Histidine 39 in the dengue virus type 2 M protein has an important role in virus assembly

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The mature flavivirus particle comprises a nucleocapsid core surrounded by a lipid bilayer containing the membrane (M) (derived from the precursor prM) and envelope (E) proteins. The formation of intracellular prM/E heterodimers occurs rapidly after translation and is believed to be important for the assembly and secretion of immature virus particles. In this study, the role of the His residue at position 39 in the M protein (M39) of dengue virus type 2 (DENV-2) in the virus life cycle was investigated. Mutations encoding basic (Arg), non-polar (Leu and Pro) and uncharged polar (Asn, Gln and Tyr) amino acids at M39 were introduced into a DENV-2 genomic-length cDNA clone and their effects on virus replication were examined. Substitution of the His residue with non-polar amino acids abolished virus replication, whereas substitution with basic or uncharged polar amino acids decreased virus replication moderately (≈2 log10 p.f.u. ml−1 decrease in viral titre for Arg and Asn) or severely (≥3.5 log10 p.f.u. ml−1 decrease in viral titre for Gln and Tyr). Selected mutations were introduced into a prM–E gene cassette and expressed transiently in COS cells to investigate whether the mutations impaired prM/E association or secretion. None of the mutations was found to disrupt the formation of intracellular prM/E heterodimers. However, the mutations that abolished virus replication prevented secretion of prM/E complexes. The results of this study pinpoint a critical residue in the M protein that potentially plays a role in viral morphogenesis, secretion and entry.

INTRODUCTION

Dengue virus (DENV) is transmitted by mosquitoes and causes the most important arthropod-borne viral disease of humans, with 50–100 million individuals infected annually worldwide (Gubler, 2002). The four serotypes of DENV (1–4) are members of the genus Flavivirus within the family Flaviviridae, along with a number of other medically important viruses that include Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and yellow fever virus (YFV) (Burke & Monath, 2001).

The mature flavivirus particle contains three structural proteins, the capsid (C), envelope (E) and membrane (M) (derived from the precursor prM) proteins, plus a lipid bilayer and a single strand of infectious RNA. The structure of the mature DENV-2 particle has been determined by fitting the X-ray crystallographic structure of the TBEV E protein (Rey et al., 1995) into a 24 Å resolution cryo-electron microscopic (cryo-EM) reconstruction of the DENV-2 particle (Kuhn et al., 2002). The structure revealed that 90 E protein dimers lying parallel to the membrane in a 'herringbone' conformation and 180 M proteins form a tightly packed outer icosahedral protein shell surrounding a lipid bilayer, which encloses a disordered nucleocapsid consisting of multiple copies of the C protein surrounding the RNA genome (Kuhn et al., 2002).

The flavivirus RNA genome is approximately 11 kb in size and encodes a large polyprotein with the gene order NH2-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. Viral and host proteases process the polyprotein to produce C, prM/M and E and at least seven non-structural proteins (Lindenbach & Rice, 2001). Translocation of prM, E and NS1 into the endoplasmic reticulum (ER) lumen occurs cotranslationally and is initiated by a signal sequence at the C terminus of the C protein. The prM and E proteins each contain two α-helical transmembrane sequences at their C termini, which serve as signal and stop anchor sequences. The production of the N terminus of the prM protein is a coordinated process that is regulated by two cleavages, one at a cytosolic site in the C-terminal region of the C protein.
by the virally encoded NS2B/3 protease and the other after the prM signal sequence by host signalase in the ER lumen (Amberg & Rice, 1999; Stocks & Lobigs, 1995). Host signalase cleavage at the prM–E and E–NS1 junctions gives rise to the N and C termini of the E protein. The prM and E proteins associate as a heterodimeric complex soon after translation (Allison et al., 1995b; Courageot et al., 2000; Lorenz et al., 2002; Wengler & Wengler, 1989). The prM protein folds rapidly and then acts as a chaperone to aid the folding and maturation of the E protein (Courageot et al., 2000; Konishi & Mason, 1993; Lorenz et al., 2002). The prM/E heterodimers then form higher-order structures (Wang et al., 1999), which potentially drive the assembly and budding of immature virus particles at the rough ER membrane (Heinz & Allison, 2003; Mackenzie & Westaway, 2001). The immature particles are transported through the secretory pathway to the cell surface by a process that is dependent on stable prM/E heterodimer formation (Konishi & Mason, 1993; Lorenz et al., 2003). The structure of immature DENV and YFV particles obtained by cryo-EM reconstruction revealed pronounced spikes, each comprising three prM/E heterodimers projecting from the virion surface (Zhang et al., 2003b). The prM proteins associate to cap each spike, covering a fusion peptide located in the E protein, a mechanism that is believed to prevent premature fusion during virion transport through the low-pH environment of the secretory pathway (Guirakhoo et al., 1991, 1992; Heinz et al., 1994). Prior to or during release of the infectious virus from cells, the host enzyme furin cleaves the prM protein (Stadler et al., 1997), a process that is believed to dissociate the prM/E heterodimer, resulting in rearrangement of the E proteins into tightly packed dimers on the surface of the mature virus particle (Heinz & Allison, 2003). The 11–15 kDa pr protein is secreted (Murray et al., 1993), whilst the ~8 kDa M protein remains anchored in the virus membrane.

Subviral particles (termed ‘slowly sedimenting haemagglutinin’ or SHA) containing the prM and E proteins, but devoid of the nucleocapsid, are released from flavivirus-infected cells in addition to infectious virions (Russell et al., 1980). Expression of the prM–E genes has been shown to result in self-assembly of these proteins into recombinant subviral particles (RSPs). The production of RSPs has been demonstrated for DENV (Chang et al., 2003; Fonseca et al., 1994; Konishi & Fujii, 2002; Raviprakash et al., 2000), YFV (Konishi et al., 1991; Mason et al., 1991), TBEV (Allison et al., 1995b; Schalich et al., 1996), WNV (Yamshchikov & Comps, 1993) and YFV (Op De Beeck et al., 2003). RSPs have proved useful tools to study prM/E interaction and assembly (Allison et al., 1995a, 1999), fusion (Allison et al., 2001) and the molecular organization (Ferlenghi et al., 2001) of empty virus-like particles.

Despite recent advances in our understanding of the structure of flaviviruses, the specific residues in the prM and E proteins that are important for the correct assembly, release and rearrangement of virions remain unknown. A comparison of flavivirus M protein sequences identified five amino acid residues that were proposed to contribute to the structural integrity of the M protein (Holbrook et al., 2001). Four of these amino acids are strictly conserved among flaviviruses, whereas the residue at position 39 in the M protein (M39) is His in DENV-1, -2 and -3 strains and Asn in all other flaviviruses. Residue M39 is located in a region that is predicted to link an extracellular α-helix and a transmembrane α-helix. In the current study, several amino acid substitutions at this residue were prepared by site-directed mutagenesis and introduced into a DENV-2 genomic-length cDNA clone to examine their effect on virus replication. A selection of these mutations was then introduced into a prM–E transient gene-expression system in an attempt to identify the role of residue M39 in the virus life cycle.

**METHODS**

**Cell lines and virus titration.** Growth of BHK-21, *Aedes albopictus* C6/36 and COS cells has been described previously (Gualano et al., 1998; Pryor et al., 1998). Stocks of DENV-2 viruses were grown and titrated by plaque assay in C6/36 cells at 28°C as described previously (Gualano et al., 1998). Viral titres were calculated as p.f.u. (ml initial virus inoculum)^-1^, incorporating 1 sd.

**Introduction of mutations into a genomic-length DENV-2 cDNA clone.** A selection of mutations encoding substitutions at M39 were introduced into the genomic-length DENV-2 strain New Guinea C (NGC) cDNA clone pDVWS501, which yields the recombinant virus MON501 (Gualano et al., 1998). Nucleotide changes that altered the His residue present at M39 were introduced into the M gene by overlap extension PCR (OE-PCR) (Ho et al., 1989). OE-PCR fragments of 1851 bp derived from pDVWS501 and encoding the amino acids Arg (CGT), Asn (AAT), Gln (CAA), Leu (CTT), Pro (CCT) or Tyr (TAC) at M39 were digested with *Bsr*G1172 and *Sph*l1384 [the numbering of DENV-2 strain NGC nucleotides and amino acids follows that described by Irie et al. (1989)] and ligated into *Bsr*G1172 and *Sph*l1384-digested pDVWS501 to produce pDVWS501M85R, pDVWS501M85L, pDVWS501M85D, pDVWS501M85E, pDVWS501M85V and pDVWS501M85Y, respectively. The PCR-derived regions of all clones were sequenced.

**Construction of pSVprM–E mutants.** pSVprM–E contained cDNA derived from pDVWS501, encoding prM and E, in the vector pSV.SPORT1 (Invitrogen). pSVprM–E was designed based on a plasmid that was used for transient expression of the TBEV prM and E genes (Allison et al., 1994). A 2083 bp DENV-2 cDNA fragment containing the last 72 nt of C, followed by the entire prM–E region, was produced by PCR using primers 7644 (5′-atgccgccgccacctATGgTGAACATCTTGAACAGGAGACG-3′), 3290 (5′-9′), changes to the pDVWS501 sequence are indicated in lower case) and 3290 (5′-agttcgtgcatccACCCCTGAGAAGCC-3′; complementary to DENV-2 nt 2407–2421). The fragment was blunt-end-ligated into the *Msal* site of pSV.SPORT1 to produce pSVprM–E. To prepare constructs with amino acid substitutions at M39, primers 7644 and 3290 were used in PCRs with the M gene by overlap extension PCR (OE-PCR) (Ho et al., 1989). OE-PCR fragments of 2083 bp from pDVWS501 and encoding the amino acids Arg (CGT), Asn (AAT), Gln (CAA), Leu (CTT), Pro (CCT) or Tyr (TAC) at M39 were digested with *Bsr*G1172 and *Sph*l1384 and ligated into *Bsr*G1172 and *Sph*l1384-digested pDVWS501 to produce pDVWS501M85R, pDVWS501M85L, pDVWS501M85D, pDVWS501M85E, pDVWS501M85V and pDVWS501M85Y, respectively. The PCR-derived regions of all clones were sequenced.

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**Production of virus from genomic-length cDNA clones.** Procedures for transcription of RNA, electroporation of BHK-21 cells and passaging of virus in C6/36 cells have been described previously (Gualano et al., 1998). In this study, following electroporation of full-length RNA transcripts into BHK-21 cells, cells were divided equally into two Petri dishes and incubated at either 33 or 37°C. Medium from BHK-21 cells incubated at both temperatures was passaged twice in C6/36 cells to produce virus stocks. To confirm the presence of mutations in the recovered viruses, total RNA was extracted from infected/transfected cells and culture supernatants by using an acid guanidium isothiocyanate method (Lewis et al., 1992) or a QiAamp Viral RNA Extraction kit (Qiagen). The viral RNA was used for RT-PCR (Gualano et al., 1998) and the resulting DNA products were sequenced over the region spanning the mutation.

**Analysis of prM–E transient gene expression.** The parental pSVprM–E and mutant constructs were introduced into COS cells for transient gene expression by using FuGENE 6 (Roche) following the manufacturer’s protocols. At 24–48 h post-transfection, COS cells were pulse–chase–radiolabelled with [35S]methionine/cysteine (Trans 35S-label; ICN) as described previously (Murray et al., 1993; Pryor & Wright, 1993). The culture medium was harvested and cell monolayers were washed in cold lysis buffer [150 mM NaCl, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 15 mM Tris/HCl, pH 7–8] and allowed to settle for 10 min at 4°C. The culture medium and cell lysate samples were centrifuged at 13,000 g for 8 min at 4°C to remove cellular debris and stored at −70°C until required for further analysis. Proteins contained in radiolabelled samples were analysed by radioimmunoprecipitation (RIP) using a mixture of anti-E mAbs followed by SDS-PAGE, as described previously (Gruenberg & Wright, 1992; Lin et al., 1994; Pryor et al., 1998). Antigen–antibody complexes were allowed to form overnight at 4°C and then captured by using protein A–Sepharose CL-4B (Amersham Biosciences). The mixture was layered over 10% sucrose in lysis buffer with half-strength detergents [150 mM NaCl, 0.25% (w/v) sodium deoxycholate, 0.5% (v/v) Triton X-100, 0.05% (w/v) SDS, 15 mM Tris/HCl, pH 7–5] and allowed to settle for 10 min at 4°C before removal of the supernatant. Precipitates were washed three times with lysis buffer with half-strength detergents, with collection by centrifugation between each wash. Radiolabelled proteins were eluted from the protein A–Sepharose by heating in denaturation buffer [0.5% (v/v) SDS, 1% (v/v) β-mercaptoethanol] at 100°C for 5 min, separated by SDS-PAGE and detected either by autoradiography (using Kodak BioMax MR film) or by PhosphorImager analysis (Molecular Dynamics). Endoglycosidase digestion of immunoprecipitated proteins was done after elution, using either N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (New England Biolabs) following the manufacturer’s protocols, before analysis by SDS-PAGE.

**Characterization of recombinant virus-like particles.** The method of virus-like particle purification was adapted from that used by Schalich et al. (1996). COS cells were transfected with pSVprM–E and radiolabelled 12 h post-transfection as described above, except that radiolabelling was done for 10 h. As a control, MON501-infected C6/36 cells were labelled for 10 h, commencing at 60 h post-infection, using the same conditions. Following labelling, the culture medium was collected and clarified by centrifugation at 12,000 g for 20 min at 4°C. Particles were pelleted by centrifugation at 142,000 g for 150 min at 5°C in a Beckman 45 Ti rotor and then resuspended in 0.4 ml Tris-buffere saline (TBSB; 0.12 M NaCl, 12 mM Tris/HCl, pH 8.0) containing 0.1% (w/v) BSA. Aggregates were removed by centrifugation at 200,000 g for 5 min at 4°C. The supernatant was layered over a 10–8 ml 5–20% linear sucrose gradient (prepared in TBS) and centrifuged at 247,000 g for 90 min at 5°C in a Sorvall TH-641 rotor. Fractions of 0.5 ml were collected from the bottom of the tubes by using a Beckman fraction-recovery system. The contents of each fraction were analysed by SDS-PAGE and PhosphorImager analysis, as described above.

**RESULTS**

**Effects of mutating M39 on virus viability**

To investigate whether a His residue at position 39 in the DENV-2 M protein (M39) was required for virus viability, nucleotide changes encoding various amino acid substitutions at M39 were engineered into the DENV-2 genome and their effects on virus recovery were examined. The amino acids chosen to substitute the basic His residue were Asn, Tyr, Arg, Leu, Pro and Gln. Asn, an uncharged polar amino acid, was found at M39 in DENV-4 and all other flaviviruses. Gln and Tyr (uncharged polar amino acids), Leu and Pro (non-polar amino acids) and Arg (a basic amino acid) were also studied to analyse the importance of the amino acid side chain at this site. The mutations were introduced into plasmid pDVWS501. pDVWS501 contains cDNA corresponding to the genome of DENV-2 strain NGC; virus produced from this plasmid is named MON501 (Gualano et al., 1998). RNA was transcribed from each of the mutant constructs in vitro and introduced into BHK-21 cells by electroporation. The BHK-21 cells were incubated at 33 and 37°C after transfection to aid the recovery of any temperature-sensitive viruses. After 4–5 days, the BHK-21 cell-culture medium was collected, passaged twice in C6/36 cells and the virus titre was determined by plaque assay on C6/36 cells. The procedures were completed at least twice for each construct and the results are summarized in Table 1. Mutant viruses were named according to the parental construct, the mutated gene and the specific mutation. Virus was only recovered from RNA transcripts containing Asn, Gln and Tyr at M39. For the mutants containing Asn (MON501-MH39N) or Arg (MON501-MH39R) at M39, the recovered viruses had titres 1–2 log10 lower than the parental virus (MON501). The titre of MON501-MH39Y recovered at 37°C appeared to be equivalent to that of MON501, whilst for recovery at 33°C, there was a 1–1 log10 decrease in virus titre. Substitution of Gln for His at M39 led to the recovery of virus (MON501-MH39Q), but had a severe effect on virus replication, with overall 5–3 log10 (33°C) or 4–2 log10 (37°C) decreases in final virus titres compared with MON501. The retention of the introduced mutations in the recovered viruses was examined by RT-PCR and sequencing (Table 1). All of the introduced mutations had been retained, with the exception of the His→Tyr substitution. It was found that the C→U mutation, specifying a His→Tyr change at M39, had reverted back to the wild-type C nucleotide. To determine how quickly the change from Tyr→His occurred, RNA was extracted from transfected BHK-21 cells and C6/36 cells at the first passage and subjected to RT-PCR and sequence analysis. By completion of the first C6/36 passage, essentially all RNA genomes contained the U→C change (data not shown). In a repeat experiment (designated § in Table 1),
Table 1. Effects of mutating M39 in the context of the virus and transient-expression systems

<table>
<thead>
<tr>
<th>Parental construct</th>
<th>M39 codon</th>
<th>M39 residue</th>
<th>Amino acid type</th>
<th>Virus titre*</th>
<th>Codon retained†</th>
<th>Requirement for reversion to His</th>
<th>Secretion of prM/E‡</th>
</tr>
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<tr>
<td>MON501</td>
<td>CAU</td>
<td>His</td>
<td>Basic</td>
<td>1 ± 0.2 × 10³</td>
<td>Yes</td>
<td>Transversion</td>
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<td></td>
<td></td>
<td></td>
<td>6.1 ± 1.0 × 10⁶</td>
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<tr>
<td>pDVWS501MH39R</td>
<td>CGU</td>
<td>Arg</td>
<td>Basic</td>
<td>2.4 ± 0.2 × 10⁵</td>
<td>Yes</td>
<td>Transition</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>4.3 ± 0.1 × 10⁴</td>
<td>Yes</td>
<td>G→A</td>
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<td>Leu</td>
<td>Non-polar</td>
<td>0</td>
<td>Yes</td>
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<td>—</td>
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<td></td>
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<td></td>
<td></td>
<td>0</td>
<td></td>
<td>U→A</td>
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<td>Pro</td>
<td>Non-polar</td>
<td>0</td>
<td>No</td>
<td>Transversion</td>
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<td>AAU</td>
<td>Asn</td>
<td>Uncharged polar</td>
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<td>Yes</td>
<td>Transversion</td>
<td>A→C</td>
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<td>6.2 ± 0.3 × 10⁴</td>
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<tr>
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<td>Gln</td>
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<td>3.5 ± 0.3 × 10³</td>
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<tr>
<td>pDVWS501MH39Y</td>
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<td>Tyr</td>
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<td>2.5 ± 0.3 × 10⁵</td>
<td>No (CAC)</td>
<td>U→C</td>
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<tr>
<td>pDVWS501MH39Y §</td>
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<td>Tyr</td>
<td>Uncharged polar</td>
<td>1 ± 0.2 × 10³</td>
<td>No (CAC)</td>
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<td>1 ± 0.3 × 10³</td>
<td>No (CAC)</td>
<td>U→C</td>
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*Virus titre in p.f.u. ml⁻¹ (± 1 SD) following electroporation of RNA transcripts into BHK-21 cells and recovery at 33 (top) or 37 (bottom) °C, followed by two subsequent passages in C6/36 cells.
†RT-PCR was performed following two passages in C6/36 cells and the sequence of the mutated region was determined. Yes indicates that the presence of the mutation was confirmed by RT-PCR; no indicates that RT-PCR revealed changes within the mutated region, as shown.
‡COS cells were transfected with transient-expression constructs containing each mutation. The amount of secreted prM/E was scored as follows: —, no secretion detected; +, low levels of secretion; + + , moderate levels of secretion; + + + , high levels of secretion; + + + + , maximum levels of secretion based on secretion of prM/E from cells transfected with pSVprM–E.
§RNA transcripts corresponding to MON501 were prepared independently and used to recover virus in a second experiment.

virus was again recovered; however, in this instance, the final titre of MON501-MH39Y after the second C6/36 passage was severely decreased (by ~ 3·8 log₁₀) in comparison with MON501 (Table 1). RNA samples were extracted from the BHK-21 cells and the C6/36 cells at the first and second passages, and from the corresponding cell-culture media samples. Analysis of the RNA samples by RT-PCR and sequencing revealed that, in this instance, the Tyr→His change was not complete until the second passage through the C6/36 cells. It was not clear from the results whether the Tyr→His reversion was a result of virus replication during the passing process or the Tyr→His change was introduced into the RNA population during the preparation of in vitro transcripts and amplified selectively during the passing process. However, as the Tyr→His change arose at the same time in viral populations that were produced from identical RNA transcripts, but recovered under different conditions (i.e. 33 and 37 °C), the latter case was more likely. Collectively, these results suggested that an amino acid with a basic charge or polarity is required at M39, as virus was not recovered from mutants containing non-polar amino acids. We identified a strong selective pressure at M39 that involved a single-site transition, resulting in a Tyr→His change. Only single-site transitions were identified in this study. For the mutants where replication was not identified, reversion to His would have required a single nucleotide transversion (Leu and Pro; Table 1).

Establishment of a prM–E transient gene-expression system

A number of the mutations introduced at M39 abolished or decreased virus replication. To investigate whether the mutations impaired particle assembly or release, a prM–E transient gene-expression system was established. The plasmid pSVprM–E, containing cDNA derived from pDVWS501 encoding the prM signal sequence and prM or decreased virus replication. To investigate whether the presence of the mutation was confirmed by RT-PCR; no indicates that RT-PCR revealed changes within the mutated region, as shown.

The kinetics of production, association and secretion of the prM and E proteins, were detected in all samples, both intra- and extracellular (Fig. 1a). The prM protein was found to associate with the E protein during the initial 1 h labelling and throughout the 6 h chase period. The amounts of prM and E proteins within cells decreased over time,
corresponding to an increase in the amounts of these proteins in the culture medium of transfected cells to a maximum between 4 and 6 h after chase. A protein with a molecular mass of 80 kDa, corresponding to the unprocessed prM–E polypeptide, was detected in the cell-lysate samples (Fig. 1a). The amount of prM–E decreased over time and was not detected in the culture medium, consistent with appropriate cleavage of this precursor protein. Endoglycosidase sensitivity confirmed that both the intracellular and extracellular prM and E proteins were glycosylated (Fig. 1b). PNGase F treatment of the intracellular and extracellular prM and E proteins altered the molecular mass of the prM and E proteins by 2–5 and 4–5 kDa, respectively, consistent with previous findings that the DENV-2 strain NGC prM protein contains one N-linked glycan and the E protein contains two N-linked glycans (Smith & Wright, 1985). The intracellular prM and E proteins were >90% Endo H-sensitive (Fig. 1b), whilst the extracellular proteins were partially (prM) or fully (E) resistant to Endo H digestion. The results suggested that the E protein expressed from pSVprM–E underwent modification of its glycans from simple high-mannose to complex glycans during the process of transport through the secretory pathway, whilst only a subpopulation of prM protein molecules underwent modification of the single N-linked glycan to the complex type before secretion.

To determine whether the prM and E proteins were secreted in a particulate form, pSVprM–E-transfected COS cells were radiolabelled and the culture supernatants were examined for the presence of RSPs by sucrose density-gradient fractionation and RIP analysis. As a comparison for RSP production, the culture medium of MON501-infected C6/36 cells was subjected to the same analysis. The largest amount of prM and E proteins from MON501-infected cells was detected in fractions close to the bottom of the gradient, representing virions, with two smaller peaks closer to the top of the gradient (Fig. 2a and c). Examination of the sedimentation properties of secreted E protein from DENV-2 strain NGC-infected Vero cells resulted in a similar three-peak profile, with no clear resolution of SHA (Konishi & Fujii, 2002). The E and prM proteins present in the culture medium of pSVprM–E-transfected cells formed a major peak corresponding to the slowest sedimenting peak from virus (Fig. 2b and c). To confirm that the proteins detected in the fractions were prM and E, peak fractions were analysed by RIP with anti-E monoclonal

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**Fig. 1.** Analysis of DENV prM and E protein processing, association and secretion from transfected COS cells. (a) COS cells transfected with pSVprM–E. At 48 h post-transfection, cells were pulsed for 1 h with [35S]methionine/cysteine and chased for 0, 1, 2, 4 and 6 h. RIP analysis of cell lysates (lanes 1–6) and culture medium (lanes 7–12) using anti-E monoclonal antisera was performed and proteins were separated by SDS-PAGE and detected by using fluorography. Arrows indicate the positions of the E, prM and prM–E proteins. (b) COS cells transfected with pSVprM–E. At 48 h post-transfection, cells were pulsed for 1 h with [35S]methionine/cysteine and chased for 6 h. Samples of the cell lysate (lanes 1–3) and culture medium (lanes 4–6) from the transfected cells were then subjected to RIP analysis using anti-E monoclonal antisera. Following RIP, each sample was divided into three and treated with Endo H (H; lanes 2 and 5), PNGase F (F; lanes 3 and 6) or left untreated (--; lanes 1 and 4). Following endoglycosidase treatment, proteins were separated by SDS-PAGE and examined by using PhosphorImager analysis. Arrows indicate the positions of the glycosylated (Egly and prMgly) and non-glycosylated (E and prM) forms of the E and prM proteins. Molecular masses of 14C protein markers are shown on the left of each gel.

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antiserum. A protein corresponding in molecular mass to the prM protein was coprecipitated with the E protein from all peak fractions (Fig. 2d). Both prM and E derived from the recombinant virus infection were smaller than the prM and E proteins derived from pSVprM–E transfection. The difference in molecular mass may be attributed to the different glycosylation patterns produced in insect and mammalian cells (Smith & Wright, 1985). In conclusion, the sedimentation, coprecipitation and glycosylation data all suggested that transient expression of the prM and E genes in COS cells resulted in the secretion of prM/E in the form of DENV-2 RSPs. Hence, the system was deemed suitable for analysis of the effects of M39 mutations on the assembly and release of RSPs.

### Analysis of the effects of mutations at M39 by transient gene expression

To determine the effects of amino acid substitutions at M39 on prM/E interaction and secretion, a selection of the mutations examined by using the DENV-2 genomic cDNA clone was introduced into pSVprM–E to generate the constructs pSVprM139R–E, pSVprM139L–E, pSVprM139P–E and pSVprM139Y–E. COS cells were transfected with each of the constructs. At 48 h post-transfection, cells were radiolabelled with [35S]methionine/cysteine for 1 h and chased for 6 h to follow the processing and secretion of the prM and E proteins. Cells were harvested immediately following labelling (0 h) and at 6 h post-chase, along with the culture supernatant, to examine the effects of each
mutation on the initial association and secretion of the prM and E proteins. The cell lysates and culture medium were subjected to RIP analysis using anti-E antiserum, as described previously (Fig. 3). RIPs were performed in duplicate on samples from each of two independent transfections and a typical result is shown. The prM protein was coprecipitated with E protein from lysates of cells transfected with each mutant construct immediately following radiolabelling (0 h post-chase) (Fig. 3a). None of the mutations appeared to affect the association between the prM and E proteins significantly. A protein with a molecular mass consistent with a prM–E precursor was also detected, as described above. Reduced amounts of intracellular prM, E and prM–E were detected at 6 h post-chase (Fig. 3b). At 6 h post-chase, secreted prM and E proteins were not detected in the culture medium of cells transfected with pSVprMH39P–E or pSVprM H39L–E (Fig. 3c, lanes 4 and 5). In contrast, the prM protein was coprecipitated with the E protein that was present in the culture medium of cells transfected with pSVprMH39Y–E and pSVprM H39R–E (Fig. 3c, lanes 2 and 3).

**DISCUSSION**

The C-terminal regions of the flavivirus prM/M and E proteins contain a number of α-helical structural elements, which play important roles in the formation and entry of virus particles. Secondary structure predictions using flavivirus prM and E amino acid sequences (Allison et al., 1999; Stiasny et al., 1996; Zhang et al., 2003a) and cryo-EM analysis of DENV-2 particles (Zhang et al., 2003a) has shown that the C-terminal ~55 and ~100 aa of the prM/M and E proteins contain three (M-H, M-TM1, M-TM2) and four (E-H1, E-H2, E-TM1 and E-TM2) consecutive α-helices, respectively. The TM1 and TM2 regions of each protein are antiparallel helices that span the membrane, whilst the remaining α-helices, termed the ‘stem regions’ (Allison et al., 1999), are weakly amphipathic and lie relatively flat on the outer viral membrane, linking the prM and E ectodomains to the transmembrane anchors (Zhang et al., 2003a). Studies with truncated E proteins cleaved from virions (Stiasny et al., 1996; Wang et al., 1999) and expressed transiently from an E gene deletion construct (Allison et al., 1995b) demonstrated that the stem–anchor region of the E protein is required for stability of prM/E heterodimers, the formation of RSPs and low pH-induced formation of E trimers. Finer mapping of the regions in the TBEV E protein stem–anchor that are required for prM interaction revealed that the H2 and TM1 regions were required for the production of stable prM/E heterodimers (Allison et al., 1999). Recent structural (Zhang et al., 2003a) and genetic (Op De Beeck et al., 2003) evidence suggests that the prM/M stem–anchor region is also likely to play a role in virion morphogenesis and cell entry. However, little is known about the specific amino acid residues that are involved in these processes.

The results of this study demonstrated that the His residue at position 39 in the DENV-2 M protein plays a critical role...
in the virus life cycle, determining virus viability. Transient expression of the prM and E genes containing various substitutions at M39 suggested that the residue at M39 most probably influences the virus-assembly process and/or release of virus particles, but may also contribute to the conformational rearrangements of the virus particle that are required for cell entry. Residue M39 is adjacent to the N terminus of M-TM1 in a 2–3 aa linker that separates M-H and M-TM1. Comparison of 40 flavivirus M protein sequences revealed that a polar amino acid was always present at M39; for DENV-1,-2 and -3 the residue at M39 is His, whereas for DENV-4 and all other flaviviruses, it is Asn (data not shown). Based on a similar comparison, Holbrook et al. (2001) suggested that the residue at M39 may be important for maintaining the structure of the M protein. Mutation of the His residue at M39 had one of three effects on virus viability and prM/E interactions. (i) Substitution with either the basic amino acid Arg or the uncharged polar residue Asn resulted in a moderate but significant (~2 log10) reduction in virus titre. Transient expression of pSVprMH39Y–E resulted in the formation of intracellular prM/E heterodimers and secretion of the prM and E proteins in a particulate form at similar levels to those of the parental construct. A His→Arg substitution at M39, in addition to two changes in the E protein, was identified in a chimeric DENV-1/YFV, which caused decreased viraemia in monkeys compared with the parental DENV-1 and YFV (Guirakhoo et al., 2001). The contribution of the M39 Arg mutation to the monkey attenuation phenotype has not been reported. (ii) Substitution of the His residue with non-polar residues (Leu or Pro) abolished virus replication. These substitutions did not disrupt intracellular prM/E heterodimer formation, yet there was no detectable secretion of the E protein or prM/E complexes. These results are similar to those obtained in a study investigating the role of the transmembrane regions of the YFV M and E proteins in particle assembly by alanine-insertion mutagenesis (Op De Beeck et al., 2003). Mutations that severely decreased the secretion of RSPs and abolished virus replication did not affect the formation and stability of prM/E heterodimers. One such mutation was an alanine insertion between residues 38 and 39 of the YFV M protein. Our results support the conclusion that the stem–anchor region of the prM protein is involved in the assembly of immature particles and/or their transport through the secretory pathway. (iii) Substitution of His with the uncharged polar amino acids Tyr and Gln severely decreased, but did not totally abolish, virus replication. In the case of the Tyr mutation, it was difficult to ascertain the absolute level of virus replication, as restoration of the parental His residue always occurred by the second C6/36 cell passage, suggesting that there was a strong selective pressure at this site. Transient expression of pSVprMI39Y–E resulted in the formation of intracellular prM/E heterodimers and appeared not to affect secretion of the prM and E proteins in a particulate form. These results suggested that the His→Tyr mutation may affect virus entry, rather than assembly and release.

Transient expression of pSVprM–E in COS cells led to the production of prM and E proteins, which formed intracellular heterodimers and were secreted as a prM/E complex. Endoglycosidase and succrose sedimentation analysis suggested that the prM/E complexes had passed through the exocytotic pathway and were secreted in a particulate form, most probably as RSPs. The kinetics of prM and E protein heterodimer formation and secretion were similar to those observed for DENV-2 strain NGC-infected Vero cells (Wang et al., 1999). In contrast to our results, Chang et al. (2003) could not detect secretion of the prM or E proteins from COS cells transiently expressing the DENV-2 prM and E genes and suggested that an ER-retention signal resides in the stem–anchor region of the E protein. However, a number of other studies have reported the secretion of DENV RSPs from mammalian cells expressing the DENV prM and E genes, either transiently (Fonseca et al., 1994; Raviprakash et al., 2000) or stably (Konishi & Fujii, 2002; Konishi et al., 2003). Previous studies have determined that furin-mediated cleavage of prM was necessary to activate the full fusogenic potential of flaviviruses (Guirakhoo et al., 1991, 1992; Stadler et al., 1997). In our experiments, the levels of secreted prM were high relative to intracellular prM levels, both from virally infected C6/36 cells and from pSVprM–E-transfected COS cells, suggesting that prM processing was impaired. Similarly, infectious DENV particles with a high proportion of prM have consistently been found to be released from infected cells (Anderson et al., 1997; He et al., 1995; Henchal et al., 1985; Murray et al., 1993; Putnak et al., 1996; Randolph & Stollar, 1990; Roehrig et al., 1998; Wang et al., 1999). Recent studies mutating the prM furin cleavage site of TBEV (Elshuber et al., 2003) and DENV-2 (Keelapang et al., 2004) have shown that, whilst cleavage of prM is essential for virus infectivity, enhanced furin cleavage of the DENV prM protein affects virus export adversely, suggesting that it may be advantageous for DENV to retain some prM on the virus surface. We have also found that mutation of the prM/M cleavage site abolished virus infectivity, but still led to the release of RSPs, by using our transient-expression system (L. Azzola, P. J. Wright & A. D. Davidson, unpublished data). Protein expression, export and subviral particle formation was not affected by the retention of prM in the TBEV mutant (Elshuber et al., 2003). In addition, the conformation of the Langat virus M protein recognized by anti-M antibodies was identical before and after furin cleavage of prM (Holbrook et al., 2001). It is therefore likely that the effects of M39 mutations on virus-particle assembly and secretion that were observed by using the transient-expression system accurately reflect those occurring in a viral infection.

Analysis of the effect of the M39 mutations in the context of the 9-5 Å resolution DENV-2 cryo-EM structure (Zhang et al., 2003a) suggests that the residues in the loop preceding the M-TM1 region, encompassing M39, may participate in a number of molecular/structural interactions, all of which may play important roles in the virus life cycle. Residues M38 and M39 are likely to be buried in the outer-membrane...
leaflet and may therefore potentially interact with the polar lipid head groups. The orientation of the prM/M ectodomain is also likely to be influenced by the amino acids present at M38 and M39. Introduction of a non-polar residue at these positions may disrupt interactions with the lipid bilayer, possibly influencing processes such as virus budding. The residue at M39 also has the potential to interact with residues either in the C-terminal region of the E-H2 element or those linking E-H2 to E-TM1. These regions are required for stabilizing prM/E heterodimers (Allison et al., 1999) and are likely to undergo major rearrangement during virus maturation and fusion (Bressanelli et al., 2004; Modis et al., 2004). The results of this study highlight the importance of the prM/M stem–anchor region in viral morphogenesis, secretion and entry and pinpoint a critical residue in the M protein that may play a role in all of these processes, either by maintaining the structure of M or by interacting with the E protein stem–anchor region.

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