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Research Article

Mutagenesis of D80-82 and G83 Residues in West Nile Virus NS2B: Effects on NS2B-NS3 Activity and Viral Replication*

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Flaviviral NS2B is a required cofactor for NS3 serine protease activity and plays an important role in promoting functional NS2B-NS3 protease configuration and maintaining critical interactions with protease catalysis substrates. The residues D^{80}DDG in West Nile virus (WNV) NS2B are important for protease activity. To investigate the effects of D^{80}DDG in NS2B on protease activity and viral replication, the negatively charged region D^{80}DD and the conserved residue G83 of NS2B were mutated (D^{80}DD/E^{80}EE, D^{80}DD/K^{80}KK, D^{80}DD/A^{80}AA, G83F, G83S, G83D, G83K, and G83A), and NS3 D75A was designated as the negative control. The effects of the mutations on NS2B-NS3 activity, viral translation, and viral RNA replication were analyzed using kinetic analysis of site-directed enzymes and a transient replicon assay. All substitutions resulted in significantly decreased enzyme activity and blocked RNA replication. The negative charge of D^{80}DD is not important for maintaining NS2B function, but side chain changes in G83 have dramatic effects on protease activity and RNA replication. These results demonstrate that NS2B is important for viral replication and that D^{80}DD and G83 substitutions prevent replication; they will be useful for understanding the relationship between NS2B and NS3.

West Nile virus; NS2B; Protease; RNA replication

West Nile virus (WNV) belongs to the *Flavivirus* genus, which is composed of a single-strand, positive-sense RNA approximately 11,000 nucleotides in length (Mukhopadhyay S, 2005) that encodes three structural proteins, capsid (C), membrane (prM/M), and envelope (E), and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The virus protease is encoded in the N-terminal portion of NS3 (Fig. 1A) and is responsible for processing the polyprotein precursor into individual functional proteins in the presence of the host proteases furin and secretase (Stadler K, 1997; Thomas G, 2002). NS3 protease cleaves the junctions at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 sites, as well as internal sites within C, NS3, and NS4A (Chappell K J, 2005; Nall T A, 2004). These cleavages are necessary for viral replication and assembly; therefore, the NS3 protease represents a potential therapeutic target (D'Arcy A, 2006; Jia F, 2010; Loughlin W A, 2004; Nall T A, 2004; Tyndall J D, 2005).

NS3 has a classical catalytic triad (H51, D75, S135), and the mutation of any one of these residues will result in the loss of protease activity (Ryan M D, 1998). Furthermore, the role of NS2B as a cofactor for NS3 protease activation has been investigated in many flaviviruses, including WNV, dengue virus (DENV), yellow fever virus (YFV), and Japanese encephalitis virus (JEV) (Chambers T J, 1993; Droll D A, 2000; Falgout B, 1991; Falgout B, 1993; Lin C W, 2007; Nall T A, 2004). The results indicate that the hydrophilic central region of NS2B 48 amino acid (aa) is essential (Leung D, 2001), and some residues are important for promoting the functional configuration of NS2B-NS3 protease and

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maintaining critical interactions with protease catalysis substrates (Chappell K J, 2008; Ciota A T, 2007; Erbel P, 2006; Mueller N H, 2007; Radichev I, 2008).

The L^{79}DDDGNF^{85} sequence in WNV NS2B is required for the productive interaction of NS2B with NS3 and for appropriate NS2B-NS3 protease viral enzyme catalytic activity (Johnston PA, 2007). The sequence alignment of NS2B in WNV, JEV, Kunjin virus (KUNV), Murray Valley encephalitis virus (MVEV), St. Louis encephalitis virus (SLEV), Zika virus (ZKV), DENV (serotypes 1, 2, 3, and 4), and YFV reveals that the G83 residue is conserved, but D^{80}DD was not highly conserved (Fig. 2A). The D^{80}DD region of NS2B forms a negatively charged patch that is important for maintaining the negative charge at the S2 pocket, and the substitution of D^{80}DD with AAA produced an inactive NS2B-NS3 protease, suggesting that the negative charge of D^{80}DD is important for protease activity (Radichev I, 2008). The crystal structure of the WNV NS2B-NS3 protease complex with inhibitor indicates that the NS2B (D82-F85) directly interacts with NS3 protease active sites. Specifically, the G83 in NS2B interacts with N152 in NS3 (Erbel P, 2006); G83 makes a hydrogen bond with the Lys residue at the P2 position of the substrate (Mueller N H, 2007). Chappell *et al*. reported that replacing G83 with Ala decreased NS2B-NS3 enzyme activity to less than 5% of

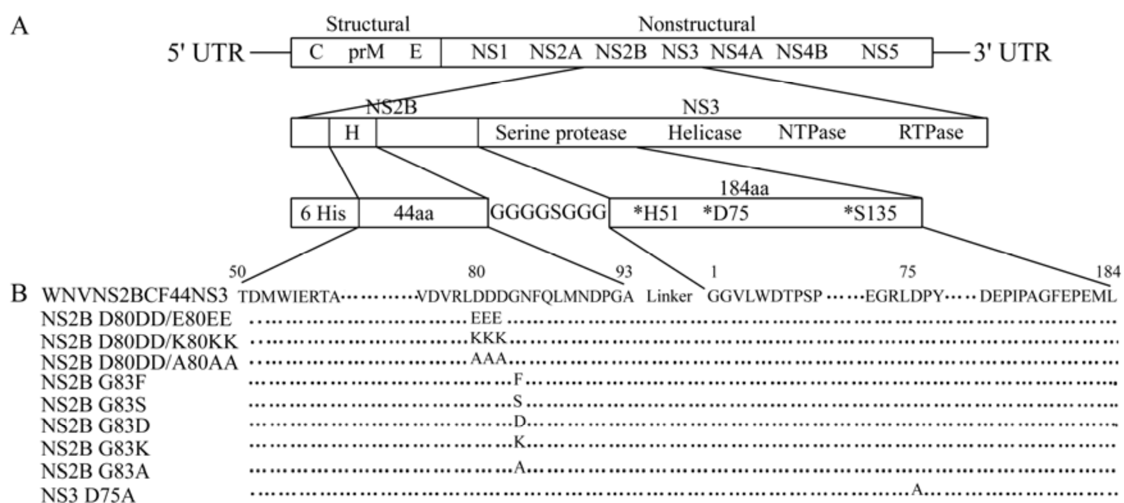

Fig. 1. Schematic of WNV polyprotein. A: The WNV genome is flanked by 5' and 3' UTRs, which encode three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). B: The protein sequences of wild-type (WNVNS2BCF44NS3) and the mutants used in this study. (E: envelope, M: membrane, UTR: untranslated region, H: hydrophilic).

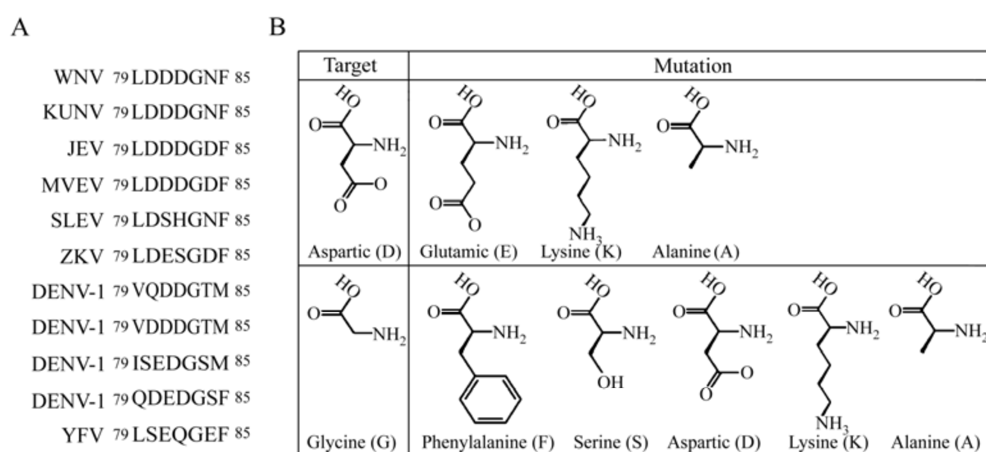

Fig. 2. Site-directed mutagenesis and alignment of partial flaviviral NS2B aa. A: Sequence alignment of partial flaviviral NS2B cofactor region. WNV, West Nile virus; KUNV, Kunjin virus; JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; SLEV, St. Louis encephalitis virus; ZKV, Zika virus; DENV1–4, dengue virus subtype 1–4; YFV, yellow fever virus. B: Mutation of D^{80}DD and G83. D^{80}DD/EEE (no change in charge), D^{80}DD/KKK (change into positive charge) and D^{80}DD/AAA (loss of charge and bulk), G83K (change to positive charge), G83D (change to negative charge), G83A G83F and G83S (no change in charge but different side chain).

that observed for wild-type NS2B-NS3 (Chappell K J, 2008). However, more details regarding the effects of $D^{80}DD$ and G83 on protease activity and the viral life cycle are needed.

In the present study, we constructed a series of mutant NB2B-NS3 proteins and WNV replicons ($D^{80}DD/E^{80}EE$, $D^{80}DD/K^{80}KK$, $D^{80}DD/A^{80}AA$, G83F, G83S, G83D, G83K, and G83A) to investigate the effects of these residues on NS3 activity and viral replication. Our results confirmed that D80-82 and G83 residues in NS2B are essential for protease activity, which is consistent with previous reports. Furthermore, we demonstrated that the negatively charged patch of $D^{80}DD$ is not absolutely required for providing the negative charge at the S2 pocket to promote substrate–protease interaction, but the side chain of the conserved residue G83 is critical for maintaining NS2B function. In addition, we found that the mutagenesis of $D^{80}DD$ and G83 prevent viral RNA replication.

MATERIALS AND METHODS

Reagents

Fluorogenic Peptide Substrate pERTKR-AMC was obtained from R&D Systems (Minneapolis, MN, USA). His-Bind Resin was purchased from Novagen (Madison, WI, USA). Plasmid pET28a-WNV-NS2BCF44NS3 (provided by Dr. Pei-Yong Shi, Wadsworth Center, New York State Department of Health, USA), contained the central portion of NS2B composed of 44 aa residues (cNS2B, residues 1424–1467 of the WNV precursor) and the NS3pro composed of 184 aa residues (cNS3, residues 1506–1689 of the WNV precursor), two of which were linked by a flexible GGGGSGGG linker. The plasmid WNV replicon cDNA encoding the NS2B–NS3 cDNA sequence from West Nile virus (strain 3356, GenBank accession No. AF404756) was also obtained from Dr. Pei-Yong Shi, Wadsworth Center, New York State Department of Health, USA. To obtain the cNS2B sequence, PCR was carried out using the primers cNS2B-F' and cNS2B-R'. To obtain the cNS3 sequence, PCR was carried out using the primers cNS3-F' and cNS3-R'. The NS2B-NS3 chimeric sequence was generated from an overlap PCR reaction with these two PCR products, digested with *Bam*H I/*Eco*R I and cloned into the same sites in pET28a. BHK21 cells were maintained in Dulbecco modified Eagle medium (DMEM) with 10% or 2% fetal bovine serum (FBS) in 5% CO_2 at 37℃.

Construction of mutant NS2B-NS3

Primers used for the site-directed mutagenesis of NS2B and NS3 were listed in Table 1. Plasmid pET28a-WNVNS2BCF44NS3 was used as template and the mutant amplifications were performed by overlap PCR reactions. The amplified PCR products were further purified separately and employed as templates in the second round PCR with the primer pairs WNVNS2BCF44NS3-F' and WNVNS2BCF44NS3-R'. These PCR products were digested with *Bam*H I and *Eco*R I and then cloned into plasmid pET28a, which was C-terminally tagged with the His × 6 tag. Nine mutants ($D^{80}DD/E^{80}EE$, $D^{80}DD/K^{80}KK$, $D^{80}DD/A^{80}AA$, G83F, G83S, G83D, G83K, G83A and D75A) were obtained and all mutant fragments were verified by DNA sequencing. NS3 D75A was designated as negative control (Fig. 1B).

Expression and purification of NS2B-NS3

Plasmids containing wild-type and mutant NS2B-NS3 recombinant DNA fragments were transformed into *Escherichia coli* BL21 (DE3), respectively. The recombinant *E. coli* were selected and cultured in 1 L Luria-Bertani (LB) broth supplemented with 30 μg/mL kanamycin and grown at 37℃ with shaking at 220 rpm. After being induced by 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside (IPTG) for 16 h at 16℃, cells were collected by centrifugation (3 000 rpm, 10 min) and stored at -70℃ for 1 h, then the pellets were thawed and resuspended in 20 mL 1×Binding Buffer (20 mmol/L Tris-HCl, 0.5 mol/L NaCl, 5 mmol/L imidazole, pH 7.9) and shaken at 4℃ for 1 h. Cells were disrupted by sonication on an ultrasound disintegrator. The pellet was removed by centrifugation (12 000 rpm, 30 min) at 4 °C and the wild-type and mutant NS2B-NS3 recombinant proteases were purified by using His-Bind Kits (Novagen) according to the procedure provided by the manufacture. The concentrations of purified proteins were determined by using BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China) and the samples were stored at -70℃ until use. All proteases were analyzed by 12% SDS-PAGE.

SDS-PAGE and Western blot

Wild-type and mutant proteases ($D^{80}DD/E^{80}EE$, $D^{80}DD/K^{80}KK$, $D^{80}DD/A^{80}AA$, G83F, G83S, G83D, G83K, G83A and D75A) in each sample were resolved on a 12% SDS-PAGE and electro-transferred to PVDF Immobilon-P membranes (Millipore, Billerica, MA, USA), blocked with 5% skim milk in TBST, and then reacted with

anti-His monoclonal antibody (1:1000 dilution; Qiagen, Valencia, CA) for 1.5 h at room temperature. The secondary anti-mouse antibody conjugated to Horseradish peroxidase (1:5000 dilution; Roche, Shanghai, CN) was applied to the blots for 1 h at room temperature after 5 times washing with TBST. Then the blots were developed with NBT/BCIP (Roche) after 5 times (10 min/one time) washing with TBST.

Enzyme characterization

The ability of wild-type and mutant NS2B-NS3 proteins to make enzymatic cleavages was tested against pentapeptide substrate pERTKR-AMC (Shiryaev S A, 2006). The total reaction volume of the assay was 0.1 mL, and the assay was carried out in 10 mmol/L Tris-HCl (pH 8.0) with 20% (v/v) glycerol. After preincubation of buffered protease and substrate in separate wells at ambient temperature (21–22 °C) for 5 min, the substrate was mixed with enzyme-buffer solution by automatic shaking for 5 s. Wild-type and mutant protease activities and initial reaction velocities were monitored at an excitation wavelength of λ_{ex}=380 nm and an emission wavelength of λ_{em}=460 nm using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT, USA). Kinetic parameters were calculated by nonlinear regression of initial velocities using nine different substrate concentrations ranging from 0 to 350 μmol/L (350, 175, 87.5, 43.75, 21.88, 10.94, 5.47, 2.73, and 0 μmol/L) assuming Michaelis–Menten kinetics using the equation $=V_{max}[S]/([S]+K_m)$, where is the initial velocity of substrate hydrolysis, $[S]$ is the concentration of substrate, V_{max} is the maximum rate of hydrolysis, and K_m is the Michaelis–Menten constant. All assays were conducted in triplicate. GraphPad Prism version 5.0 software (La Jolla, CA, USA) was used for statistical analyses, and the kinetic parameters are reported as mean ± standard error (S.E.).

Construction of mutant WNV replicon

Mutant WNV replicons were constructed based on a WNV replicon with a renilla luciferase-reporter plasmid (Lo M K, 2003) using overlap PCR reactions (primer pairs are shown in Table 1). The first round of PCR products were separately purified and served as templates for the second round of PCR amplification with the primer pairs Replicon-F' and Replicon-R', respectively. The mutated

Table 1. Primers used in this study

Name	Sequence
WNVNS2BCF44NS3-F'	5'-CCG*GGATCC* ACAGATATGTGGATTGAGAGAACG-3'
WNVNS2BCF44NS3-R'	5'-GCC*GAATTC*TTACAGCATCTCAGGTTCGAATCC-3'
NS2B D^{80}DD/E^{80}EE-R'	5'-GCTGGAAGTTTCCTTCTTCTTCAAGCCGCACATCAAC-3'
NS2B D^{80}DD/E^{80}EE-F'	5'-GTTGATGTGCGGCTTGAAGAAGAAGGAAACTTCCAGC-3'
NS2B D^{80}DD/K^{80}KK-R'	5'-GCTGGAAGTTTCCCTTCTTCTTAAGCCGCACATCAAC-3'
NS2B D^{80}DD/K^{80}KK-F'	5'-GTT GAT GTG CGG CTT AAGAAGAAGGGAAACTTCCAGC-3'
NS2B D^{80}DD/A^{80}AA-R'	5'-GCTGGAAGTTTCCAGCAGCAGCAAGCCGCACATCAAC-3'
NS2B D^{80}DD/A^{80}AA-F'	5'-GTTGATGTGCGGCTTGCTGCTGCTGGAAACTTCCAGC-3'
NS2B G83F-R'	5'-TGAGCTGGAAGTTAAAATCATCATCAAGC-3'
NS2B G83F-F'	5'-GCTTGATGATGATTTTAACTTCCAGCTCA-3'
NS2B G83S-R'	5'-AGCTGGAAGTTTC A ATC ATC ATC AAG-3'
NS2B G83S-F'	5'-CTT GAT GAT GAT TGAAACTTCCAGCT-3'
NS2B G83D-R'	5'-TGAGCTGGAAGTTGTCATCATCATCAAG-3'
NS2B G83D-F'	5'-CTTGATGATGATGACAACTTCCAGCTCA-3'
NS2B G83K-R'	5'-GAGCTGGAAGTTTTTATCATCATCAAGC-3'
NS2B G83K-F'	5'-GCTTGATGATGATAAAAACTTCCAGCTC-3'
NS2B G83A-R'	5'-GAGCTGGAAGTTTGCATCATCATCAAG-3'
NS2B G83A-F'	5'-CTTGATGATGATGCAAACTTCCAGCTC -3'
NS3 D75A-R'	5'-TAA CAA AGT CGAGCCTCCTTGACACT-3'
NS3 D75A-F'	5'-AGT GTC AAG G AGGCTCGACTTTGTTA-3'
cNS2B-F'	5'-CCGGGATCCACAGATATGTGGATTGAGAG-3'
cNS2B-R'	5'-GCCTCCACCACTACCTCCGCCCCCTGCTCCTGGATCATTCATG-3'
cNS3-F'	5'-GGGGGCGGAGGTAGTGGTGGAGGCGGAGGCGTGTTGTGGGACAC-3'
cNS3-R'	5'-GCCGAATTCTTACAGCATCTCAGGTTCGAATC-3'
Replicon-F'	5'-CCG*GCATGC*CAGCTATACTGATT-3'
Replicon-R'	5'-CCG*CGTACG*ACTTTCTGTTCAAT-3'

Note: F' means forward primer; R' means reverse primer.

Table 2. Kinetic parameters of wild-type and mutant protease

Protease	Km	Kcat	Kcat/Km
	μm	s^{-1}	$m^{-1}s^{-1}$
WNVNS2BCF44NS3	73.6 ± 2.4	2.3383 ± 0.053	31769.1 ± 718.3
NS2B D80DD/E80EE	601.8 ± 5.5	0.1438 ± 0.004	238.9 ± 7.4
NS2B D80DD/K80KK	626.8 ± 39	0.1429 ± 0.010	228 ± 15.4
NS2B D80DD/A80AA	204.4 ± 4.05	0.0906 ± 0.002	443.4 ± 10.1
NS2B G83F	68.8 ± 1.29	0.0940 ± 0.001	1365.7 ± 16.4
NS2B G83S	202.7 ± 8.33	0.0846 ± 0.002	417.2 ± 11.9
NS2B G83D	387.1 ± 12.73	0.1154 ± 0.001	298.2 ± 3.07
NS2B G83K	513.5 ± 40.94	0.1297 ± 0.007	252.7 ± 14.3
NS2B G83A	281.4 ± 34.72	0.1099 ± 0.009	390.5 ± 30.32
NS3 D75A	246.6 ± 6.05	0.0988 ± 0.002	367.6 ± 28.23

Enzyme kinetic parameters were obtained using the enzyme assay *in vitro*, and triplicate measurements were taken for each data and parameters were reported as means ± S.E.

DNA fragments were purified, digested by *Sph* I and *Bsiw* I, and engineered into WNV replicon at the *Sph* I and *Bsiw* I sites, respectively. All mutants were verified by DNA sequencing.

In vitro transcription, RNA transfection, and luciferase assay

A transient replicon assay was used to quantify viral RNA translation and replication. Briefly, all replicon cDNA templates were linearized with *Xba* I and purified using phenol-chloroform extraction and ethanol precipitation. Replicon RNA was transcribed with a mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) according to the manufacturer's protocols. All RNA transcripts (5 μg each) were electroporated into 8×10^6 BHK21 cells using a GenePulser apparatus (Bio-Rad, Hercules, CA, USA). After a 10-min recovery at room temperature, the transfected cells were resuspended in 25 mL Dulbecco's modified Eagle medium containing 10% fetal bovine serum. Upon transfection of BHK21 cells with wild-type and mutant-type Replicon RNA, 0.5 mL transfected cells were seeded per well in 12-well plates for assaying luciferase activities at 2, 4, 6, 24, 48, and 72 h post-transfection (h p.t.). Triplicate wells were seeded for each data point. Renilla luciferase (Rluc) activity was determined by using an assay kit according to the manufacturer's protocol.

RESULTS

Expression and purification of wild-type and mutant NS2B-NS3

Nine mutants (G83F, G83S, G83D, G83K, G83A, $D^{80}DD/E^{80}EE$, $D^{80}DD/K^{80}KK$, $D^{80}DD/A^{80}AA$, and D75A) were obtained, and all mutant fragments were verified by DNA sequencing. Plasmids containing wild-type or mutant NS2B-NS3 recombinant DNA fragments were transformed into *Escherichia coli* BL21 (DE3). The recombinant *E. coli* strains could produce the wild-type and mutant proteases during bacterial growth after isopropylthio-β-galacoside-induction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the purified proteases had a molecular weight of about 36 kD (Fig. 3A), and could be specifically recognized by an anti-His monoclonal antibody on western blot (Fig.3B). The migration rates of $D^{80}DD/E^{80}EE$ and

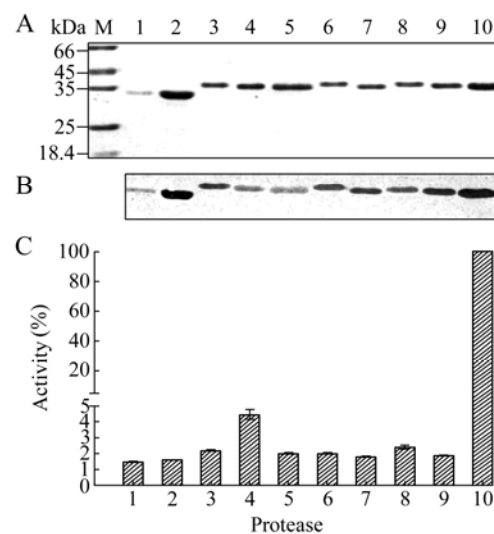

Fig. 3. Analysis of purified wild-type and mutant proteases. A: All proteins were purified from *E. coli* by Ni^{2+} metal-chelating chromatography, and protein purity was confirmed by 12% SDS-PAGE. B: All purified proteins were analyzed by western blot using a monoclonal antibody against His. C: Wild-type and mutant protease activities were tested by using substrate pERTKR-AMC. Lanes 1 to 10 represent $D^{80}DD/E^{80}EE$, $D^{80}DD/K^{80}KK$, $D^{80}DD/A^{80}AA$, G83F, G83S, G83D, G83K, G83A, NS3 D75A, and wild-type, respectively.

$D^{80}DD/K^{80}KK$ were slightly faster than the other mutants and wild type. We did not detect any proteolysis fraction by SDS-PAGE or western blot.

Catalytic activities of wild-type and mutant NS2B-NS3

Protease activities and kinetic parameters of wild-type and mutant proteases are shown in Table 2. All mutant proteases lost activity (Fig. 3C). While all proteases exhibited variations in K_m, K_{cat}, and K_{cat}/K_m, the K_m of the G83F mutation was most similar to the wild-type. The $D^{80}DD/E^{80}EE$ and $D^{80}DD/K^{80}KK$ mutations retained similar K_m, K_{cat}, and K_{cat}/K_m values. The mutagenesis of $D^{80}DD/E^{80}EE$ caused a large decrease in substrate affinity (11-fold increase in K_m) and an 18-fold decrease in K_{cat}/K_m. $D^{80}DD/A^{80}AA$ mutagenesis resulted in a 7-fold increase in K_m and a 2-fold decrease in K_{cat} to produce a 12-fold decrease in K_{cat}/K_m. The G83S, G83D, G83K, and G83A mutations reduced K_{cat} and K_{cat}/K_m, and caused minor increases in K_m, whereas G83F produced a similar K_m and a 2-fold decrease in K_{cat}, which resulted in a 3-fold decrease in catalytic efficiency (K_{cat}/K_m). The NS3 D75A protease (negative control) produced an 11-fold decrease in K_{cat}/K_m due to a 5-fold increase in K_m and a 2-fold decrease in K_{cat} versus wild-type, which was consistent with previously published results (D75 in NS3 is a protease active site) (Ryan M D, 1998).

$D^{80}DD$ and G83 mutations block viral RNA replication

We used a transient replicon system to investigate whether these mutants inhibit viral translation or viral RNA synthesis. Tilgner and Shi reported that BHK21 cells transfected with WNV replicon RNA produced two distinct Rluc signal peaks, one at 2.5 to 10 h (representing viral translation) and another at 22.5 h p.t. (representing viral RNA synthesis) (Tilgner M, 2004). The results showed that both the wild-type and the mutants have equivalent luciferase activity at 2, 4, and 6 h p.t., but all mutants suppressed the luciferase signals at 24, 48, 72 h p.t. (Fig. 4). Taken together, the results suggest that $D^{80}DD$ and G83 residues are required for viral RNA synthesis.

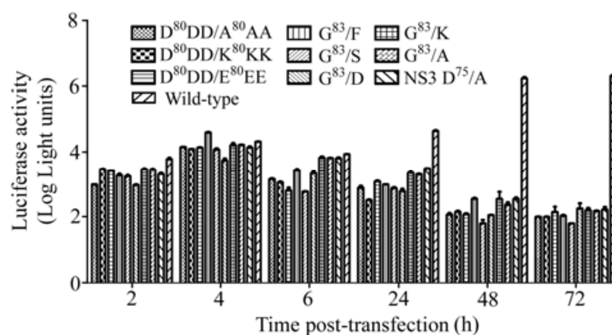

Fig. 4. The effects of $D^{80}DD$ and G83 based on viral translation and RNA synthesis based on a transient replicon assay. The luciferase signals of all mutants are equivalent with wild-type at 2, 4, and 6 h p.t. but lower than wild-type at 24, 48, and 72 h p.t. All assays were performed in triplicate.

DISCUSSION

Previous research revealed that NS2B is a required cofactor for NS3 serine protease activity and plays an important role in promoting the functional configuration of the NS2B-NS3 protease and in maintaining critical interactions with protease catalysis substrates. The $L^{79}DDDGNF^{85}$ sequence of WNV NS2B is required for productive interaction between NS2B and NS3 and for NS2B-NS3 protease viral enzymatic activity (Johnston P A, 2007). The NS2B sequence alignment of WNV, KUNV, JEV, MVEV, SLEV, ZKV, DENV (serotypes 1, 2, 3, and 4), and YFV showed that the G83 residue was conserved, but the $D^{80}DD$ patch in the NS2B was not (Fig. 2A). The negatively charged patch $D^{80}DD$ and conserved G83 residues were replaced with AAA and A, which inactivated proteases *in vitro* (Chappell K J, 2008; Radichev I, 2008). However, the underlying mechanisms are still unknown. The effects of $D^{80}DD$ and G83 residues on viral RNA synthesis are also unclear. For these reasons, we conducted a series of mutations to assess NS2B-NS3 protease activity and their effect on WNV replication.

To acquire the protease activities and kinetic parameters of different mutant proteases, eight mutant NS2B-NS3 plasmids were constructed, the wild-type and mutant proteases were expressed and purified, then all proteins were analyzed by 12% SDS-PAGE and western blotting (Fig. 3A and B). SDS-PAGE revealed that the purified proteases have a molecular weight of about 36 kD. Western blot analysis indicated that all mutant proteases were specifically recognized by an anti-His monoclonal antibody. The migration rates of $D^{80}DD/E^{80}EE$ and $D^{80}DD/K^{80}KK$ were slightly faster than other mutants and wild-type, and we did not detect a proteolysis fraction by SDS-PAGE and western blot.

All proteases exhibited variations in K_m, K_{cat}, and K_{cat}/K_m, but the K_m of the G83F mutation was similar to that observed for wild-type. The $D^{80}DD$ was replaced with EEE (no change in charge), KKK (change into positive charge) and AAA (loss of charge and bulk) to

elucidate whether a negative charge group is important for maintaining negative charge at the S2 pocket (Fig. 2B). We found that the $D^{80}DD/E^{80}EE$, $D^{80}DD/K^{80}KK$, and $D^{80}DD/A^{80}AA$ proteases lost activity, which suggests that the negatively charged patch $D^{80}DD$ is dispensable for protease activity. However, Radichev et al. (2008) reported that the negatively charged patch $D^{80}DD$ is required for maintaining the negative electrostatic potential S2 pocket. Therefore, we speculate that the negatively charged patch $D^{80}DD$ is not absolutely required for providing the negative charge at the S2 pocket to promote substrate–protease. These results also indicate that the $D^{80}DD$ patch might be variable in other flaviviruses.

Conservative mutants G83K (change to positive charge); G83D (change to negative charge); and G83A, G83F, and G83S (no change in charge but different side groups) lost activity (Fig. 3C). The G83S, G83D, G83K, and G83A mutations reduced K_{cat} and K_{cat}/K_m and caused minor increases in K_m, whereas G83F produced a similar K_m and a 2-fold decrease in K_{cat}, which resulted in a 3-fold decrease in catalytic efficiency (K_{cat}/K_m) (Table 2). Additionally, mutant G83F was greater than G83S, G83D, G83K, and G83A, but these five mutants exhibited protease activities below 5%. The NS3 D75A protease, which possesses a mutated active site, also lost activity. These results demonstrated that aa mutagenesis disrupted the interaction between G83, the P2 Lys, and N152 in the NS3 protease. Because the kinetic data showed that the G83F mutant protease has a similar substrate affinity with the wild-type protease, we speculate that the aromatic π-cation is probably important for the interaction of protese and substrate.

WNV replicons with specific mutations were constructed to assess the effects of $D^{80}DD$ and G83 on viral replication (Lo M K, 2003). Tilgner and Shi (2004) reported that BHK21 cells transfected with WNV Rluc2A-Rep RNA produce two distinct Rluc signal peaks representing viral translation and viral RNA synthesis (Tilgner M, 2004). Our results showed that all mutants inhibit viral RNA synthesis but not translation. In addition, we investigated whether the viral polyprotein was processed into individual viral proteins in the mutant replicon-transfected BHK21 cells, but no protein was detected (data not shown). The viral polyprotein precursor might not be processed into individual functional proteins due to NS2B $D^{80}DD$ and G83 mutagenesis, which induced NS2B-NS3 inactivation and resulted in viral RNA synthesis inhibition.

The results of the present study are consistent with a previous report that showed that D80-82 and G83 are essential for NS2B protease activity. Furthermore, we demonstrated that the negatively charged $D^{80}DD$ patch is not absolutely required for providing the negative charge at the S2 pocket to promote substrate–protease interaction, and that the side chain of the conserved residue G83 is required for maintaining NS2B function. Finally, we determined that $D^{80}DD$ and G83 mutagenesis effectively block viral replication. Collectively, these findings indicate that NS2B is important for viral replication and should be useful for understanding the relationship between NS2B and NS3.

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