Last 20 aa of the West Nile virus NS1′ protein are responsible for its retention in cells and the formation of unique heat-stable dimers

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West Nile virus (WNV), a mosquito-borne flavivirus, is the major cause of arboviral encephalitis in the USA. As with other members of the Japanese encephalitis virus serogroup, WNV produces an additional non-structural protein, NS1′, a C-terminal extended product of NS1 generated as the result of a −1 programmed ribosomal frameshift (PRF). We have previously shown that mutations abolishing the PRF, and consequently NS1′, resulted in reduced neuroinvasiveness. However, whether this was caused by the PRF event itself or by the lack of a PRF product, NS1′, or a combination of both, remains undetermined. Here, we showed that WNV NS1′ formed a unique subpopulation of heat- and low-pH-stable dimers. C-terminal truncations and mutational analysis employing an NS1′-expressing plasmid showed that stability of NS1′ dimers was linked to the penultimate 10 aa. To examine the role of NS1′ heat-stable dimers in virus replication and pathogenicity, a stop codon mutation was introduced into NS1′ to create a WNV producing a truncated version of NS1′ lacking the last 20 aa, but not affecting the PRF. NS1′ protein produced by this mutant virus was secreted more efficiently than WT NS1′, indicating that the sequence of the last 20 aa of NS1′ was responsible for its cellular retention. Further analysis of this mutant showed growth kinetics in cells and virulence in weanling mice after peripheral infection similar to the WT WNVKUN, suggesting that full-length NS1′ was not essential for virus replication in vitro and for virulence in mice.

INTRODUCTION

West Nile virus (WNV) is currently the major cause of arboviral encephalitis in the USA (Petersen, 2009). A member of the Japanese encephalitis virus (JEV) serogroup, this flavivirus is maintained through a transmission cycle between birds and mosquitoes, primarily Culex spp. WNV also causes incidental infections in humans and other mammals, with ~5% of symptomatic infections involving neurological symptoms, such as encephalitis and meningitis (Beckham & Tyler, 2009). Kunjin (WNVKUN) is the prevalent strain of WNV within Australia (Hall et al., 2002) and is highly attenuated compared with strains common to the USA, such as the strain responsible for the spread of WNV to the USA in 1999 (WNVNY99) (Lanciotti et al., 1999). WNVKUN has been used extensively as a model for WNV infection since it was first isolated in 1960 (Doherty et al., 1963; Westaway et al., 2002, 2003).

WNVKUN has an 11 kb positive-sense ssRNA genome encoding three structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Produced as a single polyprotein, it is cleaved by both host and viral proteases to give rise to the individual proteins (Lindenbach & Rice, 2003). NS1 is a multifunctional glycoprotein involved in the formation of the replication complex (Chu & Westaway, 1992; Khromykh et al., 1999, 2000; Lindenbach & Rice, 1997; Westaway et al., 1997; Youn et al., 2012) and the modulation of the host immune response (Avirutnan et al., 2006, 2010; Chung et al., 2006; Crook et al., 2014; Kurosu et al., 2007; Muller & Young, 2013; Schlesinger, 2006; Wilson et al., 2008). After cleavage from the polyprotein (Falgout & Markoff, 1995; Nowak et al., 1989), NS1 is glycosylated and forms a heat-labile dimer in the endoplasmic reticulum (ER) (Pryor & Wright, 1994; Winkler et al., 1988, 1989). A proportion of the dimerized protein is subsequently trafficked through the Golgi pathway and secreted from cells as a soluble hexamer (Alcon-LePoder et al., 2006; Chung & Diamond, 2008; Flamand et al., 1999; Macdonald et al., 2005).

Encephalitic flaviviruses from the JEV serogroup produce an additional non-structural protein, designated NS1′, as the result of a −1 programmed ribosomal frameshift (PRF) occurring at the beginning of the adjacent NS2A gene.
Produced in 30–50% of translation events, NS1’ consists of the entire NS1 protein with a 52 aa C-terminal extension encoding the first 9 aa of NS2A and an additional 43 aa (Firth & Atkins, 2009; Mason, 1989; Mason et al., 1987; Melian et al., 2010). We have shown previously that NS1’ is localized to the same cellular compartments as NS1 and can substitute for the function of NS1 in RNA replication (Youn et al., 2013; Young et al., 2013), indicating that NS1’ may function as an additional NS1 in viral infection. The PRF event and consequent NS1’ production have been shown to be important for the viral pathogenesis of both WNVKUN and JEV as the lack of PRF/NS1’ correlated with reduced pathogenicity (Melian et al., 2010; Ye et al., 2012). Recent work has also identified that JEV PRF/NS1’ enhanced virus production in avian cells (Takamatsu et al., 2014). However, as mutations used in the above studies abolished both the PRF event and NS1’ production (Melian et al., 2010; Takamatsu et al., 2014; Ye et al., 2012; Young et al., 2013), the role of these two events independently in virus replication and pathogenesis could not be distinguished, and the unique function for NS1’ in viral infection has therefore not been identified.

In the present study, we focused on characterizing the properties of the NS1’ protein, and its putative function in virus replication and pathogenesis. We showed that NS1’ produced a unique subpopulation of heat-stable homodimers and that the presence of the frameshifted region resulted in increased cellular retention of NS1’ compared with NS1. By using a mutant virus in which only full-length NS1’ production, but not the PRF event, was affected we showed that the last 20 aa of NS1’ were responsible for the cellular retention of WNVKUN NS1’ and that full-length NS1’ was not essential for neuroinvasiveness in mice.

RESULTS

WNV NS1’ is secreted less efficiently than NS1 from both infected and transfected cells

It has been shown previously that JEV NS1’, unlike NS1, is not secreted efficiently from infected cells (Fan & Mason, 1990; Mason, 1989). To determine whether this was also the case for WNVKUN NS1’, pulse–chase 35S-labelling experiments were carried out. A previously generated NS1’-lacking WNVKUN virus mutant (A30A’) was included in pulse–chase labelling experiments to confirm NS1’ expression from WT WNVKUN-infected cells. Radiolabelled NS1 and NS1’ were detected in both the cell monolayer and culture fluid of WNVKUN-infected Vero76 cells at 24 or 72 h post-infection (p.i.) (Fig. 1a, b). Quantification of individual protein bands and determination of the extracellular/intracellular (E/I) ratio showed that NS1’ was consistently secreted to a lower degree compared with NS1 (Fig. 1c). The same could be seen for NS1 and NS1’ expressed from pcDNA-NS1- and pcDNA-NS1’-transfected 293T cells (both co- and individually transfected) at 24 and 48 h post-transfection (Fig. 2a, b). Again, the E/I ratio showed that NS1’ (whether individually or co-transfected) was secreted to a significantly lower degree than NS1 (Fig. 2c). These results indicated that the frameshifted region of NS1’ resulted in increased cellular retention of NS1’ compared with NS1.

WNV infection produces unique heat-stable NS1’ dimers

We have shown previously that plasmid-expressed WNVKUN NS1’ retains a subpopulation of dimers when heated, whilst heating of plasmid-expressed NS1 disrupted dimers (Young et al., 2013). To confirm this stability was not an artefact from plasmid expression, lysate from WNVKUN- or A30A’ (as a non-NS1’-expressing control)-infected Vero76 cells was heated or left unheated prior to separation by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and analysed by Western blotting with an anti-NS1 mAb (4G4), which detected both NS1 and NS1’. The presence of NS1’ dimers was still seen in heated WNVKUN lysate (Fig. 3a), confirming that NS1’ produced by infected cells also formed a subpopulation of heat-stable dimers. Previous studies on JEV NS1’ did not show the presence of these heat-stable NS1’ dimers (Fan & Mason, 1990). To examine this further, lysates from JEV-, Murray Valley encephalitis virus (MVEV)-, WNVNY99- and WNVKUN-infected Vero76 cells were subjected to SDS-PAGE and Western blotting with anti-NS1 antibodies to determine the presence or absence of heat-stable NS1’ dimers. Heat-stable NS1’ dimers were only detected in heated WNV (WNVNY99 and WNVKUN) samples (Fig. 3b), but not JEV or MVEV samples, showing that these dimers were unique to WNV. The intermediate dimer band detected in all unheated samples was likely to be a NS1/NS1’ heterodimer, which has previously been shown for both JEV and MVEV (Blitvich et al., 1995; Fan & Mason, 1990; Lin et al., 1998).

WNV NS1’ dimers are resistant to heat and low pH, but susceptible to reduction

Previous work by Falconar & Young (1990) has shown that NS1 dimers were stable at low pH (pH 3.5). To assess the pH stability of NS1’ dimers, NS1- or NS1’-transfected cell lysate (Fig. 4a) and WNVKUN- or A30A’-infected cell lysate (Fig. 4b) was incubated for 1 h prior to separation by electrophoresis with 1 M glycine buffered to the indicated pH. NS1 dimers were indeed completely stable until the pH was lowered to 3.5, whilst NS1’ dimers were still partially stable at the lowest pH tested, pH 2.2. A range of temperature treatments was also tested on the same lysates to further examine heat stability (Fig. 4c, d). Whilst NS1 dimers were stable at room temperature, heating to 60 °C for 30 min was enough to completely disrupt this species. NS1’, however, formed a subpopulation of dimers that were still stable at the highest temperature tested (95 °C). The subpopulation of NS1’ that did not have this heat-stable nature had a similar stability to NS1 dimers.
with respect to both temperature and pH treatment. However, reduction of lysates with 5% β-mercaptoethanol prior to SDS-PAGE separation resulted in complete disruption of both NS1 and NS1’ dimers (Fig. 4e). Therefore, WNV NS1’ was able to form a subpopulation of heat- and low-pH-stable dimers, but these dimers were sensitive to reducing treatment during heating.

**NS1’ dimer stability resides within the first 10 aa of the last 20 aa**

As the NS1’ heat-stable dimers are distinct from the heat-labile dimers formed by NS1, the stability must be linked to the presence of the frameshifted region of NS1’. To determine the region contributing to this stability, C-terminal 10 and 20 aa truncations of NS1’ were generated by PCR mutagenesis of pcDNA-NS1’ (Fig. 5a). The presence of heat-stable dimers was determined by SDS-PAGE and Western blot analysis of heated or unheated lysates generated from HEK293T cells transfected with pcDNA-NS1, -NS1’, -NS1’del10 or -NS1’del20. NS1’ and NS1’del10 both formed a subpopulation of heat-stable dimers (Fig. 5b), whilst NS1’del20 formed only heat-labile dimers, similar to those produced by NS1. Interestingly, NS1’del20 also affected the presence of a high-molecular-mass NS1’ multimer that could be observed in both NS1’

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**Fig. 1.** NS1’ is secreted to a lower degree compared with NS1 from infected cells. (a, b) Production and secretion of NS1 and NS1’ from Vero cells infected with WNV_{KUN} or A30A’. Pulse–chase was performed at (a) 24 or (b) 72 h p.i., culture fluids were clarified by centrifugation and cell monolayers were lysed as described. Protein preparations were immunoprecipitated with anti-NS1 (4G4) using Dynabeads Protein G. Antibody-bound proteins were eluted and samples subjected to electrophoresis. Labelled proteins were transferred to nitrocellulose membranes and exposed to a phosphor screen for 1 day. (c) E/I ratios for NS1 and NS1’ at 24 or 72 h p.i. (with a 4 or 12 h chase). Results are expressed as the mean ± SEM of two independent experiments.
and NS1’del10 unheated samples (Fig. 5b), suggesting that the increased stability of NS1’ dimers was associated with the penultimate 10 aa.

To determine whether the loss of heat-stable dimers in pcDNA-NS1’del20 transfected cells was due to the specific amino acid sequence or to a minimum length requirement of the frameshifted region, aa 385–393 were mutated to Ala (Fig. 5a). SDS-PAGE and Western blot analysis of lysate from pcDNA-NS1’Ala-transfected cells showed that NS1’Ala was similar to NS1’del20, as it did not form heat-stable dimers (Fig. 5b). This confirmed that the heat-stable dimerization was linked to the specific sequence of aa 385–394, rather than to the length of NS1’.

Due to the presence of a single additional Cys residue within the mutated region of NS1’, we reasoned that an inter-chain disulfide bond may be formed between monomeric units, creating the heat stability seen. This was also supported by the fact that the dimers were sensitive to reducing treatment.
like pcDNA-NS1’del20, did not form heat-stable dimers. Immunofluorescence analysis of WNV<sub>KUN</sub> and Stop Mutant-infected cells stained with anti-NS1 (4G4) and counter-stained with anti-calnexin (ER marker) showed that the truncation of NS1’ did not alter cellular localization (Fig. 6c). To determine if the truncation of NS1’ in Stop Mutant virus affected NS1’ secretion, pulse–chase ³⁵S-labelling experiments were carried out at 24 and 48 h p.i., as before. Immunoprecipitation of cell lysate or culture fluid, harvested at various chase times following radiolabelling, with anti-NS1 (4G4) showed that Stop Mutant NS1’ was secreted during infection (Fig. 6d). Quantification of individual protein bands showed that the secretion of Stop Mutant NS1’ was in fact increased compared with WNV<sub>KUN</sub> NS1’ (Fig. 6e). The results showed that the sequence of the last 20 aa of NS1’ was responsible for the cellular retention of WNV<sub>KUN</sub> NS1’ protein.

**Truncation of NS1’ does not significantly affect virus replication in vitro or viral neurovirulence in mice**

To analyse the effect of truncation of NS1’ on virus replication *in vitro*, Vero76, C6/36 and mouse embryonic fibroblasts (MEFs) were infected with the WT WNV<sub>KUN</sub> and Stop mutant viruses at m.o.i. 1 or 0.1, and virus titres in the culture fluid were determined every 12 h (every 24 h for C6/36) for up to 120 h after infection. The results showed that truncation of NS1’ did not affect virus replication in any of the cell lines (Fig. 7a) demonstrating that full-length NS1’ protein was not required for virus replication *in vitro*. The results were similar to our previous *in vitro* findings with A30A’ mutant virus in which NS1’ production was abolished by mutation of the ribosomal frameshift (Melian et al., 2010). In the same study, we also showed that A30A’ virus was attenuated in weanling mice compared with WNV<sub>KUN</sub> (Melian et al., 2010). The attenuation of A30A’ may have been due to the absence of NS1’ itself, the elimination of the frameshift, or a combination of both. In contrast to A30A’, Stop Mutant only affected production of full-length NS1’, without affecting the ribosomal frameshift. To determine whether NS1’ production alone affected virus neurovirulence, 18-day-old mice were infected intraperitoneally with 1000 p.f.u. of either WNV<sub>KUN</sub> or Stop Mutant and monitored daily for signs of encephalitis. Infection with Stop Mutant resulted in a relatively similar level of mortality compared with the WT WNV<sub>KUN</sub> (~40% survival for Stop Mutant compared with ~20% survival for the WT WNV<sub>KUN</sub>) (Fig. 7b). This suggested that full-length NS1’ protein was unlikely to play a role in viral neurovirulence, indicating that the attenuation seen in PRF/NS1’-lacking mutants was likely due to the loss of the PRF itself.

**DISCUSSION**

We have shown here that NS1’ forms a subpopulation of heat-stable, secretable homodimers. The stability of these...
Fig. 4. Stability of NS1 and NS1\'' dimers. (a, b) Lysate from transfected (a) or infected (b) cells was incubated with 1 M glycine at the indicated pH prior to separation by electrophoresis and Western blotting with anti-NS1 (4G4). (c, d) Lysate from transfected (c) or infected (d) cells was incubated at the indicated temperature for the time shown prior to separation by electrophoresis and Western blotting anti-NS1 (4G4). Top panel is either pcDNA-NS1 transfected (a, c) or WNV KUN infected (b, d) and bottom panel is either pcDNA-NS1\'' transfected (a, c) or A30A\'' infected (b, d). (e) Anti-NS1\'' Western blot showing sensitivity of NS1\'' homodimers to reducing treatment. Lysates from WNV\_KUN-infected Vero76 cells (lanes 1 and 2) or pcDNA-NS1\''-transfected 293T cells (lanes 3 and 4) were reduced with 5\% \(\beta\)–mercaptoethanol and subsequently heat denatured or left untreated prior to Western blotting.
dimers is dependent on aa 385–394; although not specifically to the Cys reside at position 392 (Fig. 5). Whilst NS1’ is secreted from both infected and transfected cells, increased cellular retention compared with NS1 was noted (Figs 1 and 2), similar to work published previously for JEV NS1’ (Mason, 1989). We have also linked this increased cellular retention to the last 20 aa of NS1’ (Fig. 6). Finally, we have shown that C-terminal truncation of the NS1’ protein and loss of heat-stable NS1’ dimers in WNV_KUN has no effect on viral replication in vitro or viral pathogenesis in vivo (Fig. 7).

The design and use of Stop Mutant was to not only examine heat-stable dimerization in a viral context, but also to separate the function of full-length NS1’ from that of the ribosomal frameshift itself. Due to the intricate relationship between the ribosomal frameshift and the production of NS1’, it is difficult to determine whether attenuation of NS1’-lacking viruses is a result of the loss of the NS1’ protein or the frameshift itself (Melian et al., 2010; Ye et al., 2012). It is possible that the frameshift mechanism evolved to primarily control the ratio of structural to non-structural proteins (Melian et al., 2014) and its byproduct NS1’ is generated to increase the relative level of functioning NS1. The work presented here showed that truncation of NS1’ did not detrimentally affect virus growth in mammalian and insect cell culture or WNV_KUN pathogenicity in mice after peripheral inoculation (Fig. 7). These results support the hypothesis that full-length NS1’ is unlikely to have a unique biological function that contributes to viral pathogenesis in a mammalian system. This suggests that the reduced pathogenicity seen in mice for previously studied WNV_KUN viruses lacking both the PRF and NS1’ is more likely due to the loss of the PRF.

Work conducted in chicken embryonic fibroblasts and embryonated chicken eggs identified a role for JEV PRF/NS1’ in facilitating virus production in avian cells by increasing viral RNA levels (Takamatsu et al., 2014). This is in contrast to virus grown in mammalian or insect culture, which has shown that viral replication in vitro is not different between viruses lacking PRF/NS1’ and those encoding PRF and producing NS1’ (Melian et al., 2010; Ye et al., 2012). Our other recent study also found no difference in replication in avian DF-1 cells between WT and PRF-deficient mutant (A30A’) WNV_KUN viruses (Melian et al., 2014). Combined together, these results indicate that the role of PRF/NS1’ in viral replication may be virus species and perhaps host species specific.

One key characteristic examined here is the cellular retention of NS1’, despite the absence of a distinct hydrophobic region in the frameshifted sequence. This increase in cellular retention compared with NS1 was also linked to the last 20 aa of NS1’ as shown by more efficient secretion of the Stop Mutant NS1’ lacking the last 20 aa. Due to the nature of the ribosomal frameshift, and NS1’ being encoded in the −1 ORF of NS2A, it is not possible to create an Ala mutant in the viral context without affecting the coding sequence of the NS2A gene. Another way to further determine the region linked to the increase in
cellular retention would be to examine the secretion of NS1 in other flavivirus species, such as JEV and MVEV, and create similar truncation mutants. Interestingly, the sequence of the frameshifted region is not well conserved between WNV KUN, WNV NY99, JEV and MVEV viruses (Fig. 8); however, JEV NS1 has also been shown previously to be secreted inefficiently (Fan & Mason, 1990). If truncation of NS1 in other flavivirus species also results in increased NS1 secretion compared with the WT NS1, then the cellular retention of NS1 is more likely to be dependent on its length rather than specific amino acids.

The presence of heat-stable, reducing-sensitive NS1 dimers suggests the presence of a disulfide bond; however, mutagenesis of the Cys residue within the frameshifted region indicates that it is not due to a simple interaction between the C-terminal Cys residues of two monomers. It

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**Fig. 6.** The last 20 aa of NS1′ are important for cellular retention of NS1′. (a) Design of C-terminally truncated (Stop Mutant) infectious viral clone. Underlining shows the frameshifted region of NS1′ and asterisks indicate stop codons. (b) Lysates harvested from Vero76 cells infected with WNV KUN or Stop Mutant were heated (70 °C for 10 min) or left untreated and proteins were separated by PAGE. Proteins were transferred to nitrocellulose membranes and detected with anti-NS1′ antibodies. truncated NS1′ produced by Stop Mutant. (c) Immunofluorescence analysis showing localization of NS1 and NS1′ in WNV KUN- and Stop Mutant-infected cells. Infected cells were fixed and stained with anti-NS1 (4G4; green) and an antibody against calnexin (ER marker; red). (d) Production and secretion of NS1 and NS1′ from Vero cells infected with WNV KUN or Stop Mutant. Pulse–chase was performed at 24 and 48 h p.i., and samples processed as outlined in Fig. 1. (e) Quantification of secreted NS1′ band intensity from WNV KUN- or Stop Mutant-infected cells at 24 and 48 h p.i. Results are expressed as the mean ± SEM of two independent experiments and significance (**P<0.005) determined by two-way ANOVA.
has previously been suggested (M. Lobigs, personal communication) that NS1 may itself catalyse disulfide bond exchange. Viral proteins have been identified previously to contain the disulfide isomerization motif, CXXC (Li et al., 2008). NS1 contains a CXXC motif that is conserved for dengue virus, yellow fever virus and the JEV serogroup. These Cys residues (C10 and C11) have been shown previously to be important for dimer formation and NS1 secretion (Pryor & Wright, 1993). It is possible that the presence of the frameshifted region in NS1’ may in fact impede folding of this C-terminally extended NS1 protein. The heat-stable dimers observed may therefore represent folding intermediates involving intermolecular disulfide bonds between covalently linked NS1’ monomers. Although, as these heat-stable dimers appear to be secreted to some degree (Figs 1 and 2), they are unlikely to represent misfolded protein, as this would be retained in the cell. However, only a small amount of NS1’ is secreted from the

Fig. 7. The 20 aa C-terminal truncation of NS1’ does not significantly affect viral replication in cells or viral pathogenesis in mice. (a) Kinetics of viral replication of WNKN and Stop Mutant in Vero76, C6/36 or MEF cells. Cells were infected at m.o.i. 1 or 0.1 and viral accumulation was determined up to 120 h p.i. by plaque assay as described previously (Leung et al., 2008). (b) Virulence of WNKN and Stop Mutant viruses in 18-day-old weanling Swiss-outbred CD1 mice. Groups of 10 (experiment 1) and 20 (experiment 2) mice were infected intraperitoneally with 1000 p.f.u. of each virus, and monitored daily for signs of encephalitis. The graph shows survival rates calculated from the data combined from two experiments.
cell and it is therefore difficult to conclusively determine the dimer stability. Further work analysing the nature of these heat-stable NS1’ dimers and the possibility of the involvement of the NS1 part of NS1’ in disulfide bond exchange is necessary.

Whilst the sequence of the frameshifted region is not conserved between different encephalitic flaviviruses, the stop codon and consequently the length of the NS1’ extension are conserved (Fig. 8). This suggests that the PRF event leading to production of precisely 52 aa extension at the C terminus of NS1 protein may evolve to perhaps ensure that the PRF product, NS1’, could function as an additional NS1. Given efficient secretion of NS1 from cells, the ability of inefficiently secreted NS1’ to function as NS1 in infected cells may provide additional benefit to viral RNA replication. It seems highly unlikely that such a conserved mechanism as −1 PRF producing a stable NS1’ protein has evolved in a distinct group of viruses without a significant impact on viral growth/transmission properties in at least one of the vector or host systems. Relatively modest attenuation in mice of PRF- and NS1’-deficient WNVKUN virus (A30A) (Melian et al., 2014) and our data showing insignificant attenuation of Stop Mutant WNVKUN in mice suggest that mammalian hosts are unlikely to be the primary driver for the evolution of this mechanism. As mammals are only incidental hosts for these viruses, and these viruses predominantly cycle through the Culex mosquito vector and avian hosts, it is more likely that this is where the PRF mechanism evolved initially, and where it is likely to have a significant impact on virus replication and/or transmission. Recent work with WNVKUN and JEV viruses conducted in the mosquito and avian systems supports this hypothesis, with a difference in pathogenicity in birds, virus growth in avian cell culture and transmission in Culex mosquitoes observed between viruses encoding PRF and expressing NS1’ and those lacking PRF and NS1’ (Melian et al., 2014; Takamatsu et al., 2014).

In conclusion, we have shown that WNV NS1’ forms a subpopulation of heat-stable NS1’ dimers that are produced and preferentially retained both in the content of virus infection and when the NS1’ protein is produced independently from plasmid DNA. We have demonstrated that both the heat stability and cellular retention can be linked to the last 20 aa of NS1’, with dimerization being specifically linked to the penultimate 10 aa. We have also shown that a viral mutant (Stop Mutant) producing a truncated form of NS1’, but not deficient in ribosomal frameshifting, replicates with the same efficiency in cells of different origin and has a relatively similar virulence in mice to the WT WNVKUN. In combination with previous work (Melian et al., 2010, 2014), this suggests that the PRF itself (and not the NS1’ protein) is important for viral pathogenesis in the mammalian system. Further work in the mosquito and avian hosts using Stop Mutant producing truncated NS1’ will determine whether full-length NS1’ is indeed required for viral replication/transmission in this vector/host system.

**METHODS**

**Cell culture.** Baby hamster kidney (BHK) and Vero76 (African green monkey) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 5% heat-inactivated FCS, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹ and 2 mM GlutaMAX. MEFs and human embryonic kidney (HEK) 293T cells were also grown in DMEM supplemented with GlutaMAX and penicillin/streptomycin as above, with a total of 10% FCS. HEK 293T cells were also grown in 1 mM sodium pyruvate. Aedes albopictus cells (C6/36) were grown and maintained in RPMI media (Gibco), containing 10% FCS, GlutaMAX and penicillin/streptomycin as above.

**Plasmid construction.**

**pcDNA plasmids.** Plasmids for the transient expression of NS1 and NS1’ (pcDNA-NS1 and pcDNA-NS1’) were described previously (Young et al., 2013). Both plasmids contained an N-terminal signal sequence consisting of the last 26 codons of the WNVKUN E protein, and Myc- and FLAG-tags at the C terminus. Overlapping PCR mutagenesis using Pfu DNA polymerase (Promega) was carried out on pcDNA-NS1’ to generate a plasmid containing an amino acid change at residue 392 (NS1’ C392S) for Cys mutagenesis. Overlapping PCR mutagenesis was also used to delete 10 or 20 aa from the C terminus of NS1’ for truncation analysis and to introduce several point mutations to change aa 385–393 to Ala (NS1’Ala).

**FLSDX mutants.** Stop Mutant was generated by overlapping PCR mutagenesis on an intermediate E–NS2A cassette, prior to restriction enzyme digestion and ligation into a full-length WNVKUN infectious clone, FLSDX (Khromykh et al., 1998).

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**Fig. 8.** Alignment of 52 aa C-terminal extension sequences in NS1’ of the key representative viruses from the JEV serogroup. Dashes indicate amino acids consistent with WNVKUN and asterisks indicate stop codons. Virus sequences used: WNVKUN (GenBank accession number AY274504), WNVNY99 (GenBank accession number NC_009942), JEV (GenBank accession number NC_001437) and MVEV (GenBank accession number NC_000943).
Transfection conditions. 293T cells were seeded at 80–90 % confluence in antibiotic-free media 24 h prior to experiments. Transfections were carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions. A ratio of 0.8 μg DNA to 2 μl reagent for a 24-well plate was used as standard and scaled appropriately for well size.

In vitro transcription and electroporation. Full-length cDNA templates (FLSDX and mutants) were linearized with XhoI (New England Biolabs), and purified by phenol/chloroform extraction and ethanol precipitation. In vitro transcription and electroporation of BHK cells was carried out as described previously (Leung et al., 2008), and virus stocks were harvested at 2–4 days post-electroporation and titrated on BHK cells.

Virus stocks. Working virus stocks for WNVKUN, A30A’ and Stop Mutant were generated by infection of BHK cells at low m.o.i. (m.o.i. 0.1) with WNVKUN or mutant viruses harvested from electroporated BHK cells. Stocks were harvested at day 3–5 p.i. and titrated as above.

Virus infection and growth kinetics. BHK, WT MEF or C6/36 cells were seeded into six-well plates and infected with WNVKUN or mutant viruses at m.o.i. 0.1 or 1 for 2 h. Cells were washed three times and appropriate growth media was added. For growth kinetics, 100 μl per sample was harvested at the indicated times p.i., clarified by centrifugation and stored at −80 °C. Virus titres were determined by plaque assay as described previously (Leung et al., 2008).

SDS-PAGE and Western blotting. SDS-PAGE was carried out using either a Mini-PROTEAN Tetra Handcast system (Bio-Rad) or Bolt Mini-Gel system (Novex). Using the Mini-PROTEAN system, SDS-PAGE gels were prepared with a 10 % resolving and 4 % stacking gel. Bolt 4–12 % Bistris Plus Gel (Novex) were used with the Bolt Mini-gel system. To carry out SDS-PAGE, cell lysate was added to 4 × NuPAGE LDS-PAGE loading buffer (Novex) and samples were heated (70 °C for 10 min) or left untreated as indicated prior to electrophoresis for 1–2 h (as required for separation). Following SDS-PAGE, samples were transferred from the gel to a nitrocellulose membrane (GE Healthcare Hybond-ECL) using a Mini Trans-Blot system (Bio-Rad). Membranes to be immunoblotted were blocked with 2.5 % non-fat milk (Bio-Rad) in PBS overnight at 4 °C, followed by incubation with primary antibody for 1 h at room temperature. Membranes were subsequently incubated with the secondary antibody for 1 h at room temperature protected from light. Signal from membranes was detected using an Odyssey imager (Li-Cor).

Immunofluorescence. Vero76 cells were infected with either WNVKUN or Stop Mutant at m.o.i. 10 and fixed at 24 h p.i. in 80 % acetone in PBS. Fixed cells were blocked, and subsequently co-stained with 4G4 and an antibody recognizing a marker of the ER (rabbit polyclonal antibody against calnexin; Sigma-Aldrich). Alexa Fluor 488-conjugated anti-mouse and Alexa Fluor 555-conjugated anti-rabbit antibodies (Invitrogen) were used for secondary labelling. Nuclei were counterstained with DAPI and images captured using an LSM510 META confocal laser scanning microscope (Carl Zeiss).

Radiolabelling. Pulse–chase analysis was carried out in six-well plates of infected Vero cells (infected at m.o.i. 1 with WNVKUN or mutant viruses) or transfected 293T cells (transfected with pcDNA-NS1, pcDNA-NS1’ or both plasmids) at 24, 48 or 72 h. At 24 and 72 h p.i. or 24 and 48 h post-transfection, cells were starved for 30 min in Met- and Cys-free DMEM (Gibco), followed by labelling for 1.5 h with 100 μCi [35S]Met. After labelling, cells were washed once in PBS and twice in DMEM, and chased for 0, 1, 4 or 12 h in DMEM supplemented with 5 % FCS. Following radiolabelling, cell monolayers were placed on ice, and the culture fluids were removed, clarified by centrifugation at 1500 g for 5 min and mixed with equal volume of 2 × lysis buffer (20 mM Tris/HCI, pH 7.5, 150 mM NaCl, 10 mM EDTA, 2 % sodium deoxycholate, 2 % Triton X-100, 0.2 % SDS) containing 2 × Complete Protease Inhibitor Cocktail (Roche). The cell monolayer was rinsed with ice-cold PBS (pH 7.4), scraped from the plate in 1 × lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 % sodium deoxycholate, 1 % Triton X-100, 0.1 % SDS) containing protease inhibitors, incubated for 30 min on ice and clarified by centrifugation for 10 min at 14 000 g. Resulting protein preparations, both from culture fluid and cell monolayers, were immunoprecipitated with 4G4 using 25 μl Dynabeads Protein G per sample according to the manufacturer’s instructions. Eluted proteins were loaded onto SDS-PAGE gels, electrophoresed and labelled proteins were transferred to nitrocellulose membranes. Membranes were exposed to a phosphor screen and scanned on a Typhoon scanner (GE Healthcare) or exposed to X-ray film in an X-ray cassette at −80 °C and developed.

Virulence in mice. Groups of 10–20 CD1 mice (18 days old) were infected intraperitoneally with 1000 p.f.u. of either WNVKUN or Stop Mutant virus. Mice were monitored daily for signs of illness and euthanized when encephalitic symptoms were evident.

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REFERENCES


