Identification of residues in West Nile virus pre-membrane protein that influence viral particle secretion and virulence

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The pre-membrane protein (prM) of West Nile virus (WNV) functions as a chaperone for correct folding of the envelope (E) protein, and prevents premature fusion during virus egress. However, little is known about its role in virulence. To investigate this, we compared the amino acid sequences of prM between a highly virulent North American strain (WNVNY99) and a weakly virulent Australian subtype (WNVKUN). Five amino acid differences occur in WNVNY99 compared with WNKUN (I22V, H43Y, L72S, S105A and A156V). When expressed in mammalian cells, recombinant WNVNY99 prM retained native antigenic structure, and was partially exported to the cell surface. In contrast, WNKUN prM (in the absence of the E protein) failed to express a conserved conformational epitope and was mostly retained at the pre-Golgi stage. Substitutions in residues 22 (Ile to Val) and 72 (Leu to Ser) restored the antigenic structure and cell surface expression of WNKUN prM to the same level as that of WNVNY99, and enhanced the secretion of WNKUN prME particles when expressed in the presence of E. Introduction of the prM substitutions into a WNKUN infectious clone (FLSDK) enhanced the secretion of infectious particles in Vero cells, and enhanced virulence in mice. These findings highlight the role of prM in viral particle secretion and virulence, and suggest the involvement of the L72S and I22V substitutions in modulating these activities.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne virus in the Japanese encephalitis (JE) serocomplex of the genus Flavivirus, and is the causative agent of West Nile fever and West Nile encephalitis. Since the introduction of WNV to New York City in 1999 (WNVNY99), a total of 31,414 cases with 1,263 deaths have been reported in the USA (http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm, accessed 5/5/2012). In Australia, an endemic and weakly virulent strain of WNV, Kunjin virus (WNVKUN), circulates in the northern parts of the continent and has never been associated with any significant human outbreaks (Hall et al., 2002). Amino acid alignment of WNVNY99 and WNKUN reveals 97.7% similarity (Audsley et al., 2011; Lanciotti et al., 2002; Scherret et al., 2001b), and chimeric viruses of WNKUN and WNVNY99 demonstrated that the non-structural proteins, especially NS5 and NS2A, were largely responsible for the increased pathogenicity of WNVNY99 (Audsley et al., 2011). The Pro249 residue in NS3 was also similarly demonstrated to result in increased virulence of WNV (Brault et al., 2007). Additionally, the WNKUN strain lacks an N-linked glycan on the E protein, while the WNVNY99 E protein is glycosylated and has been associated with increased virulence in mice (Beasley et al., 2005; Scherret et al., 2001a). In recent years, more detailed investigations have led to the identification of specific residues and motifs in prM that have important roles in the virus life cycle. The Tyr78 residue in WNV prM was critical for particle assembly (Tan et al., 2009), and the His99 residue in Japanese encephalitis virus (JEV) prM was shown to be important for the same purpose (Lin & Wu, 2005). It was also demonstrated in dengue virus (DENV) that the His39 in M protein, corresponding to residue 131 in WNV prM, was vital for particle assembly (Pryor et al., 2004). While mutations in the GXXG motif of JEV prM, corresponding to residues 142–146 of WNV prM, resulted in reduced prM/E heterodimerization (Lin et al., 2010). Studies using tick-borne encephalitis virus (TBEV) demonstrated that the assembly and secretion of particles were affected by alanine substitutions at the residues which corresponded to Pro62, Asp64, Ile65, Asp66 and Trp68 of WNV prM (Yoshii et al., 2012). Interestingly, all the prM residues described above are conserved between WNVNY99 and WNKUN, leaving the role of prM in the pathogenicity of WNVNY99 to be poorly investigated.
Here, we evaluate the differences in antigenic characteristics between recombinant WNVKUN and WNVNY99 prM, likely to be influenced by the five amino acid differences between them. In addition, using prME expression constructs and a WNVKUN infectious clone, we show that the identified prM substitutions enhanced secretion of infectious particles in mammalian cells and increased virulence in mice.

RESULTS

The P10F8 epitope is absent on WNVKUN prM expressed independently of E

To examine potential differences in antigenic characteristics, recombinant constructs expressing WNVNY99 and WNVKUN prM were transfected in COS-7L cells and examined for the correct expression of prM in the absence of the E protein using an anti-prM mAb (P10F8). Although the binding site of P10F8 mAb has not been mapped, we have demonstrated by using reduced and unreduced WNV lysates that it binds a conformational epitope on WNVNY99 prM (Setoh et al., 2011) and WNVKUN prM (J. Hobson-Peters, unpublished data). Consistent with previous data, both recombinant prM proteins were successfully recognized when expressed in transfected cells (Fig. 1). However, when expressed independently of E, only the WNVNY99 prM protein was recognized by P10F8 (Fig. 1). Furthermore, expression of WNVKUN prMV5His containing a V5 tag for detection purposes demonstrated positive staining by using anti-V5 mAb, clearly indicating that WNVKUN prM was expressed in transfected cells (Fig. 1).

The successful recognition of WNVNY99 prM V5His by P10F8 mAb by using immunofluorescence assay (IFA) also indicated that the V5His tag did not disrupt the conformational P10F8 epitope on prM and was a suitable tag for use in this study (Fig. 1). Because the P10F8 epitope was disrupted on recombinant forms of WNVKUN prM, all subsequent WNVKUN prM constructs incorporated a C-terminal V5His tag for accurate detection and quantification of prM expression by using the anti-V5 mAb. In summary, these findings indicated that the prM protein of WNVKUN and WNVNY99 could be antigenically distinguished, and was characterized by the loss of the conformational P10F8 epitope when WNVKUN prM was expressed without E.

L72S substitution restored the P10F8 epitope on WNVKUN prM

Amino acid sequence alignment of WNVKUN (GenBank accession no. AY274504) with WNVNY99 prM (GenBank accession no. NC_009942) revealed five amino acid differences (Fig. 2a). We reasoned that the antigenic difference observed in WNVKUN and WNVNY99 prM, specifically the formation of the P10F8 epitope, was attributed directly to the amino acid sequence variation.

To test this hypothesis, the five amino acid residues of WNVKUN prM were individually substituted with those from WNVNY99 prM (I22V, H43Y, L72S, S105A and A156V). WNVKUN prM constructs bearing the respective substitutions were transfected into COS-7L cells and assessed by IFA by using the P10F8 mAb to establish the correct presentation of the epitope, and with anti-V5 mAb to confirm prM expression (Fig. 2b). The results revealed that the L72S substitution restored binding of P10F8 mAb to the recombinant WNVKUN prMV5His (Fig. 2b). To confirm that Ser72 was indeed critical for the formation of the P10F8 epitope on prM, the Ser72 residue in WNVNY99 prMV5His was substituted with Leu (WNVNY99 prMV5His S72L). The inability of P10F8 mAb to bind the WNVNY99 prMV5His S72L reverse mutant confirmed a role for Ser72 in the formation of the P10F8 epitope on prM (Fig. 2c). Consistent with the restoration of the P10F8 epitope when WNVKUN prM was expressed with E (Fig. 1), binding of P10F8 mAb to WNVNY99 prMV5His S72L was similarly restored by co-expression with E (Fig. 2c). Furthermore, binding of P10F8 mAb to the WNVNY99 prMV5His S72L reverse mutant and the wild-type WNVKUN prMV5His was restored when prM was co-expressed with either the glycosylated WNVNY99 E or the unglycosylated WNVKUN E proteins (Fig. 2d), indicating that formation of the P10F8 epitope on prM did not require the presence of the N-linked glycan on the E protein or of any other amino acid sequence differences in E between the WNVNY99 and WNVKUN strains.
Fig. 2. The L72S amino acid substitution restored P10F8 binding to WNVKUN prM. (a) Amino acid alignment of WNVKUN and WNVNY99 prM revealed five substitutions. (b) COS-7L cells transfected with constructs expressing WNVKUN prMV5His with the individual amino acid substitutions were analysed by IFA using anti-prM P10F8 mAb. Positive expression of all recombinant proteins was demonstrated by using the anti-V5 mAb. (c) COS-7L cells were transfected with constructs expressing WNVNY99 prMV5His, WNVNY99 prMV5His S72L reverse mutant, and also a co-transfection with an equal amount of WNVNY99 prMV5His S72L and WNVNY99 E constructs. The cells were fixed and probed with P10F8 and anti-V5 mAb. (d) Equal amounts of constructs expressing WNVNY99 prMV5His S72L and WNVKUN prMV5His were co-transfected with constructs expressing either glycosylated (WNVNY99) or unglycosylated (WNVKUN) forms of E protein. Correct expression of the P10F8 epitope was determined by reactivity with P10F8 mAb on IFA.
In order to assess the potential structural impact of changes in prM residue 72, a structural homology model of WNV\textsubscript{KUN} pr was generated based on the crystal structure of the DENV-2 pr (PDB 3C6E, Fig. 3) (Arnold \textit{et al.}, 2006; Li \textit{et al.}, 2008). The equivalent residue 72 in DENV-2 pr was a serine, like its counterpart in WNV\textsubscript{NY99} pr (highlighted in magenta, Fig. 3a) and was exposed on the surface of pr but juxtaposed to E in the heterocomplex. A change to the hydrophobic leucine would lead to a tendency of this residue being buried in the hydrophobic core of the protein (highlighted in magenta, Fig. 3b), leading to a local conformational change that may explain the loss of the P10F8 epitope on WNV\textsubscript{KUN} prM; whereas, in the context of a prM\textsubscript{E} heterocomplex, the leucine residue may be concealed by E, preventing this structural change from occurring.

### L72S enhances furin processing by influencing prM localization

Apart from its role in the stabilization of the epitope recognized by P10F8 mAb, Ser72 on prM also influenced furin cleavage of prM (Fig. 4). Furin cleavage efficiency was quantified by measuring the ratio of the integrated density between M and prM protein bands of transfected cell lysates by using the anti-V5 mAb on Western blot. This was possible because the C-terminal V5His tag on prMV5His remained attached to M after furin cleavage. The S72L substitution in WNV\textsubscript{NY99} prM resulted in decreased furin cleavage, while the L72S substitution in WNV\textsubscript{KUN} prM promoted furin cleavage (Fig. 4a). Since both WNV\textsubscript{KUN} and WNV\textsubscript{NY99} recombinant prM contained identical furin recognition motifs (P89-92 Arg-Ser-Arg-Arg – Fig. 2a), it was unexpected that WNV\textsubscript{NY99} prMV5His was more efficiently cleaved by furin than WNV\textsubscript{KUN} prMV5His (Fig. 4b). Because furin is predominantly localized to the trans-Golgi network (TGN) (Bosshart \textit{et al.}, 1994; Vey \textit{et al.}, 1994), and has only been shown to traffic between the TGN and the plasma membrane (Molloy \textit{et al.}, 1999; Voorhees \textit{et al.}, 1995), we hypothesized that the reduced furin cleavage in WNV\textsubscript{KUN} prMV5His was due to the retention of WNV\textsubscript{KUN} prM in the endoplasmic reticulum (ER) or at a pre-TGN stage. To test our hypothesis, cells expressing WNV\textsubscript{NY99} and WNV\textsubscript{KUN} prMV5His were lysed in furin cleavage/lysis buffer pH 7.4 and incubated at 37°C for 24 h with the addition of recombinant furin. The pH of the buffer was maintained similar to that of the ER (pH 7.1) (Kim \textit{et al.}, 1998) to eliminate the possibility that any improvement in furin cleavage of WNV\textsubscript{KUN} prM after cell lysis was due to pH-induced conformational changes in prM. Both WNV\textsubscript{NY99} and WNV\textsubscript{KUN} prM were cleaved with equal efficiencies, confirming that the furin recognition sites were identical and correctly presented on intracellular prM (Fig. 4c). Additionally, since furin was shown to be enzymically active between pH 6.0 and 8.5 (Molloy \textit{et al.}, 1992), the lack of WNV\textsubscript{KUN} prM cleavage in the cell confirms that retrograde transport of furin does not occur between the TGN and the ER. Interestingly, when the lysates were incubated in the furin cleavage/lysis buffer at 37°C for 24 h without the addition of recombinant furin, equal cleavage efficiencies were similarly observed for both WNV\textsubscript{NY99} and WNV\textsubscript{KUN} prM (Fig. 4d). Together, these data suggest that WNV\textsubscript{KUN} prM was probably retained at a pre-TGN level, and the release of furin and WNV\textsubscript{KUN} prM into the buffer during cell lysis allowed cleavage to occur (Fig. 4d).

Enhanced cleavage/export of recombinant WNV\textsubscript{KUN} prM is achieved by the L72S substitution in combination with I22V

Although the L72S substitution resulted in improved furin cleavage of WNV\textsubscript{KUN} prM, the efficiency of cleavage (K L72S – 11.6 %, Fig. 6a) was still considerably less than that of WNV\textsubscript{NY99} prMV5His (N – 21 %, Fig. 6a). To determine if additional residues were required for promoting efficient cleavage/export of the prM protein, double substitutions were introduced based on the other four amino acid differences between WNV\textsubscript{KUN} and WNV\textsubscript{NY99} prM. The results indicated that the combination of I22V and L72S substitutions increased furin cleavage of WNV\textsubscript{KUN} prMV5His (K L72S+I22V – 18.1 %, Fig. 6a) to a level similar to that of WNV\textsubscript{NY99} prMV5His (N – 18.8 %, Fig. 6a). Combinations of three amino acid substitutions...
demonstrated that no additional residues were positively influencing prM cleavage (Fig. 6a). As further evidence to support the prM export potential of the L72S and I22V substitutions, plasma membrane surface staining by IFA using anti-V5 mAb demonstrated the successful detection of wild-type WNVNY99 prMV5His and mutant WNVKUN prMV5His L72S
+ I22V, but not wild-type WNV KUN prMV5His, on the cell surface of transfected cells (Fig. 6b). Together, these data suggest that the Ser72 and Val22 residues in prM enhanced the export of the protein within the cell.

Effect of prM substitutions on prME particle secretion

To investigate the effect of the WNVKUN→WNVNY99 prM substitutions on prME particle secretion, we had to consider that WNVNY99 and WNVKUN have glycosylated and unglycosylated E, respectively. Since E glycosylation was demonstrated to enhance particle secretion (Hanna et al., 2005), a set of constructs expressing WNVNY99 and WNVKUN prME containing wild-type or prM substitutions, with or without E glycosylation were generated: (i) WNVNY99 prME CHO (+ wild-type), (ii) WNVNY99 prME CHO- (E protein glycosylation removed), (iii) WNVNY99 prM(V22I-S72L)E CHO-, (iv) WNVNY99 prM(V22I-S72L)E CHO+, (v) WNVKUN prME CHO- (wild-type), (vi) WNVKUN prME CHO+ (E protein glycosylation introduced), (vii) WNVKUN prM(I22V-L72S)E CHO-, (viii) WNVKUN prM(I22V-L72S)E CHO+.

Culture supernatant from transfected COS-7L cells was harvested 3 days post-transfection and analysed using an E antigen-capture ELISA (Hunt et al., 2002). Consistent with previous reports, the dominant requirement for efficient particle secretion was N-linked glycosylation of the E protein, demonstrated by the enhanced secretion of WNVNY99 prME and WNVKUN prME CHO+ particles (Fig. 7a). The effect of prM substitutions on particle secretion was not evident in the presence of E glycosylation (P>0.05, unpaired t-test) (Fig. 7b). However, when E

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**Fig. 4.** Ser72 promotes furin cleavage of prM intracellularly. (a) COS-7L cells were transfected with constructs expressing
WNVNY99 prMV5His (N), WNVNY99 prMV5His S72L (N S72L), WNVKUN prMV5His (K) and WNVKUN prMV5His L72S (K L72S), harvested at 48 h post-transfection and analysed by Western blot by using the anti-V5 mAb. Integrated densities of the prM and M bands were determined by using ImageJ software and the percentage M value for each lane was calculated using the formulae %M = M/(prM+M). The extra band indicated by an asterisk is the unglycosylated prM form. The furin cleavage assay was performed on transfected cell lysates under the following conditions: (b) no furin, (c) recombinant furin added, 37 °C for 24 h, (d) no furin, 37 °C for 24 h. Furin cleavage was determined by Western blot by using the anti-V5 mAb. WNVNY99 prMV5His with an abolished furin recognition motif (N furinΔ) was used as a control to account for the activity of cellular proteases.

**Fig. 5.** L72S substitution reduced the formation of prM multimers. WNVNY99 (N) and WNVKUN (K) recombinant prM with the various WNVKUN prM mutations (K I22V, K H43Y, K L72S, K S105A and K A156V) were analysed on Western blot by using anti-V5 mAb. K+DTT was prepared by heating the sample with the addition of 0.1 M DTT.

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glycosylation was absent, the effect of prM substitutions on particle secretion was significant (NprME CHO- vs NprME CHO- S72L/V22I $P=0.0024$; KprME CHO- vs KprME CHO- L72S/I22V $P=0.0002$, unpaired $t$-test), demonstrated by a 51.5% reduction in secretion of V22I+S72L WNVNY99 CHO- particles and a 65.3% increase in secretion of I22V+L72S WNVKUN CHO- particles relative to constructs with the corresponding wt prM proteins (Fig. 7c).

**Growth kinetics of prM mutant infectious clone-derived viruses**

To investigate the effect of the prM substitutions in the context of an infectious virus, the L72S and I22V substitutions were introduced into the WNVKUN infectious clone (FLSDX) (Khromykh et al., 1998) to generate the prM double mutant virus (vFLSDX2272). The prototype virus (vFLSDX) was generated directly from the FLSDX...

**Fig. 6.** L72S and I22V substitutions promoted intracellular export of recombinant prM. (a) Lysates of COS-7L cells transfected with the constructs expressing WNVNY99 prMV5His (N), WNVKUN prMV5His (K) and the respective WNVKUN prMV5His single, double or triple prM mutations were harvested at 48 h post-transfection and analysed by Western blot by using the anti-V5 mAb. %M was calculated using the formulae %M=M/(prM+M). (b) COS-7L cells seeded on glass coverslips were transfected with constructs expressing WNVNY99 prMV5His (N), WNVKUN prMV5His (K) and WNVKUN prMV5His L72S+I22V (K L72S+I22V). The cells were fixed 24 h post-transfection with 4% paraformaldehyde and probed by using the anti-V5 mAb. Three separate fields were taken per sample. Arrows indicate cell surface expression of prM. Cells were similarly stained after fixation by using 100% acetone to confirm protein expression.
enhanced export of recombinant WNVKUN prM and an WNVKUN prM; and when in combination with I22V, a substitution restored P10F8 mAb binding to recombinant 2009). Our investigation demonstrated that the L72S Yoshii et al. (2012) studies using TBEV (Goto important in assembly and secretion of virus particles in DISCUSSION

L72S and I22V prM substitutions increased WNVKUN virulence in mice

To determine the effect of L72S and I22V on virulence, Swiss weanling mice (18–19 days old, 10 mice per group) were injected with vFLSDX or vFLSDX2272 via the intra-peritoneal route at a dose of 100 p.f.u. A significant increase in virulence was observed with vFLSDX2272 compared with wt (vFLSDX) (P=0.0002, log rank Mantel–Cox algorithm), showing 100 % mortality by day 8, and a shorter median survival time of 7 days, compared with 12 days for wt (Fig. 8c). Brains from weanling mice that succumbed to encephalitis were collected for viral RNA extraction and sequencing confirmed that the prM substitutions were retained.

DISCUSSION

The prM protein has previously been demonstrated to be important in assembly and secretion of virus particles in studies using TBEV (Goto et al., 2005; Lorenz et al., 2002; Yoshii et al., 2012, 2004), DENV (Pryor et al., 2004), JEV (Lin et al., 2010; Lin & Wu, 2005) and WNV (Tan et al., 2009). Our investigation demonstrated that the L72S substitution restored P10F8 mAb binding to recombinant WNVKUN prM; and when in combination with I22V, an enhanced export of recombinant WNVKUN prM and an increased secretion of recombinant WNVKUN prME particles were observed. From in vitro analysis of prME constructs, we observed that the role of prM in the enhancement of particle secretion was masked due to the presence of the E glycan, suggesting that the prM mutations may be more significant in the context of unglycosylated WNV strains. Viral growth kinetics demonstrated that the L72S and I22V prM substitutions resulted in an earlier secretion of WNVKUN infectious particles.

The antigenic difference observed between recombinant WNVNY99 and WNVKUN prM when expressed in the absence of E suggested that the recombinant WNVKUN prM protein was differently or incompletely folded. This was indicated by the loss of reactivity with the P10F8 mAb, which recognizes a conformational epitope on prM, conserved between WNVNY99 and WNVKUN in the native virus particle (Setoh et al., 2011). In contrast, recombinant WNVNY99 prM was recognized by P10F8 mAb and as described in previous reports was capable of folding rapidly and independently of E (Konishi & Mason, 1993; Lorenz et al., 2002). Although the L72S substitution in WNVKUN prM successfully restored the epitope recognized by P10F8 mAb, it was important to note that while this epitope may be influenced by changes in residue 72, Ser72 may not necessarily be a component of the epitope itself. This was supported by the result showing successful P10F8 mAb binding when WNV prM containing a leucine at residue 72 (wild-type WNVKUN prM or WNVNY99 prM S72L) was co-expressed with the E protein. The disruption of the P10F8 epitope on WNVNY99 prM by Leu72 was probably associated with an alteration in prM structure to accommodate the hydrophobic leucine residue presented on the surface of prM.

In addition to stabilizing an antigenic site on prM, the L72S substitution was also implicated in intra-cellular transport of recombinant prM. Although the L72S substitution promoted WNVKUN prM export, thus reducing crowding in the ER, the amount of M protein detected was still lower than that observed with WNVNY99 prM,

Fig. 7. PrM substitutions affect prME secretion in the absence of E protein glycosylation. Culture supernatant from COS-7L cells transfected with the various constructs was collected at 72 h post-transfection, diluted in CoP buffer and analysed by antigen-capture ELISA. Optical densities were measured at 405 nm. (a) WNVNY99 prME (NprME) and WNVKUN prME (KprME) with or without E glycosylation (CHO +/−) analysed at a dilution of 1 : 8. (b) NprME CHO + and KprME CHO + constructs with or without prM substitutions analysed at a dilution of 1 : 16. (c) NprME CHO− and KprME CHO− constructs with or without prM substitutions analysed at a dilution of 1 : 4. Data were collected from two independent experiments; ELISA was performed in triplicate wells.

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improved the export of recombinant WNVKUN prM and I22V substitutions, similarly found in the pr segment, multiplication. Our data demonstrated that the L72S and prME. By introducing the same substitutions into the WNVKUN infectious clone, we demonstrated an enhancement in the secretion of infectious particles early in infection, particularly at a higher m.o.i. value. It must be taken into account that the reported lack of significance with viral growth kinetics at a low m.o.i. value, such as 0.1, could be attributed to a combination of factors including activity of membrane fusion, replication, particle assembly, secretion and at least an additional round of infection. At a high m.o.i. value, differences could be largely attributed to the efficiency of particle secretion, supporting the role of prM substitutions in particle secretion. Together with increased particle secretion, a significant increase in the level of virulence was also observed in mice. Although the increased virulence could be attributed to enhanced secretion, the prM mutations may potentially have a direct impact on the maturation of a virus particle, especially influencing furin-mediated maturation of an egressing virion. Specifically, the L72S mutation was demonstrated to support a more authentic recombinant prM structure. Although in vitro furin cleavage experiments demonstrated that recombinant forms of WNVNY99 and WNVKUN prM were both cleaved with equal efficiencies, the experiment neglects the structural arrangement of a prME particle, and the conformational rearrangements that occurs prior to furin cleavage (Li et al., 2008; Stadler et al., 1997). The effect of L72S mutation may influence the presentation of prM/E on a virion in a way that resulted in a more mature particle. However, it must be pointed out that the completeness of prM cleavage was shown to have an insignificant effect on the infectivity of the virus (Keelapang et al., 2004; Mukherjee et al., 2011). Nevertheless, maturation status of the prM mutant virus will be determined in a future study. In keeping with our hypothesis that the identified prM substitutions were associated with increased virulence, it was worth noting that alignment of prM sequences showed that all Kunjin isolates sequenced to date (MRM16: GenBank accession no. GQ851602, K6453: GenBank accession no. GQ851603, MRM61C: GenBank accession no. AY274504, NSW2011: JN887352) have Ile22 and Leu72, and have never been documented to be involved in significant human outbreaks. On the other hand, WNV strains that were involved in major outbreaks of human disease (Russian isolate: GenBank accession no. FJ425721, North American isolate: GenBank accession no. NC_009942, Romanian isolate: GenBank accession no. AF260969, Greece isolate: GenBank accession no. HQ537483) have Val22 and Ser72 conserved on prM.

At this point, our understanding of how the identified mutations in prM are involved in increased virulence is far from complete. In summary, evidence from our in vitro characterization experiments suggested that the L72S and I22V prM substitutions resulted in improved intracellular export of recombinant prM, and enhanced secretion of prME and infectious particles. We also highlight that the L72S and I22V prM mutations in WNVKUN resulted in increased particle secretion, a significant increase in the infectious titre of each sample was determined by TCID50 on Vero cells. The limit of detection for the assay was 2 log TCID50 ml\(^{-1}\) indicated by the horizontal dotted line. (c) The virulence of vFLSDX and vFLSDX2272 was determined by plotting the survival of weanling Swiss-outbred mice (18–19 days, 10 mice per group) following intraperitoneal (i.p.) inoculation with 100 p.f.u. of vFLSDX or vFLSDX2272. The mice were monitored for 14 days p.i. for signs of encephalitis, at which point the animals were sacrificed.

A recent study by Yoshii et al. (2012) identified a conserved region within the prM protein of TBEV, corresponding to residues 61–69 in WNV prM, which is important in several functional aspects of the virus such as a prM–E interaction, particle assembly, viral protein trafficking and viral multiplication. Our data demonstrated that the L72S and I22V substitutions, similarly found in the pr segment, improved the export of recombinant WNVKUN prM and prME. By introducing the same substitutions into the WNVKUN infectious clone, we demonstrated an enhancement in the secretion of infectious particles early in infection, particularly at a higher m.o.i. value. It must be taken into account that the reported lack of significance with viral growth kinetics at a low m.o.i. value, such as 0.1, could be attributed to a combination of factors including activity of membrane fusion, replication, particle assembly, secretion and at least an additional round of infection. At a high m.o.i. value, differences could be largely attributed to the efficiency of particle secretion, supporting the role of prM substitutions in particle secretion. Together with increased particle secretion, a significant increase in the level of virulence was also observed in mice. Although the increased virulence could be attributed to enhanced secretion, the prM mutations may potentially have a direct impact on the maturation of a virus particle, especially influencing furin-mediated maturation of an egressing virion. Specifically, the L72S mutation was demonstrated to support a more authentic recombinant prM structure. Although in vitro furin cleavage experiments demonstrated that recombinant forms of WNVNY99 and WNVKUN prM were both cleaved with equal efficiencies, the experiment neglects the structural arrangement of a prME particle, and the conformational rearrangements that occurs prior to furin cleavage (Li et al., 2008; Stadler et al., 1997). The effect of L72S mutation may influence the presentation of prM/E on a virion in a way that resulted in a more mature particle. However, it must be pointed out that the completeness of prM cleavage was shown to have an insignificant effect on the infectivity of the virus (Keelapang et al., 2004; Mukherjee et al., 2011). Nevertheless, maturation status of the prM mutant virus will be determined in a future study. In keeping with our hypothesis that the identified prM substitutions were associated with increased virulence, it was worth noting that alignment of prM sequences showed that all Kunjin isolates sequenced to date (MRM16: GenBank accession no. GQ851602, K6453: GenBank accession no. GQ851603, MRM61C: GenBank accession no. AY274504, NSW2011: JN887352) have Ile22 and Leu72, and have never been documented to be involved in significant human outbreaks. On the other hand, WNV strains that were involved in major outbreaks of human disease (Russian isolate: GenBank accession no. FJ425721, North American isolate: GenBank accession no. NC_009942, Romanian isolate: GenBank accession no. AF260969, Greece isolate: GenBank accession no. HQ537483) have Val22 and Ser72 conserved on prM.

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significantly enhanced virulence in mice. Our study emphasizes the importance of prM in viral function and suggests a role of prM-associated enhancement in particle secretion and virulence.

**METHODS**

**Construction of plasmids.** The construction of WNV$_{NY99}$ prME, prM and prMV5His recombinant proteins was as described previously (Setoh et al., 2011). WNV$_{KUN}$ prME, prM and prMV5His recombinant proteins were constructed using the same strategy. WNV$_{NY99}$ and WNV$_{KUN}$ E constructs were generated by amplifying the E genes from the prME constructs using the primer sets NY99 E Forward/NY99 E Reverse, and KUN E Forward/KUN E Reverse, and ligated into a pcDNA3.1 vector containing a modified JEV signal sequence (NH$_2$-MGKRSAGSIMWLASLAVVIACAGACOOH) to assist in ER translocation (Chang et al., 2001).

Substitutions of prM residues were performed by using the primers KUNI22V, KUNI22V antisense, KUNH43Y, KUNH43Y antisense, KUNL72S, KUNL72S antisense, KUNS105A, KUNS105A antisense, KUNA156V, KUNA156V antisense, NY99S72L, NY99S72L antisense, NY99V22I and NY99V22I antisense. The introduction or removal of the N-glycosylation site at residue 154 in the E protein was performed using the primers NY99ECHO-, NY99ECHO- antisense, KUNECHO+ and KUNECHO+ antisense. Deletion of the arginine residues 91 and 92 on WNV$_{NY99}$ prMV5His was performed using the primers del91-92 and del91-92 antisense. Deletion of the furin cleavage-deficient prM (N furin) substitutions were performed using the QuikChange Lightning Site-Directed Mutagenesis kit (Genomics Agilent). Plasmids containing the desired substitutions were confirmed by sequencing (Australian Genome Research Facility, AGRF). All primer sequences are listed in Table S1 (available in JGV Online).

**Cell culture.** COS-7L cells were maintained in RPMI 1640 (Invitrogen-Gibco) with 2 % FBS, 50 U penicillin ml$^{-1}$, 50 µg streptomycin ml$^{-1}$ and 2 mM glutamax (Invitrogen-Gibco). Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen-Gibco) with 2 % FBS, 50 U penicillin ml$^{-1}$, 50 µg streptomycin ml$^{-1}$ and 2 mM glutamax (Invitrogen-Gibco). All cells were incubated at 37°C with 5 % CO$_2$.

**Antibodies and reagents.** A mAb P10F8 that recognizes a conformational epitope on WNV prM (Setoh et al., 2011) was supplied as crude hybridoma culture supernatant and used at a 1:10 dilution. Purified anti-V5 mouse mAb (Invitrogen) that recognizes the 14-mer V5-tag (GKPIPNPLLGLDST) was used at a 1:5000 dilution. Goat anti-mouse IgG HRP (1:2000 dilution; Dako) was supplied as crude hybridoma culture supernatant and used at a 1:10 dilution. Western blot analysis. Samples were analysed by Western blot using the anti-V5 mAb. Western blot analysis was performed as described previously (Hunt et al., 2002). Briefly, Nunc MaxiSorp flat-bottom 96-well plates (Nunc) were coated with anti-E mAb 3.91D acitic fluid (1:1000 dilution) at 4°C o/n in coating buffer (50 mM Na$_2$CO$_3$, 50 mM NaHCO$_3$, pH 9.6). Culture supernatants from transfected or infected cells were diluted in CoP buffer (10 mM Tris/ HCl pH 8.2, 150 mM NaCl, 5 mM EDTA, 1 % NP-40) and added into wells in triplicate. The samples were captured by incubation for 1 h, before blocking buffer was added and incubated for 1 h. Anti-E HRP-conjugated detector mAb 6B6C-1 (1:3500 dilution; Chemicon) was added and incubated for 1 h, followed by the addition of ABTS substrate solution [0.1 M citric acid, 0.02 % (v/v) ABTS, 0.06 % (v/v) H$_2$O$_2$]. Wells were washed three times with PBS/T in between all steps. Optical densities were read on a UV plate reader at 405 nm after 30 min (Labsystems Multiscan EX type 355; PathTech).

**Antigen-capture ELISA.** Antigen-capture ELISA was performed as described previously (Hunt et al., 2002). Briefly, Nunc MaxiSorp flat-bottom 96-well plates (Nunc) were coated with anti-E mAb 3.91D acitic fluid (1:1000 dilution) at 4°C o/n in coating buffer (50 mM Na$_2$CO$_3$, 50 mM NaHCO$_3$, pH 9.6). Culture supernatants from transfected or infected cells were diluted in CoP buffer (10 mM Tris/ HCl pH 8.2, 150 mM NaCl, 5 mM EDTA, 1 % NP-40) and added into wells in triplicate. The samples were captured by incubation for 1 h, before blocking buffer was added and incubated for 1 h. Anti-E HRP-conjugated detector mAb 6B6C-1 (1:3500 dilution; Chemicon) was added and incubated for 1 h, followed by the addition of ABTS substrate solution [0.1 M citric acid, 0.02 % (v/v) ABTS, 0.06 % (v/v) H$_2$O$_2$]. Wells were washed three times with PBS/T in between all steps. Optical densities were read on a UV plate reader at 405 nm after 30 min (Labsystems Multiscan EX type 355; PathTech).

**Construction of mutant FLSDX infectious clone.** The prM and E genes from the WNV$_{KUN}$ FLSDX infectious clone (Khromykh et al., 1998) were first subcloned into the pGEM vector using the SacI and NdeI restriction sites. Amino acid substitutions in prM (L72S and L72F) were performed using the QuickChange Lightning Site-Directed Mutagenesis kit (Agilent). The KUNL72S, KUNL72S antisense, KUNI22V and KUNI22V antisense primers were used for the generation of the prM substitutions (Table S1). The pGEM subclone containing the correct substitutions was identified by sequencing, and reaction, 25 µl cell lysate in furin cleavage/lysis buffer was incubated with 1 U recombinant furin (New England Biolabs) for 24 h at 37°C. Samples were analysed by Western blot using the anti-V5 mAb.

**Cell transfection was performed using Lipofectamine 2000 following the manufacturer’s instructions (Invitrogen) with 2 % FBS, 50 U penicillin ml$^{-1}$, 50 µg streptomycin ml$^{-1}$ and 2 mM glutamax (Invitrogen-Gibco).**
the SacII/NdeI fragment containing the mutated prM and E genes was subsequently excised by restriction enzyme digestion and religated into the FLSDX infectious clone.

**Viral RNA transcription and electroporation.** Viral RNA transcripts were generated from Xhol-linearized plasmids (FLSDX and associated mutants) by using SP6 RNA polymerase as described previously (Liu et al., 2003). BHK cells electroporated with the RNA transcripts were incubated in 10% FBS/DMEM and supernatant was harvested as passage 0 virus stocks once cytopathic effects (CPE) were evident. Passage 1 stocks were prepared by infecting Vero cells with passage 0 stocks and were used in subsequent experiments. Viral RNA was extracted from passage 1 viruses and subjected to PCR amplification using the high-fidelity Phusion polymerase (Finnzymes) before sequence confirmation (AGRF). Viruses derived from infectious clones were designated with a lower case ‘v’.

**Mouse virulence.** All animal procedures had received prior approval from The University of Queensland Animal Ethics Committee and where necessary were performed under ketamine/xylazil anaesthesia. Swiss weanling mice (18–19 days old) (Animal Resources Centre, Murdoch, Western Australia, Australia) were infected via the intraperitoneal (i.p.) route with vFLSDX or vFLSDX2272 at a dose previously (Prow et al., 2011). Mice were kept on clean bedding and given food and water ad libitum. Infected animals were monitored daily for the onset of disease and culled when the first signs of encephalitis (hunching, lethargy, eye closure or hind-leg flaccid paralysis) were apparent. Surviving mice were bled by cardiac puncture at the end of the experiment (day 21) and the sera were tested for evidence of seroconversion to WNVKUN by using fixed cell ELISA (Adams et al., 1995). The significance of clinical differences between groups was determined by the Mantel–Cox test (GraphPad Prism 5.04; GraphPad Software Inc).

**REFERENCES**


