reductions in protease activity (87% and 100% respectively). These results suggest the conformation of the entire cNS2B sequence plays an important role in imparting its cofactor properties.

Table 2 Effect of alanine mutations in cNS2B on NS3 protease activity

<table>
<thead>
<tr>
<th>Residue</th>
<th>Activity (%)</th>
<th>Residue</th>
<th>Activity (%)</th>
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<tbody>
<tr>
<td>W61A</td>
<td>0</td>
<td>W61A</td>
<td>60</td>
</tr>
<tr>
<td>S70A</td>
<td>118</td>
<td>G69P</td>
<td>13</td>
</tr>
<tr>
<td>L74A</td>
<td>3.4</td>
<td>L74A/I78A</td>
<td>0</td>
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<tr>
<td>I76A</td>
<td>8.4</td>
<td>D81A</td>
<td>15</td>
</tr>
<tr>
<td>T77A</td>
<td>65</td>
<td>G82P</td>
<td>0</td>
</tr>
<tr>
<td>I78A</td>
<td>1.9</td>
<td>I86A</td>
<td>24</td>
</tr>
<tr>
<td>L74A/I78A</td>
<td>0</td>
<td>K87A</td>
<td>164</td>
</tr>
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Determination of affinity of NS3pro for cNS2B

The binding and pull-down experiments indicate that the interaction between NS3pro and cNS2B is strong. Previous attempts to dissociate cNS2B from the cNS2B/NS3pro185 complex with low pH and at high salt concentrations were not successful (Paulus Erbel, Novartis Institutes for Biomedical Research, personal communications). To estimate the affinity of NS3 protease for cNS2B, increasing concentrations of DENV-2 NS3pro were incubated with GST–DENV-2 cNS2B beads, washed and probed by Western-blot analyses to determine the amount of bound NS3pro (Figure 3). No bound NS3 protease was detected below 120 nM total NS3pro added in the mixture due to the sensitivity limit of the anti-NS3 antibodies used (results not shown). Adding 120–500 nM NS3pro to GST–cNS2B beads resulted in a corresponding increase in the amount of bound NS3pro and binding was saturated at approx. 300 nM of NS3pro. The half-saturation concentration from two independent experiments was 177 nM, indicating an equilibrium binding constant of less than 200 nM. Control experiments performed with GST alone showed negligible amounts of bound NS3pro (Figure 3).

Effect of alanine substitutions of DENV-2 cNS2B

C-terminal residues on the activity of GST–cNS2B/NS3pro complex

Previous work with a single-chain cNS2B/NS3pro complex has shown that alanine mutations of Trp61, Leu74, Ile76 or Ile78 in the C-terminal region of DENV-2 cNS2B significantly inhibited DENV-2 NS3 protease activity, while similar mutations in Ser70 and Thr77 had little or no effect (Table 2, [30]). A double alanine mutant of Leu74/Ile78 completely abolished protease activity in a single-chain NS2B–NS3 protease construct [30], while alanine substitutions of residues at the termini of cNS2B (Asp50/Glu52) had negligible effect on protease activity [31]. Despite recent

Protease activation is conserved across related members of the flavivirus genus.

DENV-2 NS3 protease activity, while similar mutations in Ser70 and Thr77 had little or no effect (Table 2, [30]). A double alanine mutant of Leu74/Ile78 completely abolished protease activity in a single-chain NS2B–NS3 protease construct [30], while alanine substitutions of residues at the termini of cNS2B (Asp50/Glu52) had negligible effect on protease activity [31]. Despite recent
including Glu\textsuperscript{95}, since deleting residues Leu\textsuperscript{93} or Glu\textsuperscript{95} abolished NS3pro activity [6,25]. However, a more recent report in which a single-chain NS2B-NS3 construct was subject to proteolysis with endoprotease Asp-N, found a C-terminal truncation in NS2B (NS2B\textsuperscript{50–80}) remained associated with NS3 protease (NS3\textsuperscript{6–179}) and retained 52% of the original protease activity [32]. To verify the role of the C-terminal cNS2B residues in protease activity, we sequentially deleted seven, nine and 11 amino acids from cNS2B (starting from Leu\textsuperscript{95}) in a single-chain DENV-2 NS2B-NS3 construct (Figure 1A). All three constructs remained highly soluble (Supplementary Figure S2 at http://www.bioscirep.org/bsr/031/bsr0310399add.htm). Removal of seven amino acids (NS2B\textsuperscript{49–88}) resulted in a 74% reduction in protease activity, while the deletion of nine (NS2B\textsuperscript{49–86}) and 11 (NS2B\textsuperscript{49–84}) amino acids led to 96% and 100% loss in NS3pro activity respectively (Table 3).

### Table 3 Protease activities of DENV-2 wild-type and C-terminal NS2B truncated NS2B-NS3 protease constructs

Assays were performed with 40 nM protease and 50 \( \mu \text{M} \) Bz-Nle-Lys-Arg-Arg-AMC substrate. The initial enzyme activity (\( \mu \text{M} / \text{s} \)) was calculated from the first 5 min of each reaction from duplicate wells. CF40(gly)-NS3pro is denoted wild-type DENV2 NS2B-NS3 protease, CF40(\( \Delta \text{C7} \)), CF40(\( \Delta \text{C9} \)) and CF40(\( \Delta \text{C11} \)) are the protease variants deleted at the C-terminal.

<table>
<thead>
<tr>
<th>DENV2 protein</th>
<th>Activity (( \mu \text{M} / \text{s} ))</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF40(gly)-NS3pro</td>
<td>0.0175 ± 0.0008</td>
<td>100</td>
</tr>
<tr>
<td>CF40(( \Delta \text{C7} ))gly-NS3pro</td>
<td>0.0046 ± 0.0001</td>
<td>26</td>
</tr>
<tr>
<td>CF40(( \Delta \text{C9} ))gly-NS3pro</td>
<td>0.0007 ± 0.0001</td>
<td>4</td>
</tr>
<tr>
<td>CF40(( \Delta \text{C11} ))gly-NS3pro</td>
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</tbody>
</table>

### Impact of an N-terminal cNS2B-specific antibody on DENV replication

The N-terminal region of cNS2B forms a \( \beta \)-strand and is sufficient to stabilize the NS3 protease domain. DENV NS3 proteins containing the first 18 amino acids of cNS2B (cNS2B\textsuperscript{49–66}) have a well-preserved protease domain and crystallize readily, but are not able to cleave the tetrapeptide substrate Bz-Nle-Lys-Arg-Arg-AMC [19,33]. To investigate the role of the N-terminal cNS2B residues on DENV replication a human Fab, 3F10, with specificity for cNS2B\textsuperscript{49–66} was utilized. We had previously isolated 3F10 from a human Fab phage library [27]. The binding epitope of the antibody has been mapped to the N-terminal 18 amino acids of NS2B and it inhibits \textit{in vitro} cleavage of Bz-Nle-Lys-Arg-Arg-AMC by a single-chain cNS2B-NS3 protease construct [27].

To study the effect 3F10 has on the interaction of cNS2B with NS3 protease, GST–DENV-4 cNS2B beads were first incubated with 3F10, followed by NS3pro. The amount of DENV-4 NS3pro bound to GST–DENV-4 cNS2B beads in the presence of 3F10 was reduced by approx. 40% compared with control beads without 3F10 (Figures 5A and 5B). As NS2B is an intracellular viral protein, to observe the effects 3F10 has on DENV replication it must be delivered across the cell membrane. HEK-293 cells were transfected with 3F10 using a protein transfection reagent and subsequently infected with DENV-2. At 48h after infection plaque assays were performed with culture supernatants. Cells transfected with 3F10 prior to DENV-2 infection showed a greater than one-log decrease in released virus compared with DENV-2-infected cells and cells transfected with control Fab (3F6). Uninfected control cells showed no viral replication (Figure 5C).

### DISCUSSION

Both the present study and a previous study [25] have shown that DENV NS3 protease can be transactivated \textit{in vitro} with a GST-fusion protein comprising the core cofactor sequence of NS2B. In the absence of a co-complexed DENV cNS2B-NS3 protease
structure, we explored DENV cNS2B-NS3 binding and activity using the transactivation system with the aim of differentiating between residues that are important in cNS2B-NS3 protease binding, and those that are required for protease activity. Pulldown DENV-2 NS3pro was active (Figure 2) and the steady-state kinetic parameters of the GST–cNS2B/NS3pro complex were very similar to those of the single-chain construct, CF40-gly-NS3pro185 (Table 1). The successful activation of NS3 protease by cNS2B provided in trans suggests that the structure of cNS2B is flexible in solution. The binding affinity of NS3pro for cNS2B is relatively strong, with an equilibrium binding constant of less than 200 nM. The interaction of cNS2B with DENV NS3 protease may be driven by residues in the N-terminal region of cNS2B initially, but in the presence of substrate, a more stable complex involving the C-terminal region of cNS2B likely forms. Indeed, crystal structures of DENV and WNV cNS2B/NS3pro reveal that the N-terminal region of cNS2B (cNS2B49–66) forms a β-strand with the N-terminal β-barrel of NS3pro [19,20,33], and by itself this region is sufficient to solubilize and stabilize the NS3 [19,33].

To determine whether the elements required for activation of the NS3 protease are conserved across the DENV serotypes and that DENV utilizes mechanisms similar to those deduced for WNV, the transactivation of DENV-2 NS3 protease by GST–cNS2B constructs from DENV-1–4 and WNV was examined. Not surprisingly DENV-1, -3 and -4 cNS2B readily transactivated DENV-2 NS3pro. Interestingly, their levels of transactivation somewhat resembled their phylogenetic relatedness [3,34] in that cNS2B from DENV-2 and -4 were able to induce activity of DENV-2 NS3pro more than cNS2B from DENV-1 and -3 serotypes (Figures 1A and 2B). WNV GST–cNS2B constructs were able to pull down similar amounts of DENV-2 NS3 protease as DENV-1–4 constructs and also transactivated the protease, confirming that the mechanisms for protease activation are conserved across members of the flavivirus genus.

Several lines of evidence for the integral role of the C-terminal cNS2B residues in DENV protease activation, but not cNS2B-NS3 binding, have emerged from the present study. C-terminal truncations of DENV-2 cNS2B in which seven, nine or 11 amino acid residues were removed (cNS2B49–88, cNS2B49–86 and cNS2B49–84 respectively) dramatically affected the activity of a single-chain NS2B-NS3 construct but not its solubility (Table 3 and Supplementary Figure S2). As previously observed [25], we also noted that GST–NS2B comprising 47 amino acids (Ala49-Leu95) possessed better cofactor activity than GST–NS2B bearing 40 amino acids (Leu53-Glu92) (results not shown). Finally, alanine mutations of single amino acids, Asp81 and Ile86, in the C-terminal region strongly affected transactivated protease activity but not binding of cNS2B to NS3pro (Figure 4 and Table 2). Our results contradict a previous report in which a truncation of DENV-2 cNS2B comprising amino acids 50–81 retained partial (52%) proteolytic activity [32]. The reason for this discrepancy may be due to the different substrates (tetra- versus depsi-peptide) and assay formats (fluorescence versus FRET) used. Nevertheless, Melino et al. [32] also concluded that amino acids 80–95 of cNS2B are highly solvent exposed and do not form strong interactions with NS3 protease.

A recent alanine mutagenesis study for WNV cNS2B50–96 using a single-chain NS2B-NS3 construct identified two
regions of the cofactor where mutagenesis greatly affected proteolytic activity [35]. These spanned cNS2B<sup>74-86</sup> and cNS2B<sup>75-87</sup> in WNV and were referred to by the authors as ‘site 1’ and ‘site 2’ (Figure 1A). The alanine mutations in the present study that map to either ‘site 1’ (Trp<sup>51</sup>) or ‘site 2’ (Leu<sup>74</sup>/Ile<sup>78</sup>, Asp<sup>81</sup>, Gly<sup>82</sup> and Ile<sup>86</sup>) also reduced proteolytic activity. Site 2 in particular has an integral role in active site formation in WNV [19–21]. It comprises a β-loop that binds the C-terminal β-barrel of NS3pro in close proximity to the substrate-binding cleft in co-complexed structures (Figure 1C). Our results, together with the mutation analysis performed by Niyomrattanaki et al. [30], are consistent with the corresponding ‘site 2’ DENV residues (cNS2B<sup>74-86</sup>) participating in NS3 protease active site formation (Table 3). Mutations in Leu<sup>74</sup>, Ile<sup>76</sup> and Ile<sup>78</sup> significantly reduced DENV protease activity of a single-chain NS2B-NS3 construct [30]. Similarly, mutations of Asp<sup>81</sup>, Gly<sup>82</sup> and Ile<sup>86</sup>, and the double mutation of Leu<sup>74</sup>/Ile<sup>78</sup> did not affect the ability of cNS2B to bind NS3 protease, but had a dramatic effect on transactivation (Figure 4 and Table 3).

Residues Asp<sup>81</sup> and Ile<sup>86</sup> are conserved in DENV-1–4 (Figure 1A) and mutation of these residues reduced NS3 protease transactivation to less than 25% of wild-type cNS2B. In WNV, the corresponding residues (Asp<sup>82</sup> and Leu<sup>87</sup>) were positioned at the distal and proximal ends of the ‘site 2’ β-loop that contributes residues to the S2- (Gly<sup>83</sup>, Asn<sup>84</sup> and Phe<sup>85</sup>) and S3-pockets (Glu<sup>86</sup> and Leu<sup>87</sup>) in the substrate-binding site (Figure 1C). Interestingly, mutating residue Lys<sup>87</sup> (conserved in DENV-1–3 and arginine in DENV-4) to alanine augmented the protease activity by nearly 2-fold (Figure 4B). The structural reason for this is not clear because the corresponding Met<sup>86</sup> residue in WNV does not participate in active site formation [19–21], and mutating this residue in WNV had very little effect on proteolytic activity [35]. This suggests that the DENV-2 active site may differ from WNV.

It is possible that a change in conformation brought about by a loss of a charged interaction or hydrogen bonding results in the amount of pulled-down NS3 protease (Figure 5B). Bindig of 3F10 to cNS2B may alter the structure and flexibility of the cofactor and, in turn, reduce the propensity for cNS2B to bind NS3 protease. Given the size of Fab molecules (50 kDa), steric effects are also likely to contribute to the reduced binding observed. Nevertheless, the disruption of the cNS2B-NS3 protease interaction observed in transactivation experiments translated to a cell-based system with 3F10 reducing DENV replication in HEK-293 cells (Figure 5C). This suggests the interaction between NS2B and NS3 can be blocked during viral replication, and provides proof-of-concept for the use of cofactor targeting inhibitors against DENV NS2B-NS3 protease. Moreover, in a very recent study, the inhibitory activity of a highly lipoophilic compound (BP2109) against DENV protease was reduced by mutations in NS2B (R55K and E80K) [37]. While the exact mechanism of action of the compound is unknown, allosteric modulation of NS2B is likely involved.

To conclude, our primary interest is in the development of protease inhibitors against dengue viral infection. One question that remains open is whether a pan-serotype inhibitor or indeed a pan-flavivirus inhibitor can be designed. We previously showed that the four dengue NS2B/NS3 proteases possessed very similar preferences for substrate cleavage sequences at their P4–P4‘ sites [18]. The present study additionally shows that the mode of activation of the flaviviral proteases is similar and that screening for allosteric inhibitors, which target residues critical for the NS2B-NS3 interaction, is a valid drug discovery strategy.

**AUTHOR CONTRIBUTION**

Siew Lim and Nicole Moreland designed and performed the experiments, analysed the data and wrote the manuscript. Wai Phong, Daying Wen and Prasad Paradkar performed the experiments. Subhash Vasudevan designed the experiments, analysed the data and wrote the manuscript.

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We thank Alex Chao for assistance in cloning the DENV-2 TSV01 pET15b-NS3pro185 construct and Andrew Davidson for the plasmid comprising DENV-2 NGC infectious clone pDVWS601. Moon Y. F. Tay and Elfin Lim are acknowledged for assistance with the preparation of DENV-4 GST–cNS2B and DENV-4 pET15b-NS3pro185 clones. We are also grateful to Dr Prida Malaisit for his gift of the

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**REFERENCES**

Flavivirus NS2B-mediated NS3 protease activation


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SUPPLEMENTARY ONLINE DATA

Dengue protease activity: the structural integrity and interaction of NS2B with NS3 protease and its potential as a drug target

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Figure S1 Refolding, purification and activity of NS3pro

(A) Coomassie Blue stained gel of the fractions of purified NS3pro185 after refolding on Ni\(^{2+}\) affinity column and passing through gel filtration. Lane 1, protein sample after Ni\(^{2+}\) affinity column; lane 2, flow through; lanes 3–4, eluted peak fractions following gel filtration. Arrows indicate NS3pro185. (B) Protease activity of refolded NS3pro in the absence of cofactor NS2B. Activity of 300 nM of purified refolded NS3pro was measured with 4 mM of substrate, Bz-L-Arg-AMC. Single-chain DV2TSV01 CF40-gly-NS3pro185 (3 \(\mu\)M) was used as a control. Protease activity assays were performed thrice and results reported as means ± S.E.M. per nmol of NS3pro.

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Figure S2 Expression and purification of DV2 CF40-gly-NS3pro185 constructs with NS2B C-terminal deletions
Proteins were resolved by SDS/12% PAGE and stained with Coomassie Blue. For each protein ΔC7 (i), ΔC9 (ii), ΔC11 (iii) lane 1, pre-induction; lane 2, post-induction with IPTG (16 h); lane 3, whole cell lysate; lane 4, lysate after clarification by centrifugation; lane 5, Ni²⁺-affinity purified proteins. M denotes protein marker. Arrow indicates position of protein.
Table 1  Primers used to generate the PCR products for cloning of the cNS2B and NS3pro constructs used in the present study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5′–3′)</th>
</tr>
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<tr>
<td>NS3pro185XhoI-F</td>
<td>ATGCTCGAGGCGCGAGATTTGTGAGGATGTC</td>
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<tr>
<td>NS3pro185BamHI-R</td>
<td>TAAATGGATCTTACCTTTTTCAGAAAGATGTCATCTCTCA</td>
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<tr>
<td>NS2BdelC7-REV</td>
<td>TCCACCACTAATTCGGCCCATTTTTATCAGATCATCTACC</td>
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<tr>
<td>NS2BdelC9-REV</td>
<td>TCCACCACTAATTCGGCCCATTTTTATCAGATCATCTACC</td>
</tr>
<tr>
<td>NS2BdelC11-REV</td>
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<tr>
<td>DEN1REV</td>
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<tr>
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<tr>
<td>DEN2(TSV01)FOR</td>
<td>GCCGATCGGCGATTTACAGCTTTTGGAACACTGAGAAAAG</td>
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<tr>
<td>DEN2(TSV01)REV</td>
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<tr>
<td>DEN3FOR</td>
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<td>cNS2BREV</td>
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Table 2  Composition of the buffers used in refolding and purification of DV2 NS3pro

<table>
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<tr>
<th>Purification step</th>
<th>Composition</th>
<th>Column volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>On column refolding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash step</td>
<td>6 M urea, 20 mM Tris/HCl, pH 8.5, 0.5 M NaCl and 20 mM imidazole</td>
<td>10</td>
</tr>
<tr>
<td>Refolding</td>
<td>20 mM Tris/HCl, pH 8.5, 0.5 M NaCl, 20 mM imidazole and 0.1% N-octyl-β-glucoside</td>
<td>30</td>
</tr>
<tr>
<td>Elution</td>
<td>20 mM Tris/HCl, pH 8.5, 0.5 M NaCl, 500 mM imidazole and 0.1% N-octyl-β-glucoside</td>
<td>–</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>50 mM Tris/HCl, pH 8.5, 0.3 M NaCl, 5% glycerol and 0.1% N-octyl-β-glucoside</td>
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</tr>
</tbody>
</table>

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