Structure-based mutagenesis identifies important novel determinants of the NS2B cofactor of the West Nile virus two-component NS2B–NS3 proteinase

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West Nile virus (WNV) is an emerging mosquito-borne flavivirus that causes neuronal damage in the absence of treatment. In many flaviviruses, including WNV, the NS2B cofactor promotes the productive folding and the functional activity of the two-component NS3 (pro)teinase. Based on an analysis of the NS2B–NS3pro structure, we hypothesized that the G22 residue and the negatively charged patch D32-DD34 of NS2B were part of an important configuration required for NS2B–NS3pro activity. Our experimental data confirmed that G22 and D32-DD34 substitution for S and AAA, respectively, inactivated NS2B–NS3pro. An additional D42G mutant, which we designed as a control, had no dramatic effect on either the catalytic activity or self-proteolysis of NS2B–NS3pro. Because of the significant level of homology in flaviviral NS2B–NS3pro, our results will be useful for the development of specific allosteric inhibitors designed to interfere with the productive interactions of NS2B with NS3pro.

West Nile virus (WNV), a member of the family Flaviviridae, is an enveloped, positive-strand, 11 kb RNA virus that is transmitted by mosquitoes (Mukhopadhyay et al., 2005; van der Meulen et al., 2005). WNV causes central nervous system damage unless specific treatment is administered (Madden, 2003; Wang et al., 2004). The genomic RNA of WNV encodes a polyprotein precursor which consists of three structural proteins (C, capsid; prM, membrane and E, envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) arranged in the order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. Polyprotein processing by the host cell signal peptidase and furin in the endoplasmic reticulum, and also by the viral two-component NS2B–NS3 proteinase (NS2B–NS3pro) is required to generate individual viral proteins (Beasley, 2005; Cahour et al., 1992; Mukhopadhyay et al., 2005). Full-length NS3 represents a multifunctional protein in which the N-terminal 184 aa residues represent NS3pro and the C-terminal sequence codes for a helicase. NS3pro is responsible for the cleavage of the C protein and at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 boundaries. In addition, the cleavage of NS4A by NS3pro is required (Lin et al., 1993) for the subsequent efficient cleavage of the NS4A/NS4B junction by the signal peptidase (Preugschat & Strauss, 1991). Inactivating mutations of the NS3pro cleavage sites in the polyprotein precursor abolished viral infectivity (Chambers et al., 1993, 2005).

In many flavivirus species including WNV, NS2B functions as a cofactor and promotes the productive folding and the activity of NS3pro. The cofactor activity of the 48 aa central portion of NS2B is roughly equivalent to that of the entire NS2B sequence (Leung et al., 2001). Structural studies suggest that NS2B–NS3pro exhibits two alternative, productive and unproductive, conformations. In the productive conformation, NS2B wraps around NS3pro, completing, in a precise and well-defined fashion, the structure of the active site (Aleshin et al., 2007; Erbel et al., 2006). In agreement, the NS2B-free NS3pro enzyme is inactive (Falguet et al., 1991, 1993). These unique cofactor–protease domain interactions are common for the multiple flaviviruses (Bessaud et al., 2005; Droll et al., 2000; Wu et al., 2003). The requirement of these interactions for catalysis raises the possibility of designing allosteric inhibitors that interfere with the NS2B fold rather than directly targeting the catalytic triad of NS3pro. The inhibitor design, however, requires the precise knowledge of the functional determinants of the cofactor that are...
essential for the catalytically productive NS2B–NS3pro interactions. Extensive efforts were expended to determine the identity of the several residue positions of mutagenesis that had a deleterious effect on the proteinase activity (Chambers et al., 1993, 2005; Chappell et al., 2006, 2007; Droll et al., 2000; Lin et al., 2007; Nall et al., 2004; Pastorino et al., 2006). These earlier studies were performed when the catalytically productive structure of the two-component NS2B–NS3pro was unknown. We extended these studies by using mutagenesis to identify the additional and novel functional determinants in the NS2B sequence. In contrast with earlier work, the knowledge of the crystal structures of NS2B–NS3pro provided structural guidance for our studies.

The hydrophilic central sequence has been identified as an essential region of the NS2B cofactor, which is required for the catalytic activity of the two-component NS2B–NS3pro (Fig. 1a). Deletion and substitution mutagenesis have already led to a substantial, albeit incomplete, understanding of the importance of the individual residues of the cofactor (Chambers et al., 1993, 2005; Chappell et al., 2006, 2007; Droll et al., 2000; Lin et al., 2007; Niyomrattanakit et al., 2004; Pastorino et al., 2006). These data in combination with the knowledge of the crystal structure of both the WNV and the dengue 2 virus NS2B–NS3pro, which has recently become available (Aleshin et al., 2007; Erbel et al., 2006), suggest that the functional determinants are localized in the α-helical (α1) and the β-strand regions (β1–3) of the flaviviral NS2B sequence. Additional functionally important residues, which are localized at the C-terminal region of the cofactor, are proximal to the NS2B–NS3pro junction region.

The structures of both the catalytically potent and inert WNV NS2B–NS3pro strain NY99 (PDB 2IJO, 2FP7 and 2GGV) and dengue 2 virus NS2B–NS3pro (PDB 2FOM) were used to model the structure of the NS2B–NS3pro mutants. The mutant residues were built using PyMOL. Energy minimization was done with PyMOL and CNS. Based on the in-depth analysis of the WNV NS2B–NS3pro atomic resolution structure, we hypothesized that because of the tight interactions existing between NS2B and NS3pro, the presence of the small size G22 residue is essential for maintenance of the productive NS2B–NS3 fold. Conversely, the insertion of a bulky side chain amino acid, instead of G22, into the NS2B–NS3pro tight interface would destabilize the productive association of the cofactor with the NS3pro domain and render the two-component NS2B–NS3pro inactive. A close analysis of the NS2B–NS3pro productive conformation also suggested that the D32DD34 sequence region of NS2B was exposed on the cofactor molecule surface and that the D32DD34 negatively charged patch does not directly contact the cleavage substrate. The presence of D32DD34 is required, however, for maintaining the negative charge at the S2 subsite that accommodates the positively charged Arg/Lys at the P2 substrate position (Fig. 1b, c). In contrast, according to our in silico modelling, D42 which is localized in the C-terminal sequence region of NS2B and,

![Fig. 1. Sequence alignment and structure of the flaviviral NS2B cofactor.](http://vir.sgmjournals.org)
therefore, is close to the scissile bond in the NS2B–NS3pro junction region rather than to the catalytic groove is likely to have no effect on either self-proteolysis in cis or on the catalytic activity of NS2B–NS3pro or both.

To acquire experimental evidence that our in silico predictions were correct, we used site-directed mutagenesis on the cofactor sequence. The 48 aa residue central portion of NS2B (residues 1393–1440 of the WNV polyprotein precursor) and NS3pro (residues 1476–1687 of the WNV precursor) sequences were linked by a flexible GGGSGGGGGG linker. This wild-type NS2B–NS3pro construct (NS2B–NS3pro-WT) was used as a template for the PCR mutagenesis to obtain the D42G mutant. The autolytic cleavage site K48G↓GGGSGGGGG in the NS2B–NS3pro junction region was inactivated by the K48A mutation (Shiryaev et al., 2007). The resulting self-proteolysis-resistant NS2B–NS3pro K48A construct was used as a template for constructing the D23DD/A32AA and G22S mutants, which have named DDD/AAA and G22S in the text below. To destroy the tight interactions involving G22, we mutated this residue to S (G22S). To eliminate the negative charge at the S2 subsite, we converted the D23DD/D34 sequence into AAA (DDD/AAA). The primers we used for the mutagenesis are listed in Supplementary Table S1 (available in JGV Online). The mutant PCR products were further amplified using the 5′-CACCATGTCACAGATATGGATGGTGATGCAGCATCTCAGG-3′ and 5′-TCAGTGATGTGATGGTACATCTCACGG-TTCGAAATCCGGC-3′ oligonucleotides as the forward and reverse primers, respectively, to obtain the final 741 bp mutant constructs, including the His-tag sequence (the sequence that encodes a His ×6 tag is underlined).

The NS2B–NS3pro constructs were then recloned into the pET101D-TOPO cloning vector (Invitrogen) to encode the recombinant sequence, which was C-terminally tagged pET101/D-TOPO cloning vector (Invitrogen) to encode the recombinant sequence. The NS2B–NS3pro constructs were then recloned into the pET101/D-TOPO cloning vector (Invitrogen) to encode the recombinant sequence. The NS2B–NS3pro constructs were then recloned into the pET101/D-TOPO cloning vector (Invitrogen) to encode the recombinant sequence.

According to the results of SDS-gel electrophoresis the original NS2B–NS3pro K48A construct and the G22S and DDD/AAA mutants were resistant to autoproteolysis at the flexible linker region (Fig. 2a, upper panel). In agreement, Western blotting demonstrated that these three constructs preserved the His×6 sequence tagged at the C-terminal end of NS3pro (Fig. 2a, lower panel). As we expected, the D42G mutant was self-proteolysed at the K48G↓GGGSGGGGG linker region and it generated the non-covalently associated NS2B and NS3pro in a manner that was similar to the NS2B–NS3pro-WT construct (Shiryaev et al., 2006).

The trans-proteolytic activity of the constructs was measured using the Boc-RVRR-AMC and Pyr-RTKR-AMC peptides (both from American Peptide Company) and the myelin basic protein (MBP; Biodesign) as substrates. MBP, a protein that is highly sensitive to NS2B–NS3pro proteolysis (Shiryaev et al., 2006), was used as a substrate to demonstrate that the activity loss of the mutant NS2B–NS3pro construct also includes the endo-proteolytic activity. The assay for NS2B–NS3pro peptide cleavage activity was performed in 0.1 ml 10 mM Tris/HCl buffer, pH 8.0, containing 20% glycerol (v/v) and 0.005% Brij 35. The cleavage peptide (either Boc-RVRR-AMC or Pyr-RTKR-AMC) and enzyme concentrations, unless indicated otherwise, were 24 μM and 10 nM, respectively. Initial reaction velocities were monitored continuously at λex=360 nm and λem=460 nm on a Spectramax Gemini electron microscopy fluorescence spectrophotometer (Molecular Devices). All assays were performed in triplicate in wells of a 96-well plate. The concentration of active proteinase was measured using a fluorescence assay by titration against a standard aprotinin solution of a known concentration (Shiryaev et al., 2006).

We determined that the specific activity of the D42G mutant was approximately 50% when compared with the NS2B–NS3pro-WT or the original NS2B–NS3pro K48A constructs against both Pyr-RTKR-AMC and Boc-RVRR-AMC (Fig. 2b). In turn, the G22S mutant retained a low, 3%, residual activity, while the DDD/AAA mutant lost 99% of its activity when compared with the WT and K48A constructs. Interestingly, the Km value of the G22S mutant decreased approximately sevenfold, while the kcat value went down ≈200-fold resulting in a 30-fold loss of the kcat/Km parameter when compared with the WT construct (Km =30 and 4 μM of WT and G22S, respectively; kcat = 2 and 0.008 s⁻¹ of WT and G22S, respectively). Aprotinin, a potent substrate-mimetic inhibitor of NS2B–NS3pro (Shiryaev et al., 2006), efficiently inhibited the proteolytic activity of both the K48A and D42G constructs, thus suggesting that the D42G mutation did not affect the active site conformation of NS3pro (data not shown).

Consistent with our previous results (Shiryaev et al., 2006), MBP was highly sensitive to NS2B–NS3pro proteolysis. MBP (4 μg; 11 μM) was co-incubated for 60 min at 37°C with the purified NS2B–NS3pro constructs (0.7 μg or...
1 μM; an enzyme:substrate ratio \( \approx 1:10 \) in 20 μl 10 mM Tris/HCl buffer, pH 8.0, containing 20 % glycerol (v/v). The reactions were stopped by adding 2 × SDS sample buffer [125 mM Tris/HCl, pH 6.8, containing 4 % SDS, 20 mM DTT, 0.005 % bromophenol blue and 20 % glycerol (v/v)]. The digested samples were resolved in 4–20 % gradient SDS-gel electrophoresis to determine the conversion of 18 kDa MBP into the 6 and 14 kDa digested fragments. Fig. 2(c) shows that 18 kDa MBP was almost totally proteolysed by the NS2B–NS3pro K48A construct generating, as a result of the proteolysis, the ~6 and ~14 kDa fragments. In contrast, both the G22S and the DDD/AAA mutants did not cleave MBP.

Circular dichroism (CD) studies confirmed the structural integrity of the DDD/AAA mutant construct, the spectrum of which did not differ significantly from that of the NS2B–NS3pro K48A construct (Fig. 2d). CD spectra (190–250 nm) of the NS2B–NS3pro purified samples (1.5–2 mg ml\(^{-1}\) in 15 mM potassium phosphate buffer, pH 7.8) were collected in a 1 mm path length quartz cuvette at ambient temperature using a 62ADC spectrometer (AVIV Instruments). The results are expressed as the [Ω]MRW (mean residue weight) ellipticity. On the other hand, the CD spectrum of the G22S mutant was significantly different. Consistent with a potential transition of this mutant to the unproductive fold (Fig. 1b), the
spectrum suggested the presence of additional \( \alpha \) and \( \beta \)-structural regions in the NS2B–NS3pro DDD/AAA samples. In agreement with our results, a similar G22A substitution resulted in a noticeable, albeit insignificant when compared to the G22S mutation, loss of activity of NS2B–NS3pro from tick-borne Alkhurma virus (Pastorino et al., 2006). Our results suggest that the activity loss was incomplete because of an insufficient increase of the residue side chain size in the Alkhurma model.

In conclusion, the in-depth analysis of the cofactor–NS3pro interactions in flaviviruses guided by the crystal structure parameters led us to identify two novel NS2B functional determinants critical for NS3pro activation. We believe this knowledge will be highly valuable for the precise understanding of the NS2B–NS3pro molecular complex formation and for the optimization of the selective allosteric small-molecule inhibitors designed to target the NS2B–NS3pro interface. We suspect that these allosteric inhibitors designed to inactivate the two-component flaviviral protease by interfering with the NS2B–NS3pro interactions, will not cross-react with host cell serine proteinases and, accordingly, they will exhibit fewer side effects when compared with the active site-targeting antagonists of the flaviviral NS2B–NS3pro (Knox et al., 2006; Lohr et al., 2007; Yin et al., 2006).

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References


