Antiviral Research 96 (2012) 305–314

Contents lists available at SciVerse ScienceDirect

Antiviral Research

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

Establishment of a robust dengue virus NS3–NS5 binding assay for identification of protein–protein interaction inhibitors

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ARTICLE INFO

Article history:
Received 29 May 2012
Revised 12 September 2012
Accepted 12 September 2012
Available online 13 October 2012

Keywords:
Dengue virus
NS3
NS5
Wheat-germ cell-free protein synthesis
AlphaScreen technology
Protein–protein interaction inhibitors

ABSTRACT

Whereas the dengue virus (DENV) non-structural (NS) proteins NS3 and NS5 have been shown to interact *in vitro* and *in vivo*, the biological relevance of this interaction in viral replication has not been fully clarified. Here, we first applied a simple and robust *in vitro* assay based on AlphaScreen technology in combination with the wheat-germ cell-free protein production system to detect the DENV-2 NS3–NS5 interaction in a 384-well plate. The cell-free-synthesized NS3 and NS5 recombinant proteins were soluble and in possession of their respective enzymatic activities *in vitro*. In addition, AlphaScreen assays using the recombinant proteins detected a specific interaction between NS3 and NS5 with a robust Z' factor of 0.71. By employing the AlphaScreen assay, we found that both the N-terminal protease and C-terminal helicase domains of NS3 are required for its association with NS5. Furthermore, a competition assay revealed that the binding of full-length NS3 to NS5 was significantly inhibited by the addition of an excess of NS3 protease or helicase domains. Our results demonstrate that the AlphaScreen assay can be used to discover novel antiviral agents targeting the interactions between DENV NS proteins.

1. Introduction

Dengue virus (DENV) belongs to the genus *Flavivirus* of the Flaviviridae family, which is a large family of enveloped, positive-stranded RNA viruses. The four serotypes of DENV (DENV-1 to DENV-4) have recently emerged as significant pathogens that can cause dengue fever (DF) and dengue hemorrhagic fever (DHF) in humans. While a majority of primary infections with one of the four DENV serotypes present as self-limited DF, subsequent secondary infections with a different serotype increases the risk of more severe forms of dengue infections such as the life-threatening DHF. However, there is currently no vaccine or specific antiviral available for DENV infection.

The genome of DENV consists of a 10.7 kb single-stranded RNA that encodes a single polyprotein, which is cleaved co- and post-translationally into three structural proteins and seven nonstructural (NS) proteins (Lindenbach and Rice, 2003). The structural proteins are required for the formation of virus particles. While NS proteins are not detectable in mature virions, they are involved in DENV RNA replication through their association with intracellular membranes (Chambers et al., 1990). Of the NS proteins, NS3 and NS5 are the best characterized and are essential for DENV replication (Bartholomeusz and Wright, 1993). NS3 is a 69 kDa protein composed of an N-terminal domain that exhibits protease activity and a C-terminal domain that has three enzymatic functions, namely nucleotide triphosphatase (NTPase), 5′ RNA triphosphatase, and RNA helicase activities (Li et al., 1999). The protease domain of NS3 serves as a trypsin-like serine protease. Although the polyprotein precursor junctions between the C to NS2A regions are cleaved by the host signal peptidase in the endoplasmic reticulum, the NS3 protease, complexed with the cofactor NS2B, cleaves the junctions between NS2A–NS2B, NS2B–NS3, NS3–NS4A, NS4B–NS5, within NS2A, NS3, NS4A, and also upstream of the C-prM junction (Falgout et al., 1991; Preugschat et al., 1990; Wu et al.,

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http://dx.doi.org/10.1016/j.antiviral.2012.09.023

2003; Zhang et al., 1992). In addition to protein processing, NS3 participates in viral RNA replication by resolving the RNA duplex through its C-terminal NTPase/helicase domain (Li et al., 1999). NS5 is the largest and the most conserved viral protein and as with NS3 it is a multifunctional protein possessing two distinct enzymatic activities, consisting of an N-terminal methyltransferase (MTase) domain that catalyzes methylation of the viral RNA cap structure (Egloff et al., 2007), and a C-terminal RNA-dependent RNA polymerase (RdRp) domain that plays a central role in viral RNA replication (Tan et al., 1996; Yap et al., 2007). Because both NS3 and NS5 are central to the function of the DENV replication complex, a molecular machine driving viral RNA synthesis in the cytoplasm, the enzymatic activities of these viral proteins are considered attractive targets for the development of antiviral drugs (Lescar et al., 2008; Sampath and Padmanabhan, 2009).

Flaviviral RNA replication is postulated to proceed through the synthesis of a negative-strand copy of viral RNA, followed by the formation of an RNA double-strand which serves as the template for the amplification of positive-strand copies of genomic RNA (Chu and Westaway, 1985, 1987). The presence of enzymatic properties necessary for RNA-dependent RNA synthesis in both the NS3 and NS5 proteins raises a possibility that both proteins may cooperate to form a replication complex, which ensures efficient amplification of the viral genome from a copy of template RNA. Indeed, *in vivo* and *in vitro* interactions between NS3 and NS5 have been reported in DENV and other flaviviruses (Chen et al., 1997; Kapoor et al., 1995). Historically, the active sites of viral enzymes and the binding pockets of cellular receptors have been targets for the development of antiviral drugs. However, growing evidence suggest that, although a challenging endeavor, targeting protein–protein interactions could be an alternative route for developing antiviral inhibitors that exhibit entirely distinct mechanisms of action (Arkin and Wells, 2004; Ryan and Matthews, 2005). In this regard, pharmacological blockage of NS3–NS5 interaction would be a promising approach for the treatment of DENV infection.

The interaction between flaviviral NS3 and NS5 proteins has been demonstrated by immunoprecipitation assay with cultured cells (Chen et al., 1997; Kapoor et al., 1995) and yeast two-hybrid assay (Johansson et al., 2001). However, such *in vivo* analyses are often time-consuming and labor-intensive, posing a critical obstacle to further adaptation to the high-throughput screening of small chemical compounds that inhibit the NS protein interactions. Although Biacore surface plasmon resonance and enzyme-linked immunosorbent assay (ELISA), in which *Escherichia coli* (*E. coli*)-derived NS5 is coupled onto a surface, have been employed to measure the DENV NS3–NS5 interaction *in vitro* (Moreland et al., 2012; Zou et al., 2011), these solid phase assays may reduce the protein's degree of freedom and thereby influence the binding activity of the protein. In the present study, in order to establish a more robust *in vitro* assay platform for monitoring the NS3–NS5 interaction in a high-throughput setting, we developed a rapid and quantitative assay by combining the wheat germ cell-free protein production system (hereinafter referred to as wheat cell-free system) and AlphaScreen detection technology in a 384-well plate format.

2. Materials and methods

2.1. Construction of template plasmid DNAs

pEU-HF vector for production of N-terminal hexahistidine (His)- and FLAG-tagged (HF) proteins was constructed by inserting a FLAG-tag sequence into the pEU-E01-His-TEV-MCS vector (pEU-His, CellFree Sciences, Japan). pEU-GST vector for production of N-terminal glutathione S-transferase (GST)-tagged and biotinylated (GB) proteins was constructed by inserting a biotin ligase-recognition site (bls) into the pEU-E01-GST-TEV-MCS vector (pEU-GST, CellFree Sciences). Then, cDNAs encoding full-length NS3, NS3 protease domain (amino acid positions 1–185 [Yusof et al., 2000]), NS3 helicase domain (amino acid positions 172–618 [Moreland et al., 2010]), and full-length NS5 proteins were amplified from a plasmid DNA containing DENV type 2 replicon cDNA (Ng et al., 2007) by PCR, and then inserted into the pEU-HF (for HF-NS3 proteins) and pEU-GB (for GB-NS5 proteins). The protease and helicase domains of NS3 were also inserted into pEU-His vector for the competition assay. cDNA encoding the co-factor region of NS2B (amino acids 49–95 [Phong et al., 2011]) was inserted into pEU-GST (for GST-NS2Bc). Bacterial dihydrofolate reductase (DHFR), used as a negative control protein for the assays, was inserted into pEU-HF, pEU-GB, pEU-His, and pEU-GST vectors (HF-DHFR, GB-DHFR, His-DHFR, and GST-DHFR).

To prepare NS3 and NS5 single amino mutant proteins, an alanine substitution was introduced into Lys-199 of NS3 and Lys-330 of NS5 in the pEU-HF-NS3 and pEU-GB-NS5 vectors, respectively.

2.2. Cell-free protein synthesis using wheat germ extract

In vitro transcription (IVT) and translation were performed according to the manufacturer's protocols. IVT was carried out in a 250 μl reaction mixture containing 25 μg of template plasmid DNA. The cell-free translation reaction (6 ml) was done with a wheat germ extracts optimized for either Ni-affinity purification (WEPRO 1240H, CellFree Sciences) or GST-affinity purification (WEPRO 1240G, CellFree Sciences). Biotinylated proteins (i.e. GB proteins) were produced as previously described (Sawasaki et al., 2008).

2.3. Purification of cell-free-expressed recombinant proteins

Batch purification of recombinant protein was performed in a robotic protein synthesizer, Protemist DTII (CellFree Sciences), according to the manufacturer's protocol with slight modifications. For purification of HF and His-tagged proteins, the cell-free reaction was mixed with Ni Sepharose High Performance beads (GE Healthcare) in the presence of 20 mM imidazole. The beads were washed three times with washing buffer (20 mM Tris–HCl, pH7.5, 500 mM NaCl, 10% glycerol) containing 40 mM imidazole. The His-tagged protein was then eluted with washing buffer containing 500 mM imidazole and further purified by a HiTrap Desalting column (GE Healthcare) with desalting buffer (20 mM Tris–HCl, pH7.5, 50 mM NaCl, 10% glycerol) to remove the imidazole.

For purification of GB and GST-tagged proteins, the cell-free reaction was mixed with 200 mM NaCl and 10 mM DTT and incubated with Glutathione Sepharose Fast Flow beads (GE Healthcare). The beads were washed three times with PBS and GST-tagged proteins were eluted with elution buffer (50 mM Tris–HCl, pH8.0, 50 mM NaCl, 10 mM reduced glutathione, 10% glycerol).

All proteins were concentrated approximately 10–20-fold using Amicon Ultra centrifugal filters (Millipore), and protein concentration was determined by the Bradford method using bovine serum albumin (BSA) as a protein standard.

2.4. Immunoblotting analysis

Proteins samples were boiled in SDS-sample buffer, separated by 4–20% gradient SDS–PAGE gel, and blotted onto PVDF Membrane. HF proteins and GB proteins were detected by probing with horseradish peroxidase (HRP)-conjugated-anti-FLAG-antibody (M2, Sigma) and HRP-conjugated-anti-biotin mouse monoclonal IgG (BN-34, Sigma) respectively, and visualized using an

ImageQuant LAS 4000 mini chemiluminescent image analyzer (GE Healthcare).

2.5. Protease assay

Two hundred nanomolar of HF-NS3 protein, its mutants, or control protein (HF-DHFR) were incubated with 50 μM Bz-Nle-Lys-Arg-Arg-AMC (Nle: norleucine, AMC: 7-amino-4-methylcoumarin, LSU Health Sciences Center) in 25 μl of reaction buffer (50 mM Tris–HCl, pH8.0, 1 mM CHAPS, 20% glycerol) in the absence or presence of 300 nM GST-NS2Bc (or GST-DHFR) at 37 °C. After 1 h incubation, the concentration of free AMC liberated by protease activity was quantified with standard AMC by an Infinite M200 (TECAN) at an excitation wavelength of 390 nm and an emission wavelength of 460 nm.

2.6. NTPase activity assay

NTPase reaction containing 100 nM of HF-NS3 protein, HF-NS3 mutants, or HF-DHFR in 20 μl of reaction buffer (20 mM Tris–HCl, pH7.5, 2 mM $MgCl_2$, 1.5 mM DTT, 0.25 ng/μl BSA, 0.05% Tween 20) was initiated by adding 5 μl of 5 mM NTPs. After incubation at 37 °C for 30 min, 70 μl of detection mixture containing 0.36% ammonium molybdate in 0.63 N H_2SO_4 and 1.05% ascorbic acid was added. After incubation at 42 °C for 20 min, the concentration of free orthophosphate released by hydrolysis of NTPs was determined by the absorbance at 820 nm using a monochrometer, Infinite M200, with NaH_2PO_4 as a standard phosphate (Lam et al., 2003).

2.7. RdRp assay

An RNA substrate, 3′UTR-U_{30} (5′-U_{30}-AAC AGG UUC UAG AAC CUG UU-3′) was incubated in TE buffer at 60 °C for 5 min and placed at room temperature for 60 min to form the intramolecular hairpin structure. The template-dependent RNA polymerization was carried out with 60 nM NS5 or control (DHFR) protein in 20 μl of reaction buffer containing 50 mM Tris–HCl, pH7.0, 2 mM DTT, 10 mM KCl, 1 mM $MnCl_2$, 0.01% Triton X-100, 100 nM 3′UTR-U_{30} and 2 μM 2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole (BBT)-conjugated ATP (BBT-ATP, Jena Bioscience GmbH) at room temperature for 1 h. Ten microliters of 1 ng/μl calf intestinal phosphatase (CIP, Promega) in 150 mM Tris–HCl, pH9.3, 300 μM $MgCl_2$ was then added to the mixture, and after 1 h incubation at room temperature, the BBT liberated by the CIP treatment was measured by an Infinite M200 at an excitation wavelength of 422 nm and an emission wavelength of 566 nm (Niyomrattanakit et al., 2011).

2.8. Immunoprecipitation assay

GB-NS5 (200 nM) was incubated with 200 nM HF-NS3 or its domain mutants in 40 μl of binding buffer (20 mM Tris–HCl, pH7.5, 40 mM NaCl, 5 mM $MgCl_2$, 1 mg/ml BSA) at 16 °C for 1 h. Then, 0.4 μg of anti-FLAG antibody (1E6, Wako) or control mouse IgG (Sigma) was added to the mixture and incubation was continued at 16 °C for 1 h. Subsequently, the reaction was mixed with 15 μl of protein A/G PLUS agarose (50% slurry equilibrated with binding buffer, Santa Cruz Biotechnology) and incubated at 4 °C for 2 h with rotation. After washing four times with binding buffer, immunoprecipitated proteins were recovered from the beads by resuspension with 20 μl of SDS-sample buffer and subjected to immunoblotting analysis. Quantification of immunoprecipitates was carried out by measuring the intensity of the immunoblot band using Image Quant LAS 4000 mini and Image Quant TL7.0 software.

2.9. AlphaScreen assay

AlphaScreen assay was performed with 384-well OptiPlates (PerkinElmer). HF-NS3 or HF-DHFR (100 nM) was incubated with serial dilutions of GB-NS5 (final concentration 25–500 nM) in 15 μl of binding mixture containing reaction buffer (20 mM Tris–HCl, pH7.5, 200 μM DTT, 5 mM $MgCl_2$, 40 mM NaCl, 1 mg/ml BSA, 8% glycerol) at 16 °C for 1 h. Then, 10 μl of the detection mixture containing 0.6 μg/ml anti-FLAG mouse monoclonal IgG (1E6, Wako Pure Chemical Industries), 0.1 μl protein A-conjugated acceptor beads, and 0.1 μl streptavidin-coated donor beads (AlphaScreen IgG detection kit, PerkinElmer) in reaction buffer was added, followed by incubation at 16 °C for 1 h. Interaction of proteins were analyzed by an EnSpire Alpha microplate reader (PerkinElmer). In other experiments, the AlphaScreen assay was performed with (i) 100 nM HF-NS3 (or HF-DHFR) and 100 nM GB-NS5 (or GB-NS5 K330A), and (ii) 100 nM GB-NS5 and serial dilutions (25–500 nM) of HF-NS3, HF-Pro, HF-Hel, or HF-DHFR. For the competition binding assay, incubation of the binding mixture was carried out with 100 nM HF-NS3 and GB-NS5 in the presence of His-Pro, His-Hel, or His-DHFR (37.5–600 nM).

2.10. Statistical analysis

Student's *t*-test was used to determine statistical significance. *P* values lower than 0.05 were considered as significant.

3. Results

3.1. Production of DENV NS3 and NS5 proteins by wheat germ cell-free system

It has been demonstrated that flaviviral NS3 proteins expressed in *E. coli* are prone to aggregation (Cui et al., 1998; Kuo et al., 1996; Zou et al., 2011). In particular, production of soluble DENV NS3 in *E. coli* could only be achieved when the hydrophilic part of NS2B, a cofactor for NS3 protease activity, was covalently fused with NS3 through a flexible linker sequence (Luo et al., 2008). In order to develop an alternative expression method, we employed the wheat germ cell-free protein production system (referred as wheat cell-free system) (Sawasaki et al., 2002). This eukaryotic cell-based *in vitro* translation method offers several advantages over *E. coli* system, allowing the production of high quality proteins that are properly folded (Goshima et al., 2008). When full-length DENV-2 NS3 (HF-NS3), its single amino acid mutant (HF-NS3 K199A), and the deletion mutants of NS3 (protease domain [HF-Pro] and helicase domain [HF-Hel]) fused with hexahistidine and FLAG tags (Fig. 1A) were produced in the wheat cell-free reaction (whole translation mixture [W], Fig. 1B, left panel), a comparable amount of the proteins could be detected in the supernatant fraction (S) after centrifugation at 17,000×*g* by an immunoblotting analysis using anti-FLAG antibody (Fig. 1B, left panel), indicating these recombinant proteins had good solubility. As is the case with HF-NS3 proteins, full-length (GB-NS5) and a single amino acid mutant of NS5 (GB-NS5 K330A), both fused with GST and biotinylated through their bls sequence (Fig. 1A), were also isolated predominantly in soluble form as indicated by a comparison between whole translation mixture and supernatant fractions in an immunoblot analysis using anti-biotin antibody (Fig. 1B, right panel). The solubilized HF and GB proteins were then purified from the wheat cell-free reaction. Affinity purification for the solubilized proteins (Fig. 1C) routinely yielded ∼10 μg HF-NS3 and ∼20 μg GB-NS5 proteins from 1 ml of wheat cell-free reaction. Other proteins used for the following experiments were also expressed and purified with same procedure (Fig. 1D).

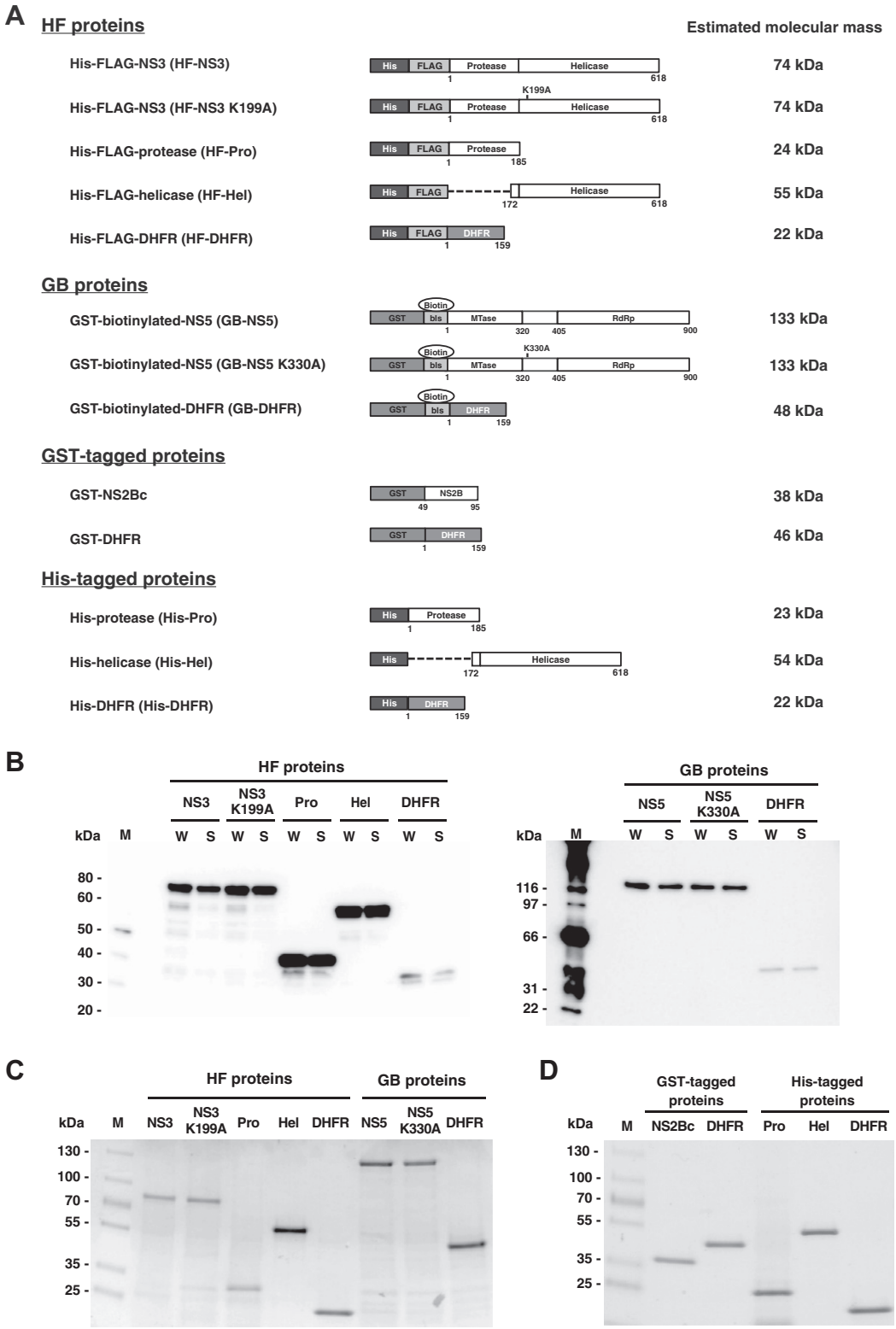

Fig. 1. Production of recombinant DENV-2 NS3 and NS5 proteins by wheat cell-free system. (A) Schematic representation of the recombinant proteins used in this study. Full-length NS3 (HF-NS3), its NTPase domain mutant (HF-NS3 K199A) and truncation mutants (protease domain [HF-Pro] and helicase domain [HF-Hel]) were produced as N-terminal hexahistidine (His)- and FLAG-tagged (HF) proteins. NS3 protease and helicase domains were also produced as His-tagged proteins for competition assay (His-Pro and His-Hel, respectively). Full-length NS5 (GB-NS5) and its single amino acid mutant in RdRp domain (GB-NS5 K330A) were produced as N-terminal GST-tagged and biotinylated (GB) proteins. As control proteins, HF, GB, GST-tagged, and His-tagged versions of DHFR were constructed. For the protease assay of HF-NS3, cofactor region of NS2B was produced as a GST-fused protein (GST-NS2Bc). The predicted molecular weight of each protein was indicated on the right. (B) Expression and solubility of the HF- and GB-proteins. Whole cell-free translation reaction (W) of each recombinant protein was centrifuged at 17,000×*g* for 10 min to obtain the supernatants (S). Four microliters of the samples were subjected to immunoblotting analysis using HRP-conjugated-anti-FLAG antibody (for HF proteins, left panel) and HRP-conjugated-anti-biotin antibody (for GB proteins, right panel). M, molecular weight marker. (C and D) Affinity purification of wheat cell-free-expressed proteins. HF (C) and His-tagged proteins (D) were batch purified using Ni Sepharose beads. Imidazole, which exhibits a strong inhibitory effect on AlphaScreen assay, was removed by further purifying HF proteins with a HiTrap Desalting column. GB proteins (C) and GST-NS2Bc (D) were purified using Glutathione Sepharose beads. Purified protein was resolved by SDS–PAGE gels and visualized by CBB staining. M, molecular weight marker.

Next, we checked whether the wheat cell-free-expressed NS3 and NS5 proteins were functional. For the biochemical activity of NS3, protease assay was performed using the fluorogenic tetrapeptide, Bz-Nle-Lys-Arg-Arg-AMC, as a substrate (Phong et al., 2011; Yusof et al., 2000). It is well known that efficient protease activity of flavivirus NS3 requires NS2B as a cofactor (Arias et al., 1993; Chambers et al., 1991; Falgout et al., 1991, 1993; Jan et al., 1995). In protease assays using wheat cell-free system-derived NS3 protein alone, no enzymatic cleavage of the substrate was observed (Fig. 2A). However, when the cofactor region of DENV-2 NS2B fused with GST (GST-NS2Bc, Fig. 1A and D) was provided *in trans*, HF-NS3 and HF-Pro were found to cleave the fluorogenic substrate (Fig. 2A), consistent with previous reports using *E. coli*-derived NS3 protease domain (Phong et al., 2011; Wu et al., 2003). On the other hand, the trans-complementation of HF-NS3 and HF-Pro was not detected with GST-DHFR, and even in the presence of GST-NS2Bc, neither HF-Hel nor a control protein (HF-DHFR) exhibited any protease activity (Fig. 2A). In addition, when the NTPase activity of the NS3 proteins were measured (Li et al., 1999), HF-NS3 and HF-Hel exhibited substantial levels of hydrolysis of NTPs, while a full-length NS3 in which a single alanine substitution had been introduced to abolish the ATPase/helicase activities (HF-NS3 K199A [Matusan et al., 2001]) as well as HF-Pro showed little NTPase activity, with activity levels equivalent to those in reactions with HF-DHFR or with no recombinant protein (Fig. 2B).

We also verified the RdRp activity of NS5. A fluorescent-based alkaline phosphate-coupled polymerase assay (FAPA, Niyomrattanakit et al., 2011) revealed that the GB-NS5 from wheat cell-free system exhibited *de novo* RNA synthesis activity (Fig. 2C). As observed in previous reports using *E. coli*-derived protein (Zou et al., 2011), a NS5 mutant in which the lysine residue in cavity A of the RdRp domain was substituted with alanine (GB-NS5 K330A) showed a substantial lower albeit significant level of RdRp activity (Fig. 2C). In contrast, no activity was detected in the assay with a negative control protein (GB-DHFR) or no protein (Fig. 2C). Taken together, these results demonstrate that the wheat cell-free system is an effective method for producing correctly folded, functional DENV-2 NS3 and NS5 proteins in a soluble form.

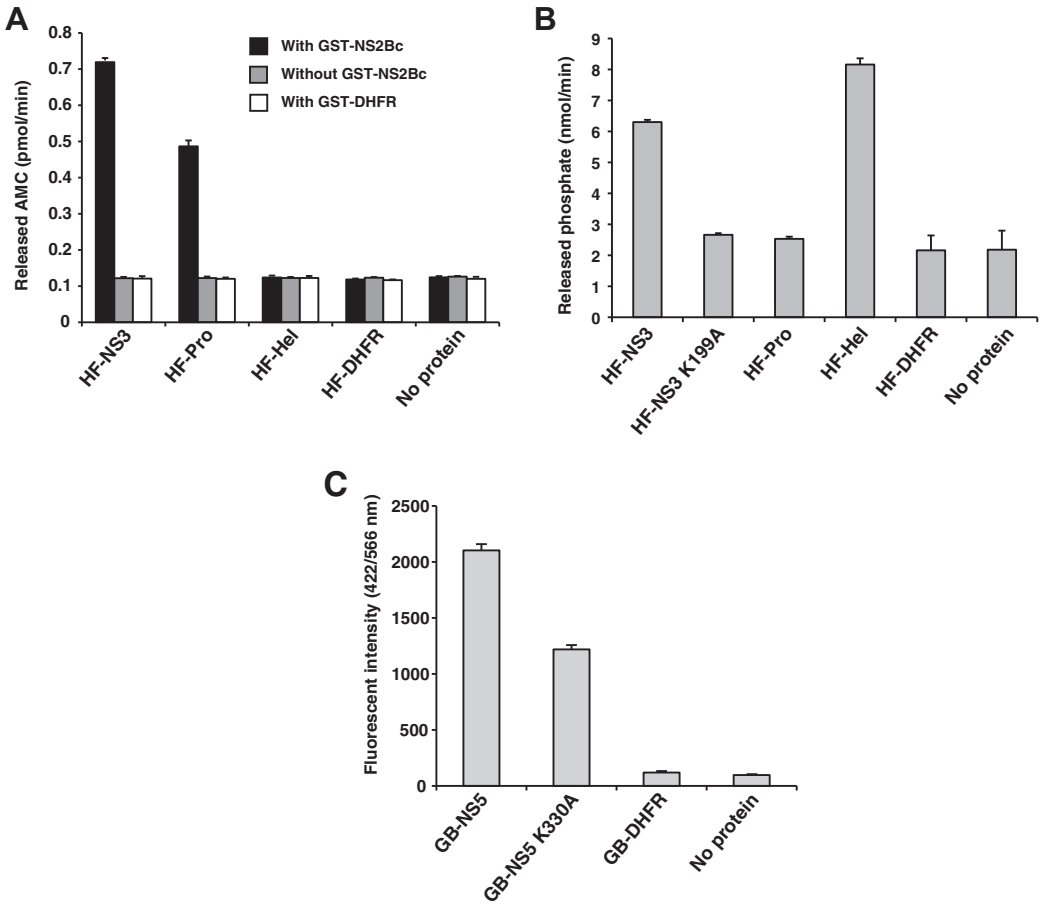

Fig. 2. Enzymatic activities of wheat cell-free system-derived NS3 and NS5 proteins. (A) Protease activity of NS3. The protease assay using a simple fluorogenic tetrapeptide substrate was carried out with 50 μM Bz-Nle-Lys-Arg-Arg-AMC and 200 nM of wheat cell-free-expressed proteins (full-length NS3 [HF-NS3], NS3 protease domain [HF-Pro], NS3 helicase domain [HF-Hel], and control protein [HF-DHFR]) in the presence (black bars) or absence (grey bars) of GST-fused NS2Bc and in the presence of DHFR (white bars). Protease activity of each protein was determined by measuring the amount of released AMC at excitation (ex) wavelength of 390 nm and an emission (em) wavelength of 460 nm. The amount of the released AMC in each reaction was quantified with an AMC standard. Enzymatic activity is represented as moles of free AMC released per minute. (B) NTPase activity of NS3. One hundred nanomolar of wheat cell-free-expressed proteins were incubated with 1 mM NTPs. After addition of ammonium molybdate, colorimetric detection of orthophosphate released by the hydrolysis of NTPs was carried out by the absorbance at 820 nm using a monochrometer. The amount of free phosphate in each reaction was quantified with a phosphate standard. Enzymatic activity is represented as moles of free phosphate released per minute. (C) RdRp activity of NS5. A fluorescence-based alkaline phosphatase-coupled polymerase assay (FAPA) was carried out with a hairpin RNA substrate containing DENV 3′UTR sequence (3′UTR-U_{30}) and BBT fluorophore group-conjugated ATP (BBT-ATP) in the presence (or absence) of 60 nM wheat cell-free-expressed full-length NS5 (GB-NS5), its single amino acid mutant (GB-NS5 K330A), and a control (GB-DHFR) protein. Subsequently, CIP was added to dephosphorylate BBT_{PPi}, which had been released from BBT-ATP by polymerization, and fluorescent BBT molecule derived from BBT_{PPi} by-products was measured at ex/em 422/566 nm. All data are expressed as mean value of three independent experiments with error bars indicating standard deviations. In (C), the *P* value was determined by Student's *t*-test.

3.2. Establishment of NS3–NS5 interaction assay using AlphaScreen technology

In order to validate the interaction between NS3 and NS5, both of which were derived from the wheat cell-free system, GB-NS5 was incubated with either HF-NS3 or a control protein (HF-DHFR), and the reaction was subjected to immunoprecipitation using anti-FLAG antibody. As with previous reports (Kapoor et al., 1995; Zou et al., 2011), GB-NS5 interacted with HF-NS3, but not with HF-DHFR (Fig. 3A, lanes 1 and 3). A parallel experiment showed that control IgG did not immunoprecipitate the NS3/NS5 complex (Fig. 3A, lane 2).

We next applied the AlphaScreen technology to measure the NS3–NS5 interaction. AlphaScreen assay is a luminescence-based binding assay, where singlet oxygen ($O_2(^1D_g)$) is generated from donor beads coupled to the target protein upon illumination, and the chemical energy of the singlet oxygen then diffuses to the acceptor beads on interaction partners, resulting in a luminescence signal (Ullman et al., 1994). In our assay, the FLAG-tag of NS3 and the biotin of NS5 were selected to be specifically recognized with anti-FLAG antibody-conjugated acceptor beads and streptavidin-coated donor beads, respectively (Fig. 3B). When a fixed concentration (100 nM) of HF-NS3 was used, an increase in the luminescent signal was observed with a GB-NS5 concentration of up to 500 nM (Fig. 3C), but the signal almost saturated once the GB-NS5 concentration exceeded 500 nM (data not shown). In comparison, the incubation of GB-NS5 with a control HF protein (HF-DHFR) resulted in a negligible background signal (Fig. 3C).

In order to assess the accuracy of this AlphaScreen assay, the value of the Z' factor was calculated, which is defined as: $Z' = 1 - (3\sigma_{c+} + 3\sigma_{c-})/|\mu_{c+} - \mu_{c-}|$, where σ and μ represent the standard deviations and the mean value, respectively, of the positive control (c^+) and the negative control (c^-) samples (Zhang et al., 1999). The Z' factor was obtained from twenty independent positive control reactions containing 100 nM HF-NS3 and 100 nM GB-NS5 and negative control reactions containing 100 nM HF-DHFR and 100 nM GB-NS5 and was determined to be 0.71 (Fig. 3D). This indicates a good overall quality for our AlphaScreen assay for the detection of the NS3 and NS5 interaction as a Z' factor value above 0.5 is indicative of a quality screen with a well-defined hit window and the required robustness for high-throughput screening applications (Zhang et al., 1999).

3.3. Disruption of NS3–NS5 interaction by NS3 domains

Several studies have shown that the NS3 helicase domain participates in the interaction with NS5, and the responsible region in NS5 was mapped to the region between amino acid residues 320 and 368 (Johansson et al., 2001; Moreland et al., 2012; Zou et al., 2011). One amino acid substitution at Lys-330 to alanine in

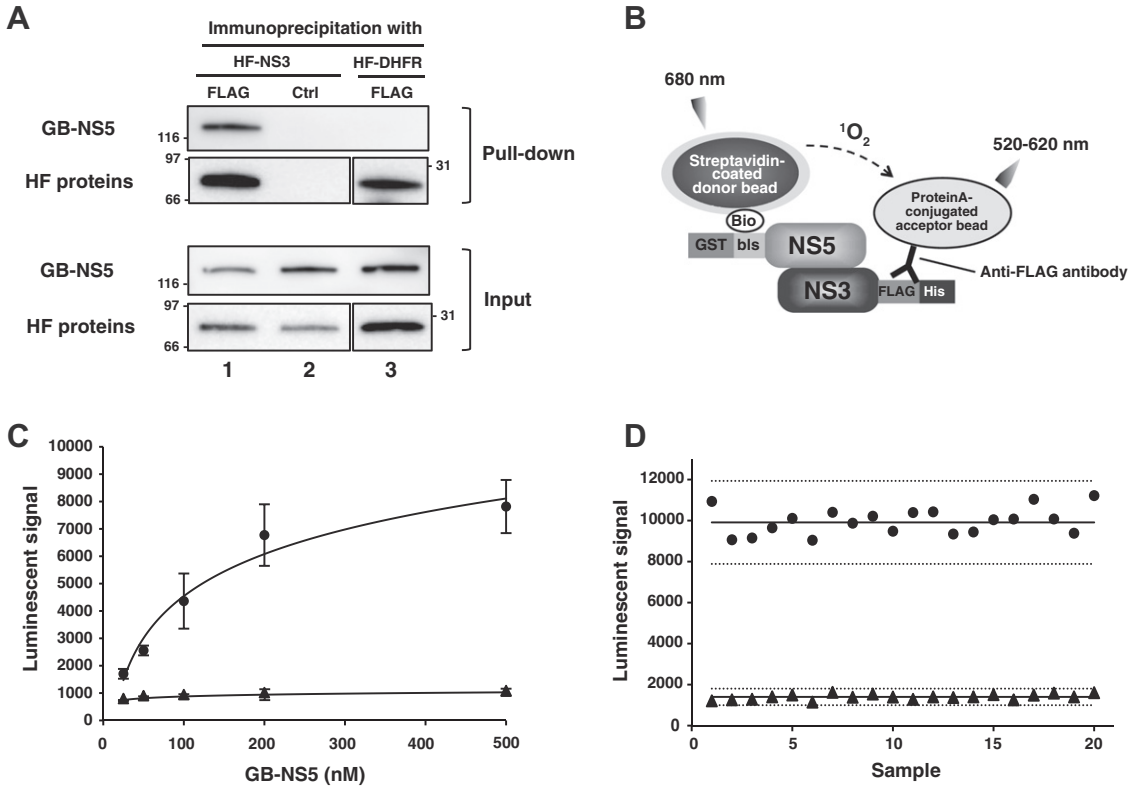

Fig. 3. AlphaScreen assay for NS3 and NS5 interaction. (A) Co-immunoprecipitation analysis of NS3–NS5 interaction. Two-hundred nanomolar of GB-NS5 was incubated with the same concentration of HF-NS3 (lanes 1 and 2) or HF-DHFR (lane 3), and subsequently immunoprecipitated using anti-FLAG antibody (lanes 1 and 3) and control mouse IgG (lane 2). HF-proteins and GB-NS5 in immunoprecipitates were detected by immunoblotting using HRP-conjugated-anti-FLAG antibody and HRP-conjugated-anti-biotin antibody, respectively. Mass of molecular weight standard in each blot is indicated at left (for NS proteins) and right (for DHFR). (B) Schematic diagram of AlphaScreen assay to detect the binding of NS3 and NS5. HF-NS3 and GB-NS5 interaction bridges the anti-FLAG antibody-conjugated acceptor bead and streptavidin-coated donor bead through the recognition of N-terminal FLAG-tag of NS3 and N-terminal biotin of NS5, respectively. Upon excitation at 680 nm, singlet oxygen molecules are produced from the donor beads, which react with the acceptor beads, resulting in light emission measured between 520 and 620 nm. (C) Determination of optimal AlphaScreen assay conditions for NS3–NS5 interaction. A fixed concentration (100 nM) of HF-NS3 (circles) or HF-DHFR (triangles) was incubated with an increasing concentration (25–500 nM) of GB-NS5 in a 384-well plate, followed by addition of anti-FLAG antibody, protein A-conjugated acceptor beads, and streptavidin-coated donor beads to detect the interaction by AlphaScreen method. The error bars represent standard deviations from three independent experiments. (D) Validation of the quality of NS3–NS5 AlphaScreen assay. The AlphaScreen assay was performed using 100 nM GB-NS5 with 100 nM HF-NS3 (positive control, $n = 20$, circles) and HF-DHFR (negative control, $n = 20$, triangles). The solid lines represent the mean of the positive and negative controls, and the dashed lines are 3 standard deviations of each control, which were used for Z' factor calculations.

the RdRp domain of NS5 was also shown to reduce its binding to NS3 and the NS3 helicase domain *in vitro* (Zou et al., 2011). When this NS5 mutant (GB-NS5 K330A) was used in our AlphaScreen assay for the interaction with full-length NS3, we observed a reduced but still substantial level of binding activity as compared to wild-type GB-NS5 (Fig. 4A), implying that there is another mode of binding for the NS3–NS5 interaction in addition to binding through the NS3 helicase domain. In order to assess the contribution of the NS3 protease domain to the interaction with NS5, the AlphaScreen assay was performed using domain mutants of HF-NS3 and wild-type GB-NS5. Although incubation of GB-NS5 with the helicase domain of NS3 (HF-Hel) exhibited AlphaScreen signal, protease domain of NS3 (HF-Pro) was also found to interact with GB-NS5 (Fig. 4B). Similar to the interaction with full-length NS3, a dose-dependent increase of the interaction signal with NS5 was observed with HF-Pro and HF-Hel at the concentrations of up to 500 nM (Fig. 4B). The luminescent signal generated with 100 nM HF-Pro was approximately 1.42-fold higher than that obtained using 100 nM HF-Hel (Fig. 4B). An immunoprecipitation assay using anti-FLAG antibody also showed that as with the case of full-length NS3 (Fig. 4C, lane 1), GB-NS5 was pulled down with HF-Pro (Fig. 4C, lane 2) as well as HF-Hel (Fig. 4C, lane 3), but

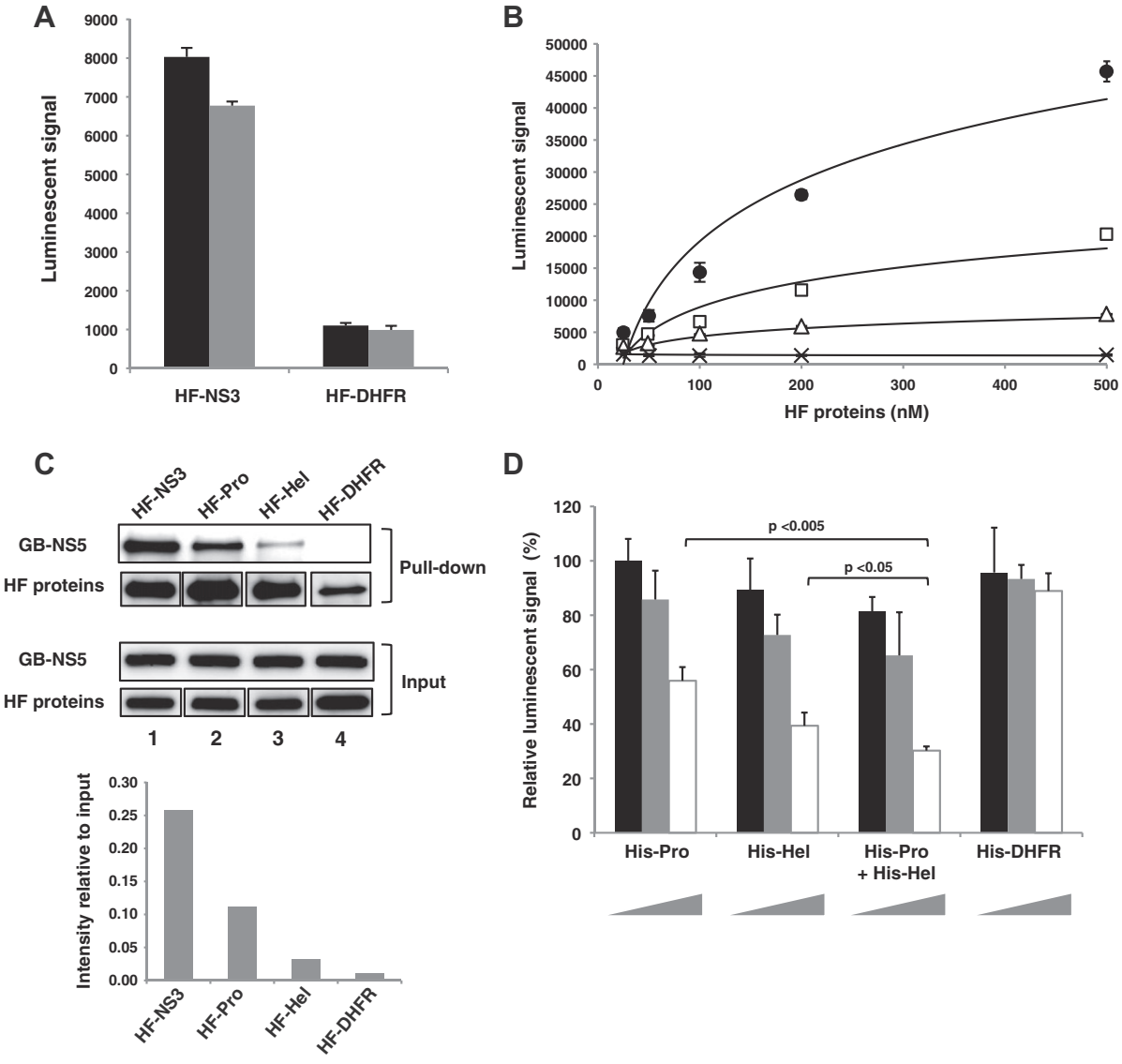

Fig. 4. Determination of binding domains of NS3 for the interaction with NS5. (A) Interaction efficiency of NS3 with an NS5 mutant. The AlphaScreen assay for the binding of full-length NS3 (HF-NS3, 100 nM) was performed with 100 nM wild-type NS5 (GB-NS5, black bars) and a single amino acid mutant of NS5 (GB-NS5 K330A, grey bars). As a control protein for HF-NS3, 100 nM HF-DHFR was used. (B) Dose-dependent binding of NS3 domains to NS5. An increasing concentration (25–500 nM) of HF-NS3 (closed circles), HF-Pro (open squares), HF-Hel (open triangles), or HF-DHFR (crosses) was incubated with 100 nM GB-NS5, and the reactions were subjected to AlphaScreen assay. The data are mean values from three independent experiments. The error bars for HF-NS3 represent standard deviations (200–1600), but in the assays with HF-Pro, HF-Hel, and HF-DHFR, all standard deviations were below 500. (C) Co-immunoprecipitation analysis of NS3 domain mutants with NS5. GB-NS5 was incubated with HF-NS3 (lane 1), HF-Pro (lane 2), HF-Hel (lane 3) and HF-DHFR (lane 4), and then subjected to immunoprecipitation assay using anti-FLAG antibody. HF-proteins and GB-NS5 in immunoprecipitates were detected by immunoblotting using HRP-conjugated-anti-FLAG antibody and HRP-conjugated-anti-biotin antibody respectively. Intensity of bands was quantified, and ratio of immunoprecipitates to input GB-NS5 is shown (bottom panel). (D) Competition of the NS3–NS5 interaction by NS3 domains. AlphaScreen assay using HF-NS3 and GB-NS5 (100 nM each) was performed in the presence of different concentrations (37.5 nM [black bars], 150 nM [grey bars], and 600 nM [white bars]) of His-tagged protease domain (His-Pro), helicase domain (His-Hel) or control protein (His-DHFR). In parallel experiments, equal molar concentrations of His-Pro and His-Hel (total 37.5, 150, and 600 nM) were simultaneously added to an AlphaScreen reaction containing HF-NS3 and GB-NS5. Results are presented as a percentage of the luminescent signal obtained by the assay without the competitor protein (i.e. AlphaScreen assay only with HF-NS3 and GB-NS5). In (A) and (D), all data are expressed as mean values of three independent experiments with error bars indicating standard deviations. The *P* value was determined by Student's *t*-test.

not with HF-DHFR (Fig. 4C, lane 4). Consistent with the results of the AlphaScreen assay, quantification of the relative amounts of GB-NS5 co-immunoprecipitated by various HF proteins revealed that NS5 precipitation by HF-Pro was more efficient than that by HF-Hel (Fig. 4C, bottom panel). These data indicate that protease and helicase domains of NS3 are coordinately involved in the binding to NS5.

Finally, we performed a competition assay to examine whether the protease and helicase domains of NS3 could serve as molecular inhibitors against the NS3–NS5 interaction. AlphaScreen assay using HF-NS3 and GB-NS5 was carried out in the presence of four different concentrations (0, 37.5, 150 and 600 nM) of NS3 protease (His-Pro) or helicase (His-Hel) domains, which did not contain the AlphaScreen-detectable FLAG-tag. The results showed that His-Pro and His-Hel significantly inhibited the interaction between HF-NS3 and GB-NS5 in a dose-dependent manner, whereas no inhibitory effect was observed with any concentration of His-DHFR (Fig. 4D) Interestingly, this inhibitory effect was augmented in the reaction containing both the His-Pro and His-Hel (Fig. 4D). This indicates some cooperative action exists between each NS3 domain to the NS3–NS5 interaction.

4. Discussion

The wheat cell-free system is shown to permit the synthesis of 'difficult-to-express' proteins that undergo proteolysis, insoluble aggregation, or codon usage bias in conventional *E. coli* expression systems (Sawasaki et al., 2002; Goshima et al., 2008). It has been reported that the N-terminal protease domain of DENV NS3 is poorly soluble in *E. coli* cells (Leung et al., 2001; Phong et al., 2011), and as such previous studies employed the fusion of either full-length NS3 or NS3 protease domain with the NS2B cofactor region (Luo et al., 2008; Moreland et al., 2010, 2012); or used refolding procedures after purification under denaturing conditions (Phong et al., 2011; Wu et al., 2003; Yusof et al., 2000). In this study, we were able to simplify the process of expressing recombinant DENV NS3 and NS5 proteins by the wheat cell-free system, which succeeded in producing the full-length and protease domains of NS3 in soluble form even without the NS2B cofactor or a denaturing step (Fig. 1). In addition, correct folding of the wheat cell-free-derived NS3 and NS5 proteins was evident by their enzymatic activity assay (Fig. 2). Although the biochemical features of the NS proteins produced by wheat cell-free system and *E. coli* expression systems were not directly compared in this study, our results suggest that the wheat cell-free system can offer certain advantages in the production of recombinant DENV proteins over conventional protein expression systems. The production of DENV NS3 by wheat cell-free system would be also favorable for structural analysis of this protein in its full-length state. So far, the crystal structure of DENV NS3 was determined by using an artificial NS3 fused with the hydrophilic region of NS2B through a flexible glycine linker due to the insoluble nature of NS3 (Erbel et al., 2006; Luo et al., 2008). Since the cofactor region of NS2B produced by wheat cell-free system was able to activate the protease activity of NS3 *in trans* (Fig. 2A [Falgout et al., 1991; Phong et al., 2011; Wu et al., 2003]), the wheat cell-free system may make it feasible to determine the structure of the active NS3 complexed with NS2B to gain further insights into the proteolytic activity of NS3.

By using the functional recombinant proteins, we have established an AlphaScreen-based binding assay to detect the NS3–NS5 interaction (Fig. 3). Robustness of the NS3–NS5 AlphaScreen assay was shown by its good Z' factor (>0.7), calculated from 20 independent reactions using HF-NS3 and GB-NS5 (Fig. 3D). As an assay with a Z' factor higher than 0.5 is considered to be suitable to high-throughput screening studies (Zhang et al., 1999), the assay platform established in this study will be used to search for inhibitors of NS3 and NS5 interactions *in vitro*. In antiviral drug discovery, the identification of small chemical compounds disrupting protein–protein interactions is considerably more challenging than identification of conventional drugs designed to target viral enzymes due to the relatively large surface area of the interaction interface between two proteins (Lo Conte et al., 1999). However, structural studies over the past two decades demonstrate that, within the entire surface, there are small subsets of the protein surface called 'hot-spots' that participate in the high-affinity binding of protein–protein interactions (Arkin and Wells, 2004). Hence, it is becoming evident that the hot-spots of protein–protein interfaces are suitable targets for novel therapeutic drugs (Arkin and Wells, 2004; Buchwald, 2010; Pagliaro et al., 2004; Ryan and Matthews, 2005) Although the X-ray structure of NS3 complexed with NS5 is not available, previous studies with truncated proteins and site-directed mutagenesis of DENV NS5 have reported that only a limited region of the protein is likely to be involved in the interaction with NS3 (Johansson et al., 2001; Zou et al., 2011). Thus, it may be possible to develop small compounds disrupting the NS3–NS5 interaction. To this end, a screening study using our AlphaScreen assay for small-chemical compound inhibitors targeting the NS3–NS5 interaction is currently underway in our laboratory. In Supplementary Fig. 1, we have shown a pilot screen using a collection of small compounds, including US Food and Drug Administration (FDA)-approved molecules. Taken together with the competitive action of NS3 domains on the NS3–NS5 interaction (Fig. 4D), the present study provides a proof-of-concept of the application of our AlphaScreen assay to the discovery of antiviral agents disrupting DENV NS interaction.

One question to ponder is which domains of the DENV NS3 are involved in the NS3–NS5 complex formation? A previous report using yeast two-hybrid assay revealed that the binding regions are mapped to the C-terminal half of NS3 (amino acid residues 303–618) and the N-terminal part of the NS5 RdRp domain (amino acid residues 320–368) (Johansson et al., 2001). However, the report did not detect any binding activity with the N-terminal half of NS3 (amino acid residues 1–303) containing the protease domain (Johansson et al., 2001). In addition, a recent study using ELISA-based binding assay showed that when the NS5 RdRp domain was coated on an immunoplate, NS3 protease domain fused with the hydrophilic cofactor region of NS2B ($NS2B_{CF47}NS3_{pro}$) did not bind to the truncated version of NS5 protein, while the interaction of NS5 RdRp domain with full-length NS3 could be detected (Moreland et al., 2012). It is possible that additional residues from 171 to 303 in the N-terminal half of NS3 and the artificially linker NS2BCF47 sequence in $NS2B_{CF47}NS3_{pro}$ may have inhibited the interaction in the yeast two-hybrid and ELISA-based assay. On the other hand, the contribution of the protease domain to the NS3–NS5 interaction has been also proposed. A surface plasmon resonance assay by Zou et al. revealed that the dissociation rate constant (K_D) of the interaction of full-length NS5 with NS3 helicase domain was 4.4-fold higher than that of the interaction with full-length NS3, indicating that the binding affinity of NS3–NS5 interaction is enhanced by another domain (i.e. protease domain) of NS3 (Zou et al., 2011). Interestingly, reduced DENV-2 replication caused by three amino acids substitutions in the MTase domain of NS5 (Glu-192, Lys-193, and Glu-195), which retained sufficient levels of its enzymatic activity *in vitro*, was restored by an additional single amino acid substitution (Ala-70) in the protease domain of NS3 (Kroschewski et al., 2008). This also indicates a genetic interaction between the NS3 protease and NS5 MTase domains. Given the previous results, one could envisage that the NS3 protease domain and NS5 MTase domain form parts of the binding regions for the NS3–NS5 interaction (Kroschewski et al., 2008; Zou et al., 2011). Supporting this possibility, our AlphaScreen assay demonstrates that the N-terminal protease domain as well as

C-terminal helicase domain of NS3 is able to interact with NS5 (Fig. 4A). Involvement of both NS3 domains in the NS3–NS5 interaction was evident in an immunoprecipitation assay (Fig. 4C). More importantly, an AlphaScreen-based competition assay using His-Pro and His-Hel showed an augmented inhibition of the full-length NS3–NS5 interaction by co-incubation with competitive protease and helicase domains of NS3 (Fig. 4D). Taken together, it can be speculated that the NS3–NS5 complex is organized by two distinct (N-terminal and C-terminal) domains in the respective NS protein: interactions through (i) NS3 protease domain and NS5 MTase domain, and (ii) NS3 helicase domain and NS5 RdRp domain. As expected, when the MTase domain of NS5, produced by wheat cell-free system, was used for AlphaScreen assay with NS3 domains, considerable levels of luminescent signal were obtained with NS3 protease domain as well as full-length NS3, but not with the helicase domain, indicating the direct binding of NS3 protease domain and NS5 MTase domain (Supplementary Fig. 2). Further experiments will unveil the molecular detail of the interaction between DENV NS3 and NS5. In addition, it will be intriguing to examine by our AlphaScreen assay whether the Glu-192/Lys-193/Glu-195 mutations in NS5 MTase domain leads to reduced interaction with NS3, which would be restored by the second site mutation (Ala-70) in NS3 protease domain (Kroschewski et al., 2008).

Aside from the binding to NS5, DENV NS3 has also been shown to associate with NS2B (Arias et al., 1993) and NS4B (Umareddy et al., 2006). Hence, these NS proteins may affect the function of the NS3–NS5 complex via interaction with NS3. Of particular interest is that NS4B is reported to dissociate NS3 from single-stranded RNA, and subsequently elevate the helicase activity of NS3 (Umareddy et al., 2006). Given the previous data showing that the NS3's NTPase activity, which is a part of helicase function, is increased by its interaction with NS5 (Cui et al., 1998; Yon et al., 2005), it is possible that NS4B and NS5 collaborate to form a complex with NS3 in order to enhance the efficiency of viral RNA synthesis during DENV replication. If this is the case, the association of NS2B with NS3 may induce a conformational change in NS3 (or the NS3–NS5 complex), leading to an activation of the protease activity of NS3 that is required for subsequent processing of the viral polyprotein precursor. Therefore, the influence of NS2B and NS4B on the regulation of the NS3–NS5 complex would be a fascinating issue to investigate. Additionally, these interactions could also be attractive targets for the development of new antivirals against DENV, to which our AlphaScreen assay should be applicable. In conclusion, our new assay system is not only helpful in elucidating a better understanding of the molecular mechanisms underlying the NS3–NS5 interaction, but also useful for future high-throughput screenings to develop protein–protein interaction inhibitors targeting the DENV NS proteins.

Acknowledgements

We thank Yaeta Endo (Ehime University) for support in the wheat cell-free protein synthesis and AlphaScreen assay, and Danny N.P. Doan (Duke-NUS) for support in the DENV experiments. We are also grateful to Wei Xin Chin for proofreading on the manuscript and to members of laboratory for helpful discussions. This work was supported by NUS SoM Start-up Grant (R-182-000-160-733, R-182-000-160-133) to NY, and partly supported by National Medical Research Council, Singapore (http://www.nmrc.gov.sg) under grant (NMRC/1315/2011) to SGV.

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.antiviral.2012.09.023.

References

Arias, C.F., Preugschat, F., Strauss, J.H., 1993. Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology 193, 888–899.
Arkin, M.R., Wells, J.A., 2004. Small-molecule inhibitors of protein–protein interactions: progressing towards the dream. Nat. Rev. Drug Discov. 3, 301–317.
Bartholomeusz, A.I., Wright, P.J., 1993. Synthesis of dengue virus RNA *in vitro*: initiation and the involvement of proteins NS3 and NS5. Arch. Virol. 128, 111–121.
Buchwald, P., 2010. Small-molecule protein–protein interaction inhibitors: therapeutic potential in light of molecular size, chemical space, and ligand binding efficiency considerations. IUBMB Life 62, 724–731.
Chambers, T.J., Grakoui, A., Rice, C.M., 1991. Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J. Virol. 65, 6042–6050.
Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.
Chen, C.J., Kuo, M.D., Chien, L.J., Hsu, S.L., Wang, Y.M., Lin, J.H., 1997. RNA-protein interactions: involvement of NS3, NS5, and 3′ noncoding regions of Japanese encephalitis virus genomic RNA. J. Virol. 71, 3466–3473.
Chu, P.W., Westaway, E.G., 1985. Replication strategy of Kunjin virus: evidence for recycling role of replicative form RNA as template in semiconservative and asymmetric replication. Virology 140, 68–79.
Chu, P.W., Westaway, E.G., 1987. Characterization of Kunjin virus RNA-dependent RNA polymerase: reinitiation of synthesis *in vitro*. Virology 157, 330–337.
Cui, T., Sugrue, R.J., Xu, Q., Lee, A.K., Chan, Y.C., Fu, J., 1998. Recombinant dengue virus type 1 NS3 protein exhibits specific viral RNA binding and NTPase activity regulated by the NS5 protein. Virology 246, 409–417.
Egloff, M.P., Decroly, E., Malet, H., Selisko, B., Benarroch, D., Ferron, F., Canard, B., 2007. Structural and functional analysis of methylation and 5′-RNA sequence requirements of short capped RNAs by the methyltransferase domain of dengue virus NS5. J. Mol. Biol. 372, 723–736.
Erbel, P., Schiering, N., D'Arcy, A., Renatus, M., Kroemer, M., Lim, S.P., Yin, Z., Keller, T.H., Vasudevan, S.G., Hommel, U., 2006. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. Nat. Struct. Mol. Biol. 13, 372–373.
Falgout, B., Miller, R.H., Lai, C.J., 1993. Deletion analysis of dengue virus type 4 nonstructural protein NS2B: identification of a domain required for NS2B–NS3 protease activity. J. Virol. 67, 2034–2042.
Falgout, B., Pethel, M., Zhang, Y.M., Lai, C.J., 1991. Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J. Virol. 65, 2467–2475.
Goshima, N., Kawamura, Y., Fukumoto, A., Miura, A., Honma, R., Satoh, R., Wakamatsu, A., Yamamoto, J., Kimura, K., Nishikawa, T., Andoh, T., Iida, Y., Ishikawa, K., Ito, E., Kagawa, N., Kaminaga, C., Kanehori, K., Kawakami, B., Kenmochi, K., Kimura, R., Kobayashi, M., Kuroita, T., Kuwayama, H., Maruyama, Y., Matsuo, K., Minami, K., Mitsubori, M., Mori, M., Morishita, R., Murase, A., Nishikawa, A., Nishikawa, S., Okamoto, T., Sakagami, N., Sakamoto, Y., Sasaki, Y., Seki, T., Sono, S., Sugiyama, A., Sumiya, T., Takayama, T., Takayama, Y., Takeda, H., Togashi, T., Yahata, K., Yamada, H., Yanagisawa, Y., Endo, Y., Imamoto, F., Kisu, Y., Tanaka, S., Isogai, T., Imai, J., Watanabe, S., Nomura, N., 2008. Human protein factory for converting the transcriptome into an *in vitro*-expressed proteome. Nat. Methods 5, 1011–1017.
Jan, L.R., Yang, C.S., Trent, D.W., Falgout, B., Lai, C.J., 1995. Processing of Japanese encephalitis virus non-structural proteins: NS2B–NS3 complex and heterologous proteases. J. Gen. Virol. 76, 573–580.
Johansson, M., Brooks, A.J., Jans, D.A., Vasudevan, S.G., 2001. A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-β and the viral helicase, NS3. J. Gen. Virol. 82, 735–745.
Kapoor, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ebner, K.E., Padmanabhan, R., 1995. Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J. Biol. Chem. 270, 19100–19106.
Kroschewski, H., Lim, S.P., Butcher, R.E., Yap, T.L., Lescar, J., Wright, P.J., Vasudevan, S.G., Davidson, A.D., 2008. Mutagenesis of the dengue virus type 2 NS5 methyltransferase domain. J. Biol. Chem. 283, 19410–19421.
Kuo, M.D., Chin, C., Hsu, S.L., Shiao, J.Y., Wang, T.M., Lin, J.H., 1996. Characterization of the NTPase activity of Japanese encephalitis virus NS3 protein. J. Gen. Virol. 77, 2077–2084.
Lam, A.M., Keeney, D., Eckert, P.Q., Frick, D.N., 2003. Hepatitis C virus NS3 ATPases/helicases from different genotypes exhibit variations in enzymatic properties. J. Virol. 77, 3950–3961.
Lescar, J., Luo, D., Xu, T., Sampath, A., Lim, S.P., Canard, B., Vasudevan, S.G., 2008. Towards the design of antiviral inhibitors against flaviviruses: the case for the multifunctional NS3 protein from Dengue virus as a target. Antiviral Res. 80, 94–101.
Leung, D., Schroder, K., White, H., Fang, N.X., Stoermer, M.J., Abbenante, G., Martin, J.L., Young, P.R., Fairlie, D.P., 2001. Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors. J. Biol. Chem. 276, 45762–45771.
Li, H., Clum, S., You, S., Ebner, K.E., Padmanabhan, R., 1999. The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. J. Virol. 73, 3108–3116.

Lindenbach, B.D., Rice, C.M., 2003. Molecular biology of flaviviruses. Adv. Virus Res. 59, 23–61.
Lo Conte, L., Chothia, C., Janin, J., 1999. The atomic structure of protein–protein recognition sites. J. Mol. Biol. 285, 2177–2198.
Luo, D., Xu, T., Hunke, C., Gruber, G., Vasudevan, S.G., Lescar, J., 2008. Crystal structure of the NS3 protease–helicase from dengue virus. J. Virol. 82, 173–183.
Matusan, A.E., Pryor, M.J., Davidson, A.D., Wright, P.J., 2001. Mutagenesis of the Dengue virus type 2 NS3 protein within and outside helicase motifs: effects on enzyme activity and virus replication. J. Virol. 75, 9633–9643.
Moreland, N.J., Tay, M.Y., Lim, E., Paradkar, P.N., Doan, D.N., Yau, Y.H., Geifman Shochat, S., Vasudevan, S.G., 2010. High affinity human antibody fragments to dengue virus non-structural protein 3. PLoS Negl. Trop. Dis. 4, e881.
Moreland, N.J., Tay, M.Y., Lim, E., Rathore, A.P., Lim, A.P., Hanson, B.J., Vasudevan, S.G., 2012. Monoclonal antibodies against dengue NS2B and NS3 proteins for the study of protein interactions in the flaviviral replication complex. J. Virol. Methods 179, 97–103.
Ng, C.Y., Gu, F., Phong, W.Y., Chen, Y.L., Lim, S.P., Davidson, A., Vasudevan, S.G., 2007. Construction and characterization of a stable subgenomic dengue virus type 2 replicon system for antiviral compound and siRNA testing. Antiviral Res. 76, 222–231.
Niyomrattanakit, P., Abas, S.N., Lim, C.C., Beer, D., Shi, P.Y., Chen, Y.L., 2011. A fluorescence-based alkaline phosphatase-coupled polymerase assay for identification of inhibitors of dengue virus RNA-dependent RNA polymerase. J. Biomol. Screen. 16, 201–210.
Pagliaro, L., Felding, J., Audouze, K., Nielsen, S.J., Terry, R.B., Krog-Jensen, C., Butcher, S., 2004. Emerging classes of protein–protein interaction inhibitors and new tools for their development. Curr. Opin. Chem. Biol. 8, 442–449.
Phong, W.Y., Moreland, N.J., Lim, S.P., Wen, D., Paradkar, P.N., Vasudevan, S.G., 2011. Dengue protease activity: the structural integrity and interaction of NS2B with NS3 protease and its potential as a drug target. Biosci. Rep. 31, 399–409.
Preugschat, F., Yao, C.W., Strauss, J.H., 1990. *In vitro* processing of dengue virus type 2 nonstructural proteins NS2A, NS2B, and NS3. J. Virol. 64, 4364–4374.
Ryan, D.P., Matthews, J.M., 2005. Protein–protein interactions in human disease. Curr. Opin. Struct. Biol. 15, 441–446.
Sampath, A., Padmanabhan, R., 2009. Molecular targets for flavivirus drug discovery. Antiviral Res. 81, 6–15.
Sawasaki, T., Ogasawara, T., Morishita, R., Endo, Y., 2002. A cell-free protein synthesis system for high-throughput proteomics. Proc. Natl. Acad. Sci. USA 99, 14652–14657.
Sawasaki, T., Kamura, N., Matsunaga, S., Saeki, M., Tsuchimochi, M., Morishita, R., Endo, Y., 2008. Arabidopsis HY5 protein functions as a DNA-binding tag for purification and functional immobilization of proteins on agarose/DNA microplate. FEBS Lett. 582, 221–228.
Tan, B.H., Fu, J., Sugrue, R.J., Yap, E.H., Chan, Y.C., Tan, Y.H., 1996. Recombinant dengue type 1 virus NS5 protein expressed in *Escherichia coli* exhibits RNA-dependent RNA polymerase activity. Virology 216, 317–325.
Ullman, E.F., Kirakossian, H., Singh, S., Wu, Z.P., Irvin, B.R., Pease, J.S., Switchenko, A.C., Irvine, J.D., Dafforn, A., Skold, C.N., Wagner, D.B., 1994. Luminescent oxygen channeling immunoassay: measurement of particle binding kinetics by chemiluminescence. Proc. Natl. Acad. Sci. USA 91, 5426–5430.
Umareddy, I., Chao, A., Sampath, A., Gu, F., Vasudevan, S.G., 2006. Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA. J. Gen. Virol. 87, 2605–2614.
Wu, C.F., Wang, S.H., Sun, C.M., Hu, S.T., Syu, W.J., 2003. Activation of dengue protease autocleavage at the NS2B–NS3 junction by recombinant NS3 and GST-NS2B fusion proteins. J. Virol. Methods 114, 45–54.
Yap, T.L., Xu, T., Chen, Y.L., Malet, H., Egloff, M.P., Canard, B., Vasudevan, S.G., Lescar, J., 2007. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J. Virol. 81, 4753–4765.
Yon, C., Teramoto, T., Mueller, N., Phelan, J., Ganesh, V.K., Murthy, K.H., Padmanabhan, R., 2005. Modulation of the nucleoside triphosphatase/RNA helicase and 5′-RNA triphosphatase activities of Dengue virus type 2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. J. Biol. Chem. 280, 27412–27419.
Yusof, R., Clum, S., Wetzel, M., Murthy, H.M., Padmanabhan, R., 2000. Purified NS2B/NS3 serine protease of dengue virus type 2 exhibits cofactor NS2B dependence for cleavage of substrates with dibasic amino acids *in vitro*. J. Biol. Chem. 275, 9963–9969.
Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen. 4, 67–73.
Zhang, L., Mohan, P.M., Padmanabhan, R., 1992. Processing and localization of Dengue virus type 2 polyprotein precursor NS3–NS4A–NS4B–NS5. J. Virol. 66, 7549–7554.
Zou, G., Chen, Y.L., Dong, H., Lim, C.C., Yap, L.J., Yau, Y.H., Shochat, S.G., Lescar, J., Shi, P.Y., 2011. Functional analysis of two cavities in flavivirus NS5 polymerase. J. Biol. Chem. 286, 14362–14372.