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Functional determinants of NS2B for activation of Japanese encephalitis virus NS3 protease

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Abstract

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus, causing severe central nerve system diseases without specific treatments. The NS2B–NS3 protease of flaviviruses mediates several cleavages on the flavivirus polyprotein, being believed to be a target for antiviral therapy. NS2B is the cofactor of the viral serine protease, correlating with stabilization and substrate recognition of the NS3 protease. In this study, we investigate the functional determinants in the JEV NS2B for the activation of the NS3 protease. *Cis*- and *trans*-cleavage assays of the deletions at the N-terminal of NS2B demonstrated that the NS2B residues Ser_{46} to Ile_{60} were the essential region required for both *cis* and *trans* activity of the NS3 protease. In addition, alanine substitution at the residues Trp53, Glu55, and Arg56 in NS2B significantly reduced the *cis*- and *trans*-cleavage activities of the NS3 protease. Sequence alignment and modeled structures suggested that functional determinants at the JEV NS2B residues Ser46 to Ile60, particularly in Trp53, Glu55 and Arg56 could play an important configuration required for the activity of the flavivirus NS3 protease. Our results might be useful for development of inhibitors that block the interaction between NS2B and NS3.

Keywords: Japanese encephalitis virus; NS2B–NS3 protease; Functional determinant; *Cis*-cleavage; *Trans*-cleavage

1. Introduction

Japanese encephalitis virus (JEV), a member of the *Flaviviridae* family, is a mosquito-borne neurotropic flavivirus (Burke and Monath, 2001; Lindenbach and Rice, 2001). JEV causes severe central nerve system diseases such as poliomyelitis-like acute flaccid paralysis, aseptic meningitis and encephalitis (Solomon et al., 2000). Because of no specific treatment for the disease, approximately 50,000 JE cases with 10,000 deaths are reported annually in Asian countries (Solomon et al., 2000). JEV contains a single-stranded, positive-sense RNA with about 11,000 nucleotides in length (Lindenbach and Rice, 2001). The JEV genome encodes for three structural proteins (capsid (C), membrane (prM/M), and envelope (E)) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The NS2B–NS3 protease of flaviviruses, including yellow fever virus (YFV), West-Nile virus (WNV), dengue virus (DENV) and JEV mediates several cleavages on the NS2A–NS2B, NS2B–NS3, NS3–NS4A and NS4B–NS5 junctions of the flavivirus polyprotein (Arias et al., 1993; Jan et al., 1995; Bessaud et al., 2006). In addition, flavivirus NS2B–NS3 protease is involved in the cleavages that occur in C, NS2A, and NS4A (Stocks and Lobigs, 1998; Kummerer and Rice, 2002; Lin et al., 1993). Therefore, the NS2B–NS3 protease plays a critical role in the flavivirus replication, being believed to be a target for antiviral therapy.

The N-terminal one-third (180 residues) of NS3 contains the protease active sites at His51, Asp75, and Serl35 (Ryan et al., 1998). NS2B is the cofactor of the viral serine protease (Chambers et al., 1993; Falgout et al., 1993), showing a central hydrophilic region surrounded by three hydrophobic regions

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Table 1
The primers used in this study

Plasmid constructions	Forward primer	Reverse primer
$NS2B_{FL}$–$NS3_{180}$	5′-A TCC ***GAA TTC*** GGG GTG GCC AGC TAC TGA G-3′	5′-G GTG ***CTC GAG*** GGT GTA AGC TTC TGG GAC TGG TTC-3′
$NS2B_{Ndel45}$–$NS3_{180}$	5′-A TCC ***GAA TTC*** TCA GGA AAA GCA ACA GAT ATG -3′	5′-G GTG ***CTC GAG*** GGT GTA AGC TTC TGG GAC TGG TTC-3′
$NS2B_{Ndel60}$–$NS3_{180}$	5′-A TCC ***GAA TTC*** AGC TGG GAG ATG GAT GCT-3′	5′-G GTG ***CTC GAG*** GGT GTA AGC TTC TGG GAC TGG TTC-3′
$NS2B_{Ndel45(W53\rightarrow A)}$–$NS3_{180}$	5′-A TCC ***GAA TTC*** TCA GGA AAA GCA ACA GAT ATG ***GCG*** CTT GAA CGG-3′	5′-G GTG ***CTC GAG*** GGT GTA AGC TTC TGG GAC TGG TTC-3′
$NS2B_{Ndel45(E55\rightarrow A)}$–$NS3_{180}$	5′-A TCC ***GAA TTC*** TCA GGA AAA GCA ACA GAT ATG TGG CTT ***GCG*** CGG GCC GCC-3′	5′-G GTG ***CTC GAG*** GGT GTA AGC TTC TGG GAC TGG TTC-3′
$NS2B_{Ndel45(R66\rightarrow A)}$–$NS3_{180}$	5′-A TCC ***GAA TTC*** TCA GGA AAA GCA ACA GAT ATG TGG CTT GAA ***GCG*** GCC GCC GAC-3′	5′-G GTG ***CTC GAG*** GGT GTA AGC TTC TGG GAC TGG TTC-3′

(Bessaud et al., 2005). Deletion analysis of DENV NS2B indicated that the central hydrophilic domain of NS2B is sufficient for co-factor (Falgout et al., 1993; Yusof et al., 2000; Leung et al., 2001). In addition, the proteolytic activity of WNV NS3 with the NS2B hydrophilic region (the residues 50–97) has been demonstrated using the expression system of *Escherichia coli* (*E. coli*) (Nall et al., 2004). Mutation at the residue Trp62 in the DENV-2 NS2B completely abolishes the *cis*-cleavage ability at the NS2B/NS3 site, while alanine substitutions at the residues Leu75, Ile77 and Ile79 in NS2B significantly decrease the proteolytic activity (Niyomrattanakit et al., 2004). Recently, crystal structures of a dengue NS2B–NS3pro complex and a West Nile virus NS2B–NS3pro complex reveal that the NS2B residues 51–57 are important for the stabilization of the NS3 protease fold, and the residues 82–85 are involved in substrate recognition (Erbel et al., 2006). The NS2B residues 51–57 consisting of a β-strand to the N-terminal barrel of NS3 are in part different from identified critical determinants of NS2B for the flavivirus protease activation.

In our previous study, the *cis*-cleavage activity of the JEV NS3 protease with the full length NS2B has been demonstrated, and the NS2B–NS3 protease-interacting proteins with the dibasic residue motif were identified using phage display human cDNA brain library (Lin et al., 2006). Because there are rare reports on functional interactions of the JEV NS2B with NS3 protease, we investigate the functional determinants in the JEV NS2B for the activation of the NS3 protease in this study. Deletion analysis and site-directed mutagenesis of NS2B were performed for identification of the region and key residues involved in the cofactor. *Cis*-cleavage at the NS2B/NS3 site and *trans*-cleavage with fluorogenic peptide substrate reveal functional determinants of NS2B in the NS3 active enzyme.

2. Materials and methods

2.1. Expression and purification of deleted and mutation forms of NS2B–NS3 protease

Vial gene encoding full-length NS2B and the residues 1–180 of NS3 that has been constructed (Lin et al., 2006) is used as the template in this study. Polymerase chain reaction was performed to amply deleted and mutant forms of NS2B using indicated oligonucleotide primers listed in Table 1. The PCR product was cloned into the *Eco*RI–*Xho*I site of the bacterial expression vector pET24a (Novagen). Expression and purification of each complex of the NS3 with deleted/mutant forms of NS2B was carried out as previously described (Lin et al., 2006).

2.2. SDS-PAGE and Western blotting

The samples from each purification step were dissolved in 2× SDS-PAGE sample buffer without 2-mercaptoethanol, and boiled for 10 min. Proteins were resolved on 12% SDS-PAGE gels, and then electrophoretically transferred to nitrocellulose paper. The resultant blots were blocked with 5% skim milk, and then reacted with the 1:1000 dilution of Anti-His Tag monoclonal antibody (mAb) (Serotec) for 3-h incubation. After three-time washing with TBST (Tris-buffered saline, pH 7.5, 0.1% Tween-20), the blots were overlaid with a 1:1000 dilution of rabbit anti-mouse IgG antibodies conjugated with alkaline phosphatase (KPL). Following another 1-h incubation at room temperature, the blots were developed with NBT/BCIP (Invitrogen).

2.3. Trans-cleavage of fluorogenie peptide substrate

The fluorogenic peptide substrate tBoc-Gly-Lys-Arg-4-methylcoumaryl-7-amide (GKR-AMC) (Bachem) was used for the *trans*-cleavage assay. The NS2B–NS3 protease (1 μM) was incubated with the peptide substrate (20 μM) in 100 mM Tris–HCl (pH 9.0) at 37 °C for 30 min. Finally, the fluorescence change resulted from the reaction was measured using a fluorescent plate reader CytoFluor (PerSeptive Biosystems) with excitation at 355 nm and emission at 460 nm. The fluorescence variation, indicative of the rate of product released by the proteolytic cleavage, was calculated as the *trans*-cleavage ability of NS3 protease with each deleted and mutant form of NS2B.

2.4. Molecular modeling of a JEV NS2B–NS3 complex

The modeled structure for the NS2B protein of JEV T1P1 strain was based on X-ray crystallographic structure of the WNV