Determinants of attenuation in the envelope protein of the flavivirus Alfuy

Natalie A. Prow,¹ † Fiona J. May,¹ † Daniel J. Westlake,¹ † Robert J. Hurrelbrink,² Rebecca M. Biron,¹ Jason Y. Leung,¹ Peter C. McMinn,² † David C. Clark,¹ § John S. Mackenzie,³ Mario Lobigs,⁴ Alexander A. Khromykh¹ and Roy A. Hall¹

¹Centre for Infectious Disease Research, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, QLD 4072, Australia
²Division of Virology, Telethon Institute for Child Health Research, Roberts Road, Subiaco, WA 6008, Australia
³Australian Biosecurity Cooperative Research Centre, Faculty of Health Sciences, Curtin University of Technology, GPO U1987, Perth, WA 6845, Australia
⁴John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia

Murray Valley encephalitis virus (MVEV) is a mosquito-borne flavivirus endemic to Australia and Papua New Guinea. Most strains of MVEV cause potentially fatal cases of encephalitis in humans and horses, and have been shown to be highly neuroinvasive in weanling mice. In contrast, the naturally occurring subtype Alfuy virus (ALFV) has never been associated with human disease, nor is it neuroinvasive in weanling mice, even at high doses. To identify viral factors associated with ALFV attenuation, a chimeric infectious clone was constructed containing the structural genes premembrane (prM) and envelope (E) of ALFV swapped into the MVEV genome. The resulting virus (vMVEV/ALFVstr) was no longer neuroinvasive in mice, suggesting that motifs within prM–E of ALFV confer attenuation. To define these motifs further, mutants were constructed by targeting divergent sequences between the MVEV and ALFV E proteins that are known markers of virulence in other encephalitic flaviviruses. MVEV mutants containing a unique ALFV sequence in the flexible hinge region (residues 273–277) or lacking the conserved glycosylation site at position 154 were significantly less neuroinvasive in mice than wild-type MVEV, as determined by delayed time to death or increased LD50. Conversely, when the corresponding MVEV sequences were inserted into the vMVEV/ALFVstr chimera, the mutant containing the MVEV hinge sequence was more neuroinvasive than the parental chimera, though not to the same level as wild-type MVEV. These results identify the hinge region and E protein glycosylation as motifs that contribute to the attenuation of ALFV.

INTRODUCTION

The Japanese encephalitis serogroup of flaviviruses comprises a number of mosquito-borne viruses that cause neurological disease in humans and animals (Mackenzie et al., 2002). These include Japanese encephalitis virus (JEV), West Nile virus, St. Louis encephalitis virus and Murray Valley encephalitis virus (MVEV). MVEV is enzootic in northern regions of Australia, with occasional epizootics in more southerly areas resulting in sporadic outbreaks of human neurological disease (Broom et al., 2002). Annual cases of severe and sometimes fatal encephalitis in aboriginal children in northern Australia are often associated with MVEV infection (Broom et al., 2002). This is consistent with observations that isolates of MVEV obtained from clinical material or mosquito samples are highly neuroinvasive when inoculated into weanling mice (Lobigs et al., 1988). In contrast, Alfuy virus (ALFV), which shares a high degree of genetic and antigenic similarity with (May et al., 2006) and is...
classified as a subtype of MVEV (Thiel et al., 2005), has not been associated with human disease despite sharing ecology, prevalence and distribution similar to MVEV (Mackenzie & Williams, 2009). Furthermore, isolates of ALFV have been shown to be poorly neuroinvasive in weanling mice when compared with MVEV (May et al., 2006).

Flaviviruses are positive-sense RNA viruses, containing a genome of approximately 11 kb. The genome encodes a single polyprotein which is co- and post-translationally processed into three structural proteins, capsid (C), premembrane/membrane (prM/M) and envelope (E), along with seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Mature virions possess a lipid envelope with the viral M and E proteins embedded, and a nucleocapsid comprised of the viral RNA and C protein (Mukhopadhyay et al., 2005). The E protein is a class II fusion protein involved in virus entry into cells by receptor-mediated endocytosis (Chen et al., 1996; Heinz & Allison, 2003; Gollins & Porterfield, 1985) and low-pH-dependent fusion of the virus envelope with endosomal membranes (Allison et al., 1995; Heinz et al., 1994; Kimura et al., 1986; Randolph & Stollar, 1990; Vorovitch et al., 1991). The flavivirus E protein ectodomain can be divided into three domains (I, II and III), with virulence determinants having been identified within each of these, including the N-linked glycosylation motif in domain I (Shirato et al., 2004), the fusion peptidase domain II (Allison et al., 2001) and the RGD motif in domain III (Lobigs et al., 1990).

Sequence alignment revealed 85% amino acid identity between the polyproteins of ALFV and MVEV with 92 amino acid changes in the prM and E proteins. The most notable differences occurred in the E protein, including: (i) a stable substitution resulting in abolition of the conserved glycosylation site in domain I of ALFV E protein from positions 154 to 156 and (ii) a stretch of five consecutive amino acid substitutions within the flexible hinge region of positions 273 to 277 (May et al., 2006). The role of E protein glycosylation in flavivirus virulence is unclear, with some studies on WNV showing that removal of the glycosylation site results in decreased neuroinvasion (Beasley et al., 2004a; Beasley et al., 2005; Shirato et al., 2004), while others report that acquisition of glycosylation decreased neuroinvasion (Chambers et al., 1998; Haley et al., 1994). The hinge region of E protein is a flexible region between domains I and II that is involved in the pH-induced conformational change that occurs during fusion with endosomal membranes (Bressanelli et al., 2004; Gollins & Porterfield, 1985). Mutations in this region of MVEV caused decreased neuroinvasiveness, most probably by affecting interaction of the mutant E protein with the cell surface or with endosomal membranes (Hurrelbrink & McMinn, 2001). Mutations within the hinge region may also indirectly disrupt the receptor–ligand interaction, causing premature release of the virion from the endosomal membrane prior to fusion (Cecilia & Gould, 1991; Hurrelbrink & McMinn, 2001; McMinn et al., 1995, 1996).

To determine the effect that the conserved glycosylation site and the flexible hinge region have on the attenuation of ALFV, a panel of mutant MVEV and ALFV infectious clones was constructed. The results show that the lack of neuroinvasiveness associated with ALFV can be attributed to motifs within the E protein, including the absence of glycosylation and unique substitutions in the flexible hinge region.

**RESULTS**

**Generation and characterization of chimeric virus, vMVEV/ALFVstr**

To assess whether determinants in the prM and E genes of ALFV are responsible for virus attenuation, the chimeric infectious clone, pMVEV/ALFVstr, was constructed by inserting the ALFV prM and E genes into an MVEV infectious clone (Fig. 1a). Sequencing of the genomic RNA of progeny virus (vMVEV/ALFVstr) and staining of infected cells with virus-specific mAbs to the E and NS1 proteins, which were then detected by immunofluorescence, confirmed the chimeric composition of the virus (Fig. 1b).

The growth characteristics of vMVEV/ALFVstr were assessed in a range of cell lines and were compared with the parental viruses MVEV and ALFV. The chimeric virus, vMVEV/ALFVstr, showed growth kinetics similar to MVEV and ALFV in cell lines that are used to propagate flaviviruses, including porcine stable equine kidney cells (PSEK; Fig. 1c), mouse neuronal cells (2.3D17; Fig. 1d), human adrenal carcinoma cells (SW-13) and baby hamster kidney (BHK) cells (data not shown). In the C6/36 mosquito cell line, vMVEV/ALFVstr growth appeared delayed, producing lower titres than ALFV and MVEV during the first 2 days of infection (Fig. 1e), although peak titres at 72 h post-infection (h p.i.) reached levels that differed by less than twofold from those for parental MVEV and ALFV. In summary, vMVEV/ALFVstr growth kinetics are similar to parental MVEV in mammalian cells, indicating that insertion of the ALFV prM–E genes into the MVEV genome had a negligible effect on virus replication. Although replication of vMVEV/ALFVstr in mosquito cells was lower during early infection, peak levels were comparable to the parental ones by 72 h p.i.

**The inability of ALFV to invade the central nervous system (CNS) can be mapped to prM–E**

Previously, we have shown that ALFV fails to invade the CNS of weanling mice and produce neurological disease after peripheral inoculation (May et al., 2006). To investigate the association between ALFV prM–E and this lack of neuroinvasion, 3-week-old Swiss mice were inoculated with vMVEV/ALFVstr via the intraperitoneal (i.p.) route. The majority of mice inoculated with a high dose of $10^7$ infectious units (IU) of vMVEV/ALFVstr did not develop neurological symptoms during the first 14 days of...
the observation period (Fig. 2), similar to our previous observations with ALFV (May et al., 2006). In comparison, all mice infected with vMVEV at the same dose succumbed within 5 days and as few as 1 IU of MVEV produced neurological symptoms in 50% of mice and 10^3 IU resulted in 100% mortality (LD50 = 0.2; Table 1). These data suggest an association between the ALFV prM–E genes and the lack of neuroinvasion and virulence attenuation.

**Generating mutants with altered E protein glycosylation and/or hinge motifs**

Sequence analyses of the MVEV and ALFV prM–E genes reveal two distinctly divergent motifs, both of which have been linked with neuroinvasiveness in encephalitogenic flaviviruses: a conserved glycosylation site in the MVEV E protein that is absent in ALFV (position 154 in domain I), and a unique amino acid sequence between residues 273
and 277 in the flexible hinge region of the ALFV E protein (Hurrelbrink & McMinn, 2001; May et al., 2006; McMinn et al., 1995). To evaluate the role of these motifs as determinants of neuroinvasiveness, a panel of mutant viruses was constructed (Table 2). All mutant constructs produced infectious virus except for the MVEV mutant containing both the glycosylation and hinge motifs of ALFV, PMVEV-CHO−H\textsuperscript{ALFV}, which was non-viable when electroporated in BHK cells.

**Glycosylation status and fusion activity of mutant viruses in vitro**

To ensure glycosylation-site mutations created or abolished a functional glycosylation motif, the E protein glycosylation status of each virus was evaluated by N-glycosidase F (PNGaseF) digestion and Western blot analysis. As expected, mutants vMVEV/ALFVstr-CHO\textsuperscript{+} and vMVEV/ALFVstr-H\textsuperscript{MVEV} contained a carbohydrate moiety on the E protein, demonstrated by the E protein band migrating more rapidly following PNGaseF digestion (Table 3, Supplementary Fig. S1, available in JGV Online). In comparison, vMVEV-CHO\textsuperscript{−} virus, lacking a glycosylation motif, displayed no difference in E protein migration after PNGaseF digestion (Table 3, Supplementary Fig. S1).

The effect of mutations in the E protein hinge region upon membrane fusion was assessed by haemagglutination (HA) assay. In flaviviruses, HA is a pH-dependent interaction between viral and erythrocyte membranes that serves as a measure of the ability of a virus to fuse efficiently with host cell membranes during pH-dependent fusion events (Hurrelbrink & McMinn, 2001; Porterfield & Rowe, 1960). While the optimal pH for gander red cell agglutination was approximately pH 6.6 for all parental and mutant viruses tested, a markedly narrower pH range of pH 6.6–6.8 was observed for the chimeric virus and its derivatives compared with the parental strains (pH 5.8–6.8; Table 3). This appears to be independent of the amino acid sequences in the hinge region that were exchanged during this study, and may simply be a by-product of chimerization.

To further characterize mutant viruses *in vitro*, the plaque morphology of parental and mutant viruses was assessed. vMVEV and associated mutants, vMVEV-CHO\textsuperscript{−} and vMVEV-H\textsuperscript{ALFV}, produced large plaques of 1.8–3 mm, compared with smaller plaques seen for ALFV, vMVEV/ALFVstr and associated mutants, vMVEV/ALFstr-CHO\textsuperscript{+}, vMVEV/ALFstr-H\textsuperscript{MVEV} and vMVEV/ALFstr-CHO\textsuperscript{+}H\textsuperscript{MVEV}, which were all of ~1 mm (Table 3).

**Growth kinetics of mutant viruses in PSEK cells**

The growth kinetics of mutant viruses were assessed in PSEK cells and compared with wild-type (vMVEV and ALFV) and chimeric (vMVEV/ALFVstr) viruses (Fig. 3). All mutant viruses replicated with similar efficiency (less than twofold difference) to their parental virus, while ALFV, vMVEV/ALFVstr and its associated mutants (vMVEV/ALFstr-CHO\textsuperscript{+}, vMVEV/ALFstr-H\textsuperscript{MVEV} and vMVEV/ALFstr-CHO\textsuperscript{+}H\textsuperscript{MVEV}) demonstrated a delay in replication of approximately 12 h compared with vMVEV (Fig. 3). These data show that substitution of the glycosylation and hinge motifs in MVEV E protein with corresponding sequences from ALFV does not significantly affect virus growth in PSEK cells, but that substitution of the entire prM–E region causes a 12 h delay in virus propagation.

**Effect of E protein glycosylation and hinge motifs on neuroinvasion in mice**

To assess the influence of glycosylation status and hinge region motifs on neuroinvasion, 3-week-old Swiss mice were inoculated via the i.p. route with parental and mutant viruses at a range of doses. Insertion of the ALFV-like CHO\textsuperscript{−} motif into the E protein of MVEV significantly increased survival rates at doses of 10\textsuperscript{3} and 10\textsuperscript{4} IU (*P*<0.05) compared with the parental vMVEV. This mutant also resulted in an eightfold increase in LD\textsubscript{50} (Table 1). Surprisingly, inoculation with this mutant at the higher doses of 10\textsuperscript{3} and 10\textsuperscript{4} IU resulted in less mortality compared with the lower doses of 10\textsuperscript{2} and 10 IU. This ‘prozone’ phenomenon has been seen previously with other flaviviruses (Monath et al., 1980). The ALFV-like QMDS hinge sequence inserted into MVEV also significantly improved survival, as measured by increased time to death, but only at a single dose of 10\textsuperscript{3} IU (*P*<0.05; Table 1). Interestingly, neither mutant vMVEV-CHO\textsuperscript{−} nor vMVEV-H\textsuperscript{ALFV} was attenuated to the same level as ALFV or vMVEV/ALFVstr. Conversely, only mutants that contained the MVEV-like FSSST hinge sequence in the E protein of vMVEV/ALFVstr, vMVEV/ALFstr-H\textsuperscript{MVEV} and vMVEV/ALFstr-CHO\textsuperscript{+}H\textsuperscript{MVEV} resulted in significantly higher mortality rates than those observed for the parental virus (*P*<0.05). Together these results confirm that the absence of
glycosylation and/or substitutions in the hinge region of the E protein contribute to loss of neuroinvasion and attenuation of ALFV.

**ALFV, chimeric and mutant viruses produce lower levels of infectious virus in peripheral and CNS tissues of infected mice than MVEV**

To determine whether lack of neuroinvasion was caused by a lack of peripheral replication, levels of infectious virus were measured in tissue homogenates from peripheral and CNS tissues of mice infected with wild-type, chimeric and mutant viruses by titration on PSEK cells. Mice were infected with 10^3 IU for MVEV and associated mutants, and 10^4 IU for the chimera and associated mutant viruses. Infectious virus was detected by ELISA in the sera of vMVEV-infected mice on days 1, 3 and 5, with peak levels of 10^3 IU ml\(^{-1}\) on day 3 p.i. (Fig. 4a). In comparison, vMVEV/ALFVstr, ALFV and all mutants produced no detectable viraemia at all time points tested, even though antibodies were detected in the sera at day 21 in all surviving mice inoculated with each dose (data not shown). Infectious virus was detected in heart, spleen, kidney and liver of vMVEV-infected mice, with peak titres detected on day 5 p.i (data not shown). With the exception of vMVEV-H\(^{ALFV}\), which produced trace levels of virus in the spleen homogenates, all other viruses produced no detectable infectious virus in peripheral tissues. In CNS tissues, mice infected with wild-type vMVEV virus produced levels between 10^5 and 10^6 IU ml\(^{-1}\) on days 5 and 7 p.i (Fig. 4b, c). In comparison, the MVEV mutants containing ALFV motifs in the E protein produced significantly less virus at the same time p.i., between 10^2 and 10^3 IU ml\(^{-1}\). Of the

<table>
<thead>
<tr>
<th>Virus</th>
<th>Dose (IU)*</th>
<th>Mortality (%)</th>
<th>Mean survival time† (days)</th>
<th>LD(_{50}) (IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMVEV</td>
<td>10 000</td>
<td>10/10 (100%)</td>
<td>5.9</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>10/10 (100%)</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9/10 (90%)</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8/10 (80%)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>8/10 (80%)</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>3/10 (30%)</td>
<td>12.1</td>
<td></td>
</tr>
<tr>
<td>vMVEV-CHO(^-)</td>
<td>10 000</td>
<td>7/10 (70%)</td>
<td>10†</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>6/10 (60%)</td>
<td>10.1†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8/10 (80%)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8/10 (80%)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>4/10 (40%)</td>
<td>12.5†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0/10 (0%)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>vMVEV-H(^{ALFV})</td>
<td>10 000</td>
<td>10/10 (100%)</td>
<td>6.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>9/10 (90%)</td>
<td>10†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>8/10 (80%)</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>8/10 (80%)</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>7/10 (70%)</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>4/10 (40%)</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>vMVEV/ALFVstr</td>
<td>100 000</td>
<td>1/10 (10%)</td>
<td>7</td>
<td>&gt;100 000</td>
</tr>
<tr>
<td></td>
<td>10 000</td>
<td>1/9 (11%)</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0/5 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0/5 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0/5 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>vMVEV/ALFVstr-CHO(^+)</td>
<td>100 000</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>vMVEV/ALFVstr-H(^{MVEV})</td>
<td>100 000</td>
<td>2/10 (20%)</td>
<td>18.7</td>
<td>&gt;100 000</td>
</tr>
<tr>
<td></td>
<td>10 000</td>
<td>5/10 (50%)</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>4/10 (40%)</td>
<td>17.2</td>
<td>100 000</td>
</tr>
<tr>
<td>vMVEV/ALFVstr-CHO(^+) H(^{MVEV})</td>
<td>100 000</td>
<td>6/10 (60%)</td>
<td>8.5†</td>
<td></td>
</tr>
<tr>
<td>ALFV</td>
<td>100 000</td>
<td>1/10 (10%)</td>
<td>20</td>
<td>63 106</td>
</tr>
<tr>
<td></td>
<td>10 000</td>
<td>1/10 (10%)</td>
<td>20</td>
<td>&gt;100 000</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0/10 (0%)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

*IU, Infectious units.
†Significant differences between mutant virus and matching parental virus using a log rank (Mantel–Cox) test. Only statistically significant \(P\) values are provided.

**Table 1. Virulence of wild-type, chimeric and mutant viruses in 3-week-old Swiss outbred mice after i.p. inoculation**

N/A, Not applicable, no animals succumbed to disease.
remaining viruses, only vMVEV/ALFVstr-HMVEV and vMVEV/ALFVstr-CHO +HMVEV produced detectable virus levels in the brain or spinal cord (data not shown).

Virus was not detected in serum or liver samples from animals infected with ALFV, vMVEV/ALFVstr or the associated mutants using virus titration on PSEK cells with a limit of detection of 10 infectious units. Therefore, real-time PCR was used to determine whether trace amounts of viral RNA were present in tissues where infectious virus could not be detected. These results confirmed an absence of virus in these tissues (data not shown).

In summary, these data suggest that the efficient neuroinvasion and induction of neurological symptoms in mice infected with wild-type vMVEV are associated with moderate levels of virus in serum and peripheral organs prior to entry into the brain, while infection with the attenuated viruses (ALFV, vMVEV/ALFVstr and vMVEV/ALFVstr-CHO +) or partially attenuated viruses (vMVEV/ALFVstr-HMVEV, vMVEV/ALFVstr-CHO +HMVEV and vMVEV-CHO −) resulted in undetectable or only trace levels of virus in these tissues. Furthermore, only viruses that showed significant levels of neurological disease in mice following i.p. inoculation could be detected in the CNS after infection.

**DISCUSSION**

Despite their being genetically closely related, ALFV displays a number of important phenotypic differences when compared with the virulent MVEV (May et al., 2006). Most importantly, ALFV is unable to invade the CNS of 3-week-old Swiss mice after peripheral inoculation, while MVEV displays a highly neuroinvasive phenotype in this mouse model (Lee & Lobigs, 2002; Lobigs et al., 1988, 1990; May et al., 2006). This study aimed to determine the genetic basis for the poor neuroinvasiveness of ALFV by using an engineered chimeric virus containing the structural genes of ALFV in the MVEV genome. The chimeric virus was designed to incorporate putative attenuation determinants within the structural genes of ALFV into a virulent MVEV backbone. We have previously shown that the E gene of ALFV contains substitutions in

---

**Table 2. Summary of MVEV, MVEV/ALFVstr and mutant constructs in the E protein**

<table>
<thead>
<tr>
<th>Virus/plasmid name</th>
<th>Glycosylation region*</th>
<th>Hinge region†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALFV</td>
<td>DYS</td>
<td>QMDS</td>
</tr>
<tr>
<td>pMVEV</td>
<td>NYS</td>
<td>EFSSS</td>
</tr>
<tr>
<td>pMVEV/ALFVstr</td>
<td>DYS</td>
<td>QMDS</td>
</tr>
<tr>
<td>pMVEV/ALFVstr-CHO +</td>
<td>NYS</td>
<td>EFSSS</td>
</tr>
<tr>
<td>pMVEV/ALFVstr-HMVEV</td>
<td>DYS</td>
<td>QMDS</td>
</tr>
<tr>
<td>pMVEV/ALFVstr-CHO +HMVEV</td>
<td>NYS</td>
<td>EFSSS</td>
</tr>
<tr>
<td>pMVEV-CHO −</td>
<td>DYS</td>
<td>QMDS</td>
</tr>
<tr>
<td>pMVEV-HALFV</td>
<td>NYS</td>
<td>EFSSS</td>
</tr>
<tr>
<td>pMVEV-CHO −HMVEV</td>
<td>DYS</td>
<td>QMDS</td>
</tr>
</tbody>
</table>

*Glycosylation region represents the N-linked glycosylation site at residues 154–156 of the E protein.
†Hinge region represents residues 273–277 of the E protein, based on the MVEV sequence. Residue 277 is deleted in ALFV.
‡Did not produce infectious virus.

---

**Table 3. Phenotype of wild-type, chimeric and mutant viruses in vitro**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean plaque size in PSEK cells (mm)</th>
<th>Optimal haemagglutination pH (pH range)</th>
<th>Glycosylation status*</th>
</tr>
</thead>
<tbody>
<tr>
<td>vMVEV</td>
<td>2.75</td>
<td>6.6 (5.8–6.8)</td>
<td>+</td>
</tr>
<tr>
<td>vMVEV-CHO −</td>
<td>3</td>
<td>6.6 (5.8–6.8)</td>
<td>–</td>
</tr>
<tr>
<td>vMVEV-HALFV</td>
<td>1.8</td>
<td>6.6 (5.8–6.8)</td>
<td>+</td>
</tr>
<tr>
<td>vMVEV/ALFVstr</td>
<td>1</td>
<td>6.6 (6.6–6.8)</td>
<td>–</td>
</tr>
<tr>
<td>vMVEV/ALFVstr-CHO +</td>
<td>1</td>
<td>6.6 (6.6–6.8)</td>
<td>+</td>
</tr>
<tr>
<td>vMVEV/ALFVstr-HMVEV</td>
<td>1</td>
<td>6.6 (6.6–6.8)</td>
<td>–</td>
</tr>
<tr>
<td>vMVEV/ALFVstr-CHO +HMVEV</td>
<td>1</td>
<td>6.6 (6.6–6.8)</td>
<td>+</td>
</tr>
<tr>
<td>ALFV</td>
<td>1</td>
<td>6.6 (6.6–6.8)</td>
<td>–</td>
</tr>
</tbody>
</table>

* Determined by PNGaseF digestion (Supplementary Fig. S1.)
two regions thought to be important in the virulence of flaviviruses: (i) the conserved glycosylation site, and (ii) a string of unique amino acids in the flexible hinge region, which is relatively conserved among other members of the Japanese encephalitis complex of viruses (May et al., 2006). To define the role of these putative attenuation determinants further, a series of mutant viruses were constructed to incorporate the glycosylation and hinge region motifs of MVEV and ALFV into infectious clones.

The conserved glycosylation site in the E protein of the JEV serogroup is thought to protect the fusion peptide from exposure to low pH (Rey et al., 1995) and is important for secretion of the virus (Lorenz et al., 2003), but its presence reduces the infectivity of MVEV, WNV and dengue virus in mosquito cells by approximately tenfold (Lee et al., 2010). Accordingly, the opposing influences of infectivity and release on virus growth, modulated by the glycan, may explain the plasticity of E protein glycosylation among the flaviviruses. Several studies involving West Nile and dengue virus strains have shown that changes at this site can cause attenuation in mice (Beasley et al., 2004b, 2005; Chambers et al., 1998; Haley et al., 1994; Kawano et al., 1993; Shirato et al., 2004). We have shown that viruses lacking an N-linked glycosylation motif at positions 154–156 of the E protein, specifically ALFV and the chimeric virus vMVEV/ALFVstr, are non-neuroinvasive in mice. Furthermore, when the glycosylation motif was abolished in MVEV (vMVEV-CHO−), a decrease in neuroinvasiveness in mice was observed, represented by improved survival and reduced viral load in CNS tissues compared with the parental vMVEV. However, the ALFV E protein was glycosylated in vMVEV/ALFVstr-CHO+, this mutant did not display increased virulence. These results would suggest that viruses naturally glycosylated in E (e.g. ALFV), no improvement in virulence was observed. Indeed, the unglycosylated virus may have an altered structure to compensate for the absence of the glycan. Thus, introduction of this site did not enhance viral growth or virulence.

The hinge region of the E protein in ALFV has a number of amino acid substitutions in comparison with the corresponding region of MVEV and other JEV serogroup viruses. This region of the protein undergoes a low-pH-induced conformational change during fusion of the virus envelope with the endosomal membrane of the host cell (Zhang et al., 2004). Furthermore, changes in the size and hydrophobicity of the amino acid side chains in this region affected flexibility and impaired the fusion of E in an infectious clone of MVEV (McMinn et al., 1996), resulting in decreased neuroinvasiveness and reduced growth in cell culture (Cecilia & Gould, 1991; Hurrelbrink & McMinn, 2001; McMinn et al., 1995). We have shown that substitution of the hinge region of ALFV into the MVEV backbone (to produce vMVEV-HALFV) resulted in a decrease in neuroinvasion in mice, observed as extended time to death and reduced viral load in peripheral and CNS tissues compared with the parental vMVEV. In the reverse situation in which the MVEV hinge region was substituted into the ALFV backbone (to produce vMVEV/ALFVstr-HMVEV), an increase in virulence was observed, as demonstrated by a decrease in survival. However, this substitution did not increase peripheral replication to detectable levels.

Both the glycosylation and hinge motifs were required for maximum virulence of MVEV, with the latter having a more pronounced effect on enhancing the virulence of the chimeric virus (Table 1). However, when MVEV-like motifs were inserted into the structural genes of vMVEV/ALFVstr, this did not enhance neuroinvasion to levels seen for wild-type vMVEV, suggesting that additional

---

Fig. 3. Growth kinetics of vMVEV, ALFV, vMVEV/ALFVstr and associated mutants in PSEK cells. (a) ALFV-like mutants. Cells were infected with an m.o.i. of 0.1 and the titre of the infectious virus in the supernatant was determined by TCID₅₀ on PSEK cells every 12 h until 72 h p.i.
motifs located within the structural genes contribute to neuroinvasion.

We previously showed that ALFV grows poorly in the periphery of mice compared with MVEV after i.p. inoculation, as indicated by a lower rate of seroconversion in infected Swiss outbred mice and a delayed time to death in interferon-α-receptor-deficient mice (May et al., 2006). In contrast, all mice inoculated with the chimeric virus developed virus-specific antibodies at all doses, as detected by ELISA (Table 1), indicative of a more productive peripheral infection. However, our inability to detect infectious virus in the serum or peripheral organs of vMVEV/ALFVstr-infected mice at all times post-infection suggests that insufficient levels of virus circulating in the blood contribute to a lack of neuroinvasion, as seen in other flaviviruses (Beasley et al., 2005; Huang & Wong, 1963; Monath et al. 1980). Interestingly, when ALFV-like motifs were substituted into the MVEV backbone, a significant reduction in detectable virus in both the periphery and CNS tissues was observed (Fig. 4), compared with wild-type MVEV. Collectively, these data suggest that ALFV-like sequences or motifs are either hampering viral replication or promoting increased viral clearance in the mouse model.

Previously Lee & Lobigs (2002) have shown that substitution at residue 390 (aspartic acid to glycine) in the conserved RGD motif in the E protein of MVEV promotes high-affinity binding to glycosaminoglycans (GAGs) on cellular surfaces, resulting in the rapid removal of infectious virus from the blood by GAG-rich tissues and loss of neuroinvasiveness in mice. Although the RGD sequence is also conserved in ALFV, the presence of positively charged residues on the surface of domain III, in close proximity to the RGD motif (e.g. lysine at residue 328), might similarly explain the absence of virus in the blood of mice infected with ALFV and the chimeric virus, as has been shown for JEV (Lee & Lobigs, 2002). This is currently being investigated.

In summary, our results demonstrate that glycosylation and hinge motifs in the E protein significantly affect the neuroinvasive phenotype of MVEV and ALFV, but are not the sole determinants of virulence or attenuation in these viruses, respectively. Further elucidation of the differing events of infection displayed between these closely related viruses at the molecular and cellular level will provide a better understanding of the mechanisms of pathogenesis of encephalitic flaviviruses.

**METHODS**

Cell culture and virus production. PSEK (Gorman et al., 1975), 2.3D17 (Bartlett et al., 1988), SW-13 (ATCC CCL-105) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco). Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.

ALFV strain MRM3929 and MVEV strain 1-51 were obtained from laboratory stocks. Virus stocks were produced by infecting a subconfluent monolayer of PSEK cells with virus at an m.o.i. of 0.1 in DMEM containing 2 % FBS (2 %FBS/DMEM) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco).

Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.

ALFV strain MRM3929 and MVEV strain 1-51 were obtained from laboratory stocks. Virus stocks were produced by infecting a subconfluent monolayer of PSEK cells with virus at an m.o.i. of 0.1 in DMEM containing 2 % FBS (2 %FBS/DMEM) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco). Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.

ALFV strain MRM3929 and MVEV strain 1-51 were obtained from laboratory stocks. Virus stocks were produced by infecting a subconfluent monolayer of PSEK cells with virus at an m.o.i. of 0.1 in DMEM containing 2 % FBS (2 %FBS/DMEM) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco). Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.

ALFV strain MRM3929 and MVEV strain 1-51 were obtained from laboratory stocks. Virus stocks were produced by infecting a subconfluent monolayer of PSEK cells with virus at an m.o.i. of 0.1 in DMEM containing 2 % FBS (2 %FBS/DMEM) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco). Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.

ALFV strain MRM3929 and MVEV strain 1-51 were obtained from laboratory stocks. Virus stocks were produced by infecting a subconfluent monolayer of PSEK cells with virus at an m.o.i. of 0.1 in DMEM containing 2 % FBS (2 %FBS/DMEM) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco). Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.

ALFV strain MRM3929 and MVEV strain 1-51 were obtained from laboratory stocks. Virus stocks were produced by infecting a subconfluent monolayer of PSEK cells with virus at an m.o.i. of 0.1 in DMEM containing 2 % FBS (2 %FBS/DMEM) and BHK cells (ATCC CCL-10) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen-Gibco) supplemented with 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ (Invitrogen-Gibco). Cells were cultured at 37 °C in a 5 % CO₂ atmosphere. Aedes albopictus salivary gland cells (C6/36) (ATCC CRL-1660) were cultured in RPMI 1640 medium (Invitrogen-Gibco) containing 10 % FBS, 50 U penicillin ml⁻¹ and 50 μg streptomycin ml⁻¹ at 28 °C.
Viral growth kinetics. 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 an m.o.i. of 0.1, diluted in 2%FBS/DMEM. After 1 h at 37 °C, virus was removed and the cells were washed before the addition of 1 ml 2%FBS/DMEM. Samples were removed every 12 or 24 h p.i. and stored at −70 °C. The virus titre in each sample was determined by measuring the TCID₅₀ on PSEK cells by the method of Reed and Muench (1938).

Plaque assays. PSEK cells in six-well plates were infected with a series of tenfold dilutions as described for the TCID₅₀ assays above. Cells were overlaid with 0.75% low-melting-point agarose in 2%FBS/DMEM and incubated at 37 °C. After 4 days, monolayers were fixed with 10% formaldehyde and stained with 0.2% crystal violet.

Construction of pMVEV/ALFVstr. The infectious clone, pMVEV/ALFVstr (Fig. 1) was constructed by exchanging the prM and E genes of the pMVEV₁₋₅₁ plasmid reported elsewhere (Hurrelbrink et al., 1999) with the prM and E genes of ALFV, obtained by PCR from viral RNA. To facilitate construction, a NotI site was engineered at the C–prM junction of pMVEV₁₋₅₁ and a SpIi site was engineered at the E–NS1 junction. A NotI site was introduced at the C–prM junction and the natural SpIi site was utilized at the E–NS1 junction of ALFV. These two products were digested with NotI and SpIi and ligated together. The XbaI site used to linearize the pMVEV₁₋₅₁ clone was changed to a BsaI6I site because of the presence of an Xbal site in the ALFV E gene.

Construction of mutant plasmids. Mutations in the glycosylation and hinge regions were introduced by overlap extension PCR mutagenesis using Phusion polymerase (Finnzymes) as described by Hall et al. (1999). The nucleotide sequences of primers used for mutagenesis are shown in Supplementary Table S1 (available in JGV Online). First, mutations were introduced into two overlapping fragments using Dynazyme (Finnzymes). To introduce the MVEV-like motif in pMVEV/ALFVstr, glycosylation-mutant primers were designed to replace Asp at residue 154 in the E gene with the Asn like motif in pMVEV/ALFVstr, glycosylation-mutant primers were designed to replace Asp at residue 154 in the E gene with the Asn silent mutation for identification purposes. The construct changed the glycosylation motif from unglycosylated (DYS) to glycosylated (NYS) and therefore was designated pMVEV/ALFstr-CHO²⁻. In pMVEV₁₋₅₁, the reverse mutation was introduced via a silent mutation with an XmnI site introduced for identification purposes. This construct changed the glycosylation motif of E from glycosylated (NYS) to unglycosylated (DYS) and therefore was designated pMVEV-CHO⁻. Hinge-mutant primers were designed to introduce the MVEV-like motif into the pMVEV/ALFVstr E gene, resulting in the introduction of the Glu-Phc-Ser-Ser-Ser (EPSSS) motif from codons 273–277 and an Xhol site, via a silent mutation, for identification purposes. This construct changed the hinge region from an ALFV-like motif to an MVEV-like motif and therefore was designated pMVEV/ALFVstr-Hattern. The ALFV-like hinge motif was introduced in pMVEV₁₋₅₁, resulting in the introduction of the Gln-Met-Asp-Ser (QMDS) motif from codons 273–276, with an AatII site, introduced via a silent mutation, for identification purposes. This construct changed the hinge region from an MVEV-like motif to an ALFV-like motif and therefore was designated pMVEV-Halff. Plasmids containing the desired mutations were confirmed by sequence analysis (Australian Genome Research Facility, The University of Queensland, Australia) and are summarized in Table 2.

Viral RNA transcription and electroporation. RNA transcripts were prepared from BsaI6I-linearized plasmids (pMVEV/ALFVstr and associated mutants) or XbaI6I-linearized plasmids (pMVEV₁₋₅₁ and associated mutants) by using T7 RNA polymerase as previously described (Khromykh et al., 1998). Electroporated BHK cells were incubated in 10%FBS/DMEM and supernatant was harvested once CPE was evident. Viruses derived from plasmids were designated by naming with a lower case ‘v’.

Evaluation of glycosylation status. Glycosylation of the E protein was examined by digestion with endoglycosidase F. Viral proteins in the culture supernatant were heat denatured in the presence of 1% SDS (w/v) at 98 °C for 3 min. After addition of EDTA (to 10 mM) and N-octyl-β-D-glucopyranoside (to 1%), the mixture was digested overnight at 37 °C with 1 U PNaseF (Roche). Proteins were separated and analysed by Western blot. Samples were loaded with non-reducing SDS-PAGE LDS sample buffer (Invitrogen) on a 12% NuPage gel (Invitrogen). Electrophoresed proteins were electroblotted onto nitrocellulose paper (Hybond C; Amersham) and immunostained with mAb4G2 (anti-E) at a dilution of 1:5, as previously described (Adams et al., 1995).

HA assay. Virus from infected PSEK supernatants was used as a source of haemagglutinin. HA assays were performed by using a modified protocol of Clarke & Casals (1958), as previously described (McMinn et al., 1995). Titres were recorded as the reciprocal of the highest dilution which yielded complete agglutination of gander red blood cells. The optimal haemagglutination pH was recorded as being the pH yielding peak titres, and the pH range for haemagglutination was determined as being the pH values across which haemagglutination occurred.

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. Three-week old Swiss outbred mice (Animal Resources Centre, Murdoch, Western Australia, Australia) were infected via the i.p. route with vMVEV/ALFVstr, vMVEV, ALFV or associated mutant viruses at a range of doses determined by TCID₅₀ and expressed in terms of IU. 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 heart puncture at the end of the experiment (day 21) and the sera were tested for evidence of seroconversion to MVEV and ALFV by using a fixed cell ELISA as previously described (Clark et al., 2007). The significance of clinical differences between groups was calculated by Kaplan–Meier analysis where noted (GraphPad Prism version 5.0; GraphPad).

Titrations of virus in tissue samples. To measure the amount of infectious virus in the brain, spinal cord, liver, kidney, heart and spleen, tissues were extracted, weighed, snap frozen on dry ice and stored at −80 °C until viral titration assays were performed. At the time of these titrations, 10% (w/v) homogenates of each sample were prepared in 2%FBS/DMEM and serial tenfold dilutions of each homogenate were assayed by TCID₅₀ on monolayers of PSEK cells. The results presented are the mean ± SEM of the log₁₀ IU per gram of tissue derived from three animals at each time point. The amount of infectious virus in serum was also determined. In this case, blood samples were collected via cardiac heart puncture and stored on ice until the blood had separated and serum could be collected and stored at −80 °C. Infectious virus in serum samples was assayed on PSEK cells as described above.

RNA extraction from tissues. Livers were homogenized (10% w/v in 2% FBS/DMEM), clarified and stored at −80 °C. A 200 µl volume of homogenized sample was added to 600 µl of RLT buffer (Qiagen) containing 1% β-mercaptoethanol and vortexed vigorously. After 5 min at room temperature, the mixtures were transferred to Qiashredder columns (Qiagen) and centrifuged for 2 min at
13 000 r.p.m. A 200 µl volume of each of the filtrates containing target RNA was then extracted using an EZ1 virus mini kit v2.0 (Qiagen) according to the manufacturer’s protocol. After lysis, binding buffer containing magnetic particles was mixed with the lysate, allowing the nucleic acids allowed to bind to magnets. Contaminants that did not bind to magnetic particles were then washed away with wash buffers 1 and 2, followed by an ethanol wash. RNA was then eluted in AVE buffer. RNA was also extracted from serum samples as described above.

Real-time PCR. Primer and probe oligonucleotide sequences derived from the non-structural protein NS5 of ALFV strain MRM3929 (GenBank accession number AY898809) were designed and prepared for real-time TaqMan RT-PCR as previously described by Pyke et al. (2004). Sequences and relative nucleotide positions for the primers and dual-labelled probe were as follows: forward primer (ALFlor) 5′-GGATGGAGGTT-CCAGACA-3′, reverse primer (ALFrev) 5′-AAGTAGAGGAG-AGCCACATCTGG-3′ and probe (ALFProbe) 5′-FAM-AGCTT-GCTTGGCAAAGGCCTATGC-TAMRA-3′.

The Taqman RT-PCR was performed by using an ABI 7500 RT-PCR system (PE Applied Biosystems). Detection of ALFV RNA and amplification of the 68 bp product were carried out using a single-tube, one-step RT-PCR format in a final reaction volume of 25 µl. The reaction mixture and cycling conditions were as described by Pyke et al. (2004). The threshold cycle number (Ct) was determined for each sample and a negative result, indicating no RNA detection, corresponded to any value of Ct ≥ 40 cycles (Pyke et al., 2004).

ACKNOWLEDGEMENTS

The authors would like to thanks Kim Pham, Kelly Reddan, Jannelle Bentz, Sonia Hall-Mendelin, Russell Simmons, Doris Genie, Judy Northill and Natasha Kondratieva for technical assistance during this project. The cell line 2.3D17 was initially obtained from the Walter and Eliza Hall Institute, Melbourne, Australia, as a gift from Professor Perry Bartlett. This work was supported by funding from the National Health and Medical Research Council grant 456126.

REFERENCES


