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 WNVKUN (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, WNVKUN 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 WNVKUN prM to the same level as that of WNVNY99, and enhanced the secretion of WNVKUN prME particles when expressed in the presence of E. Introduction of the prM substitutions into a WNVKUN infectious clone (FLSDX) 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 WNVKUN reveals 97.7% similarity (Audsley et al., 2011; Lanciotti et al., 2002; Scherret et al., 2001b), and chimeric viruses of WNVKUN and WNVNY99 demonstrated that the non-structural proteins, especially NS5 and NS2A, were largely responsible for the increased pathogenicity of WNV (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 WNVKUN 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 GXXGX 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 WNVKUN, leaving the role of prM in the pathogenicity of WNVNY99 to be poorly investigated.
Here, we evaluate the differences in antigenic characteristics between recombinant WNV<sub>KUN</sub> and WNV<sub>NY99</sub> prM, likely to be influenced by the five amino acid differences between them. In addition, using prME expression constructs and a WNV<sub>KUN</sub> 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 WNV<sub>KUN</sub> prM expressed independently of E

To examine potential differences in antigenic characteristics, recombinant constructs expressing WNV<sub>NY99</sub> and WNV<sub>KUN</sub> 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 WNV<sub>NY99</sub> prM (Setoh et al., 2011) and WNV<sub>KUN</sub> prM (J. Hobson-Peters, unpublished data). Consistent with previous data, both recombinant prM proteins were successfully recognized when expressed in conjunction with E (Fig. 1). However, when expressed independently of E, only the WNV<sub>NY99</sub> prM protein was recognized by P10F8 (Fig. 1). Furthermore, expression of WNV<sub>KUN</sub> prM<sub>V5His</sub> containing a V5 tag for detection purposes demonstrated positive staining by using anti-V5 mAb, clearly indicating that WNV<sub>KUN</sub> prM was expressed in transfected cells (Fig. 1). The successful recognition of WNV<sub>NY99</sub> prM V5His by P10F8 mAb was restored by co-expression with E (Fig. 1). To confirm that Ser72 was indeed critical for the formation of the P10F8 epitope on prM, the Ser72 residue in WNV<sub>NY99</sub> prM was substituted with Leu (WNV<sub>NY99</sub> prM<sub>S72L</sub>). The inability of P10F8 mAb to bind the WNV<sub>NY99</sub> prM<sub>S72L</sub> 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 WNV<sub>KUN</sub> prM was expressed with E (Fig. 1), binding of P10F8 mAb to WNV<sub>NY99</sub> prM<sub>S72L</sub> was similarly restored by co-expression with E (Fig. 2c). Furthermore, binding of P10F8 mAb to the WNV<sub>NY99</sub> prM<sub>S72L</sub> reverse mutant and the wild-type WNV<sub>KUN</sub> prM<sub>V5His</sub> was restored when prM was co-expressed with either the glycosylated WNV<sub>NY99</sub> E or the unglycosylated WNV<sub>KUN</sub> 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 WNV<sub>NY99</sub> and WNV<sub>KUN</sub> strains.

L72S substitution restored the P10F8 epitope on WNV<sub>KUN</sub> prM

Amino acid sequence alignment of WNV<sub>KUN</sub> (GenBank accession no.AY274504) with WNV<sub>NY99</sub> prM (GenBank accession no. NC_009942) revealed five amino acid differences (Fig. 2a). We reasoned that the antigenic difference observed in WNV<sub>KUN</sub> and WNV<sub>NY99</sub> prM, specifically the formation of the P10F8 epitope, was attributed directly to the amino acid sequence variation.

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**Fig. 1.** The P10F8 epitope is absent on recombinant WNV<sub>KUN</sub> prM. Comparison of antibody reactivity by IFA in COS-7L cells transfected with WNV<sub>KUN</sub> and WNV<sub>NY99</sub> prM, prME and prM<sub>V5His</sub> constructs. Cells were fixed in 100 % ice-cold acetone at 24 h post-transfection and probed with P10F8 and anti-V5 mAb.

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To test this hypothesis, the five amino acid residues of WNV<sub>KUN</sub> prM were individually substituted with those from WNV<sub>NY99</sub> prM (I22V, H43Y, L72S, S105A and A156V). WNV<sub>KUN</sub> prM constructs bearing the respective substitutions were transfected into COS-7L cells and assessed by IFA 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 WNV<sub>KUN</sub> prM<sub>V5His</sub> (Fig. 2b). To confirm that Ser72 was indeed critical for the formation of the P10F8 epitope on prM, the Ser72 residue in WNV<sub>NY99</sub> prM was substituted with Leu (WNV<sub>NY99</sub> prM<sub>S72L</sub>). The inability of P10F8 mAb to bind the WNV<sub>NY99</sub> prM<sub>S72L</sub> 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 WNV<sub>KUN</sub> prM was expressed with E (Fig. 1), binding of P10F8 mAb to WNV<sub>NY99</sub> prM<sub>S72L</sub> was similarly restored by co-expression with E (Fig. 2c). Furthermore, binding of P10F8 mAb to the WNV<sub>NY99</sub> prM<sub>S72L</sub> reverse mutant and the wild-type WNV<sub>KUN</sub> prM<sub>V5His</sub> was restored when prM was co-expressed with either the glycosylated WNV<sub>NY99</sub> E or the unglycosylated WNV<sub>KUN</sub> 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 WNV<sub>NY99</sub> and WNV<sub>KUN</sub> strains.
Fig. 2. The L72S amino acid substitution restored P10F8 binding to WNV\textsubscript{KUN} prM. (a) Amino acid alignment of WNV\textsubscript{KUN} and WNV\textsubscript{NY99} prM revealed five substitutions. (b) COS-7L cells transfected with constructs expressing WNV\textsubscript{KUN} 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 WNV\textsubscript{NY99} prMV5His, WNV\textsubscript{NY99} prMV5His S72L reverse mutant, and also a co-transfection with an equal amount of WNV\textsubscript{NY99} prMV5His S72L and WNV\textsubscript{NY99} E constructs. The cells were fixed and probed with P10F8 and anti-V5 mAb. (d) Equal amounts of constructs expressing WNV\textsubscript{NY99} prMV5His S72L and WNV\textsubscript{KUN} prMV5His were co-transfected with constructs expressing either glycosylated (WNV\textsubscript{NY99}) or unglycosylated (WNV\textsubscript{KUN}) 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_KUN pr was generated based on the crystal structure of the DENV-2 pr (PDB 3C6E, Fig. 3) (Arnold et al., 2006; Li et al., 2008). The equivalent residue 72 in DENV-2 pr was a serine, like its counterpart in WNV 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_KUN prM; whereas, in the context of a prME 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 of WNV NY99 prM resulted in decreased furin cleavage, while the L72S substitution in WNV_KUN prM promoted furin cleavage (Fig. 4a). Since both WNV_KUN and WNV NY99 recombinant prM contained identical furin recognition motifs (P89-92 Arg-Ser-Arg-Arg – Fig. 2a), it was unexpected that WNV NY99 prMV5His was more efficiently cleaved by furin than WNV_KUN prMV5His (Fig. 4b). Because furin is predominantly localized to the trans-Golgi network (TGN) (Bosshart et al., 1994; Vey et al., 1994), and has only been shown to traffic between the TGN and the plasma membrane (Molloy et al., 1999; Voorhees et al., 1995), we hypothesized that the reduced furin cleavage in WNV_KUN prMV5His was due to the retention of WNV_KUN prM in the endoplasmic reticulum (ER) or at a pre-TGN stage. To test our hypothesis, cells expressing WNV NY99 and WNV_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 et al., 1998) to eliminate the possibility that any improvement in furin cleavage of WNV_KUN prM after cell lysis was due to pH-induced conformational changes in prM. Both WNV NY99 and WNV_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 et al., 1992), the lack of WNV_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 NY99 and WNV_KUN prM (Fig. 4d). Together, these data suggest that WNV_KUN prM was probably retained at a pre-TGN level, and the release of furin and WNV_KUN prM into the buffer during cell lysis allowed cleavage to occur (Fig. 4d).

In addition, the WNV_KUN prM L72S mutant displayed a considerable reduction in prM aggregates that migrated as high molecular mass prM multimers by immunoblot. Boiling of the samples in the presence of SDS failed to dissociate the prM multimers, with successful dissociation only achieved after the addition of the reducing agent DTT (Fig. 5). This suggested that wild-type WNV_KUN prM and the other four prM mutants (I22V, H43Y, S105A and A156V) were probably retained in the ER, and the prM multimers were the result of prM aggregates linked by intermolecular disulphide bonds. We propose that the L72S substitution promote the export of recombinant WNV_KUN prM within the cell, and that furin cleavage is a good indicator of recombinant prM export.

**Enhanced cleavage/export of recombinant WNV_KUN prM is achieved by the L72S substitution in combination with I22V**

Although the L72S substitution resulted in improved furin cleavage of WNV_KUN prM, the efficiency of cleavage (K L72S – 11.6%, Fig. 6a) was still considerably less than that of WNV 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_KUN and WNV NY99 prM. The results indicated that the combination of I22V and L72S substitutions increased furin cleavage of WNV_KUN prMV5His (K L72S + I22V – 18.1%, Fig. 6a) to a level similar to that of WNV NY99 prMV5His (N – 18.8%, Fig. 6a). Combinations of three amino acid substitutions

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**Fig. 3.** Crystal structure and homology model of pr-E. (a) The residue Ser72 on pr is highlighted in magenta on the DENV-2 pr-E crystal structure. (b) Homology model of WNV_KUN pr based on the DENV-2 pr model, highlighting the equivalent residue Leu72 in magenta.
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...
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

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**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 et al. (Lin et al., 2010; Lin & Wu, 2005) and WNV (Tan et al., 2004), JEV (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,

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 intraperitoneal 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.

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**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.
I22V substitutions, similarly found in the pr segment, multiplication. Our data demonstrated that the L72S and particle assembly, viral protein trafficking and viral functional aspects of the virus such as a prM–E interaction, residues 61–69 in WNV prM, which is important in several region within the prM protein of TBEV, corresponding to 1972 indicating that WNVKUN prM export was still limited by Y. X. Setoh and others virion. Specifically, the L72S mutation was demonstrated impact on the maturation of a virus particle, especially secretion, the prM mutations may potentially have a direct increased virulence could be attributed to enhanced 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 prME 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...
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 WNVNY99 prME, prM and prMV5His recombinant proteins was as described previously (Setoh et al., 2011). WNVKUN prME, prM and prMV5His recombinant proteins were constructed using the same strategy. WNVNY99 and WNVKUN 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 (NH₂-MGKRASGSIMWLASLAVIACAGACOOH) 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, NY999S27L, NY999S27L_antisense, NY999V21L and NY999V21L_antisense. The introduction or removal of the N-glycosylation site at residue 154 in the E protein was performed using the primers NY999ECOH-, NY999ECOH_antisense, KUNECHO+ and KUNECHO+ _antisense. Deletion of the arginine residues 91 and 92 on WNY99 prMV5His was performed using the primers del91-92 and del91-92_antisense to generate a furin cleavage deficient prM (N furinΔ). All modifications 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 and 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 % CO₂.

**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 used for the detection of mouse primary antibodies. Incubation of all antibodies was performed at room temperature.

**Antigen preparation.** Cell transfection was performed using Lipofectamine 2000 following the manufacturer’s instructions (Invitrogen). Enough blocking buffer was added to cover the cells and incubated for 1 h, followed by 1 h incubation with the respective primary antibodies diluted in blocking buffer. The cells were then washed with PBS/T, followed by 1 h incubation with Alexa Fluor 488-conjugated anti-mouse IgG in the dark (1:500 dilution in blocking buffer; Invitrogen). A nuclear stain (Hoechst 33342; Invitrogen) was added at a dilution of 1 : 1000 in PBS and incubated for 5 min. A final wash with PBS/T was performed before mounting the coverslips onto glass microscope slides by using ProLong Gold Antifade Reagent (Invitrogen). Immunofluorescence imaging was performed by using the ZEISS LSM 510 META confocal microscope.

**Western blot analysis.** Cell lysates were diluted in 4 × NuPage LDS sample buffer (Invitrogen) and heated at 95 °C for 5 min with or without the addition of 0.1 M DTT. The proteins were separated by SDS-PAGE (4–12 % precast gradient polyacrylamide gels; Invitrogen) at 170 V for 30 min, and transferred onto a nitrocellulose membrane (Amersham Biosciences) at 30 V for 1 h. Membranes were blocked using blocking buffer [0.05 M Tris/HCl pH 8.0, 1 mM EDTA, 0.15 M NaCl, 0.05 % (v/v) Tween 20, 0.2 % (w/v) casein] for 1 h at room temperature or overnight (o/n) at 4 °C to reduce background, followed by the addition of diluted mAbs or horse serum for 1 h. The blots were washed three times with PBS containing 0.05 % Tween 20 (PBS/T) and then incubated for 1 h with the relevant HRP-conjugated secondary antibody. After a further three washes, the blots were developed by using DAB substrate solution [1.5 mM 3,3'-diaminobenzidine, 0.06 % (v/v) H₂O₂, in PBS, pH 7.2] for 15 min. The reaction was terminated by washing with PBS. The developed blots were air-dried and scanned, and the integrated densities of bands were determined by using the ImageJ image processing and analysis software (http://rsweb.nih.gov/jj).

**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 (1:1000 dilution) at 4 °C o/n in coating buffer (50 mM Na₂CO₃, 50 mM NaHCO₃, 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) H₂O₂]. 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 WNVNY99 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 I22V) were performed using the QuikChange Lightning Site-Directed Mutagenesis kit (Genomics 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
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 for 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’.

**Viral growth kinetics.** Vero cells were cultured in 24-well plates, allowing one well for each sample collected. All time points were collected in triplicate. When the cells were 70% confluent they were infected with virus at a m.o.i. of 0.1 or 1 by dilution in 2% FBS/DMEM. After 1 h at 37 °C, virus inoculum was removed and the cells were washed three times with PBS before the addition of 1 ml fresh medium (2% FBS/DMEM). Samples were collected at 2, 6, 12, 24, 48 and 72 h p.i. and stored at −70 °C. The infectious titre in each sample was determined by measuring the TCID₅₀ on Vero cells by the method of Reed & Muench (1938).

**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 of 100 p.f.u. determined by plaque assay using Vero cells as described 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 collected for viral neutralization and antigenic variation among Kunjin virus isolates. Surviving mice were bled by cardiac puncture at the end of the experiment (day 21) and the sera were collected for virus isolation.

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