Post-translational regulation and modifications of flavivirus structural proteins

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Flaviviruses are a group of single-stranded, positive-sense RNA viruses that generally circulate between arthropod vectors and susceptible vertebrate hosts, producing significant human and veterinary disease burdens. Intensive research efforts have broadened our scientific understanding of the replication cycles of these viruses and have revealed several elegant and tightly co-ordinated post-translational modifications that regulate the activity of viral proteins. The three structural proteins in particular – capsid (C), pre-membrane (prM) and envelope (E) – are subjected to strict regulatory modifications as they progress from translation through virus particle assembly and egress. The timing of proteolytic cleavage events at the C-prM junction directly influences the degree of genomic RNA packaging into nascent virions. Proteolytic maturation of prM by host furin during Golgi transit facilitates rearrangement of the E proteins at the virion surface, exposing the fusion loop and thus increasing particle infectivity. Specific interactions between the prM and E proteins are also important for particle assembly, as prM acts as a chaperone, facilitating correct conformational folding of E. It is only once prM/E heterodimers form that these proteins can be secreted efficiently. The addition of branched glycans to the prM and E proteins during virion transit also plays a key role in modulating the rate of secretion, pH sensitivity and infectivity of flavivirus particles. The insights gained from research into post-translational regulation of structural proteins are beginning to be applied in the rational design of improved flavivirus vaccine candidates and make attractive targets for the development of novel therapeutics.

Flaviviruses

The genus Flavivirus in the family Flaviviridae comprises small, enveloped viruses with non-segmented genomes consisting of single-stranded, positive-sense RNA. These RNA genomes are flanked by 5’ and 3’ untranslated regions (UTRs) and encode a single polyprotein that is cleaved co- and post-translationally to generate between 10 and 11 viral proteins. The 5’ UTR contains a type 1 m7GpppNm cap structure and the 3’ UTR lacks a polyadenylated tail. Structural elements within the UTRs regulate genome replication, translation and host immune responses. Viral proteins are divided between structural proteins, which form the virion, and non-structural proteins, which are involved in genomic replication and modulating host immunity (Fig. 1a) (Lindenbach et al., 2007; Roby et al., 2012).

Flaviviruses are maintained predominantly in natural transmission cycles between vertebrate reservoir species (normally mammalian or avian) and haematophagous arthropod vectors (mosquitoes or ticks). Notable exceptions are the viruses that are maintained solely in mosquito hosts (insect-specific flaviviruses) and viruses with no known invertebrate vector (Cook et al., 2012; Mackenzie et al., 2004). As of 2013, the International Committee on Taxonomy of Viruses has classified 53 different viral species as belonging to the genus Flavivirus; however, many newly identified flaviviruses are being isolated each year and remain to be formally described (Adams et al., 2013; King et al., 2012). Of these 53 separate species, 40 have been associated with infection in humans such as dengue virus (DENV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) and West Nile virus (WNV). Several flavivirus species including JEV, WNV and louping ill virus also cause economically important diseases of livestock and poultry (Gubler, 2012; van der Meulen et al., 2005). In light of the heavy disease burden attributed to flaviviruses, the generation of effective vaccines and therapeutics remains a significant goal within the medical research community.

The replicative cycle of flaviviruses is an elegantly regulated process and has been described in detail elsewhere (Roby et al., 2012). Briefly, replication consists of seven discrete stages (Fig. 2): (i) receptor-mediated virus entry; (ii)
low-pH-induced fusion of the viral envelope with the host endosome, uncoating of the nucleocapsid and initial polyprotein translation; (iii) asymmetrical semi-conservative replication of genomic RNA within endoplasmic reticulum (ER)-derived vesicle packets (VPs); (iv) virion assembly on ER membranes directly opposed to the VP pore; (v) trafficking of immature virions to the Golgi apparatus for glycan maturation; (vi) furin cleavage of pre-membrane protein (prM) within the trans-Golgi network and low-pH-mediated rearrangement of the envelope proteins on the virion surface; and (vii) secretion of mature progeny virus and dissociation of the cleaved pr peptide.

Throughout this replication cycle, the three flavivirus structural proteins, capsid (C), prM and envelope (E), are subjected to strict post-translational control in order to regulate efficient virion assembly, secretion and infectivity. Co-ordination between dual cleavage events at the C–prM transmembrane junction of the flavivirus polyprotein has been demonstrated to be important in the effective incorporation of nucleocapsid into nascent virions (Lobigs & Lee, 2004; Lobigs et al., 2010). Heterodimeric interaction between prM and E has been revealed as necessary for the correct conformational folding of the envelope and the prevention of premature virus fusion during secretory transit (Mukhopadhyay et al., 2005). Indeed, the protective role of prM is so efficient that cleavage by the host enzyme furin is required in the final stages of virion secretion in order to promote infectivity of progeny virus. The addition of complex glycans to the envelope proteins prM and E within the ER and Golgi has also been demonstrated to act in regulating the rate of virion secretion, sensitivity to low-

**Fig. 1.** Flavivirus genomic organization and membrane topology. (a) Organization of the flavivirus genome. The positive-sense ssRNA of ~11 kb encodes a single ORF flanked by 5′ and 3′ UTRs. Translation of the ORF produces a single polyprotein, which is cleaved co- and post-translationally to form each of the mature viral proteins. (b) Schematic demonstrating the membrane topology of flavivirus structural proteins. Crystal structures of the Kunjin virus C (Dokland et al., 2004) and DENV pr peptide and E protein (Li et al., 2008) have been used to enhance visualization of the polyprotein, rather than to imply that these mature conformations are adopted prior to cleavage. Sites of proteolytic processing of the polyprotein are indicated, as is the signal sequence (ss) between C and prM. Trans-membrane domains and membrane-associated helices are represented as cylinders.
Flavivirus structural proteins

C protein

The flavivirus C protein is a small (approx. 14 kDa) cytosolic protein with affinity for both lipid membranes and nucleic acids (Dokland et al., 2004; Ivanyi-Nagy et al., 2008; Jones et al., 2003; Khromykh & Westaway, 1996; Ma et al., 2004; Markoff et al., 1997). It is translated in cis at the N terminus of the flavivirus polyprotein (Fig. 1b) and undergoes maturation to a soluble protein by post-translational cleavage from the prM signal sequence by cytosolic viral NS2B/3 protease (see ‘Polyprotein processing’ below and Fig. 3). Nuclear magnetic resonance and X-ray crystallography experiments (Dokland et al., 2004; Ma et al., 2004) have revealed that the C protein consists of four structurally conserved α-helices and contains a conserved internal hydrophobic domain. The C protein forms dimers in solution mediated by association of the internal hydrophobic regions, and in particular a conserved tryptophan residue (W69 in WNV) in α-3 (Bhuvanakantham & Ng, 2005; Kierrmayr et al., 2004). Recent evidence indicates that the α-4 helix may also play a crucial role in dimerization (Teoh et al., 2014). Indeed, this is supported by experiments that demonstrate that WNV can tolerate complete deletion of the α-2 and α-3 helices (including the hydrophobic domain and residue 69) and retain the ability to package RNA (Schlick et al., 2009). Small internal C-protein deletions in TBEV infectious clones have also pH environments and the infectivity of mature virus particles (Beasley et al., 2005; Goto et al., 2005; Guirakhoo et al., 1993; Hanna et al., 2005; Lee et al., 1997, 2010; Li et al., 2006; Mondotte et al., 2007; Scherret et al., 2001). Exploitation of these post-translational control measures has facilitated the continuing development of novel vaccines and therapeutics for use against flavivirus infections (Goto et al., 2005; Keelapang et al., 2013; Ohtaki et al., 2010; Pang et al., 2014; Roby et al., 2011, 2013, 2014; Zhang et al., 2011).

Flavivirus structural protein regulation

Fig. 2. Flavivirus genomic organization and replication cycle. Canonical model of the replication cycle of flaviviruses: (i) receptor-mediated entry of virus; (ii) low-pH-induced fusion of the viral envelope with endosomes and release of genomic RNA; (iii) formation of the replication complex (RC) within endoplasmic reticulum (ER)-derived vesicle packets (VPs) and subsequent asymmetrical semi-conservative replication of genomic RNA; (iv) progeny genomic RNA associates with C and evagination into the ER lumen generates the viral envelope containing prM and E heterodimer trimeric spikes; this process also occurs asymmetrical semi-conservative replication of genomic RNA; (v) immature virions and prM/E particles are trafficked in individual vesicles to the Golgi apparatus for glycan maturation; (vi) low pH induces conformational rearrangement of prM/E heterodimers, and cleavage of prM by host furin occurs within the trans-Golgi network; (vii) mature virions and prM/E particles are secreted from the infected cell by exocytosis, and the associated pr peptide is released.
(a) Efficient NS2B/3 cleavage

Efficient signalase cleavage

High-titre virions
High-titre prM/E particles

(b) Efficient signalase cleavage

Very inefficient NS2B/3 cleavage

No or low-titre virions
High-titre prM/E particles

(c) Efficient, simultaneous NS2B/3 and signalase cleavage

Efficient, simultaneous NS2B/3 and signalase cleavage

Low-titre virions
High-titre prM/E particles
Fig. 3. Co-ordinated proteolytic cleavages at the C–prM junction. (a) Flavivirus polyprotein cleavage in the natural state. Cytosolic C protein is first cleaved by the viral NS2B/3 protease. This initial cleavage induces a change at the ER lumenal signal sequence site in prM allowing efficient cleavage. The outcome of the sequential events is a delay in prM accumulation and concomitant efficient nucleocapsid incorporation. (b) Introduction of the PQAQA mutation upstream of the prM signal sequence cleavage site, which removes the dependence on prior cleavage of C. Signal sequence cleavage of prM renders a change at the NS2B/3 cleavage site at the end of C, making subsequent C release very inefficient. The outcome of uncoupling signal sequence cleavage is that no or very little nucleocapsid incorporation occurs though prM/E particle secretion continues. (c) The introduction of a GS dipeptide and S→Y mutation adjacent to the NS2B/3 cleavage site allows C to be processed irrespective of prior signal sequence cleavage of prM. The outcome of completely uncoupling the co-ordinated cleavages is a low titre of nucleocapsid-containing virions and efficient prM/E particle secretion. Note that crystal structures of the KUNV C (Dokland et al., 2004) and DENV pr peptide (Li et al., 2008) were used to enhance visualization of the polyprotein.

demonstrated that the x-1 and x-2 helices are dispensable, as these mutants either were able to functionally package infectious virions (1–16 aa within x-1 and x-2) (Kofler et al., 2002) or developed compensatory mutations elsewhere within C that restored encapsidation (19–30 aa in x-2) (Kofler et al., 2003).

The x-4 helices of C associate to form a positively charged surface that has been proposed to act alongside the flexible N terminus in stabilizing the interaction with RNA (Dokland et al., 2004; Khromykh & Westaway, 1996; Ma et al., 2004). The flexible N terminus of DENV C has also been demonstrated to mediate lipid droplet association (Martins et al., 2012). Highly charged basic residues found at the N and C termini (aa 1–32 and 82–105 in WNV) associate with nucleic acids, particularly with the 5’ and 3’ UTRs of flavivirus genomic RNA (Khromykh & Westaway, 1996; Khromykh et al., 1998), and nucleic acid interaction is dependent on dephosphorylation of the C protein (Cheong & Ng, 2011). Phosphorylation of WNV C has conversely been shown to promote nuclear localization and thus removes the protein from sites of genomic RNA interaction (Bhuvanakantham et al., 2010). The Kunjin virus (KUNV; a naturally attenuated strain of WNV) and DENV C structures place x-1 in different orientations; thus, it is hypothesized that this helix rotates in order to change the position of the flexible N terminus for optimal RNA interaction (Dokland et al., 2004; Ma et al., 2004).

The C protein localizes to three distinct cellular compartments: the ER membrane and lipid droplets in the cytoplasm, as well as the nucleus (specifically the nucleolus) (Bhuvanakantham et al., 2010; Bulich & Aaskov, 1992; Colpitts et al., 2011; Martins et al., 2012; Mori et al., 2005; Samsa et al., 2009; Westaway et al., 1997). The primary role of the C protein in the flavivirus life cycle is in the assembly of the nucleocapsid and its incorporation into nascent virions, whilst other functions of C, including those performed in the nucleus, are less explored.

**prM protein**

The prM protein of flaviviruses is an approximately 19–21 kDa (the M protein is approximately 10 kDa after furin cleavage) membrane glycoprotein that is translocated into the ER lumen during polyprotein translation by an N-terminal signal sequence immediately downstream from C (Fig. 1b) (Lobigs et al., 2010; Lorenz et al., 2002; Setoh et al., 2012). Liberation of prM from this signal sequence by host cell signalase is tightly co-ordinated and is dependent on prior cleavage of C by viral NS2B/3 protease (see ‘Polyprotein processing’ below and Fig. 3). Flavivirus prM also contains two C-terminal transmembrane helices anchoring the protein to the ER membrane, the second of which acts as a signal sequence for luminal translocation of the E protein (Hsieh et al., 2011). Nascent prM is initially glycosylated in the ER lumen at between one and three sites within the pr portion of the protein, with glycan maturation occurring during transit through the Golgi apparatus (Mackenzie & Westaway, 2001).

Whereas the structure of the mature M portion of prM has yet to be solved beyond cryo-electron microscopy reconstructions (Zhang et al., 2013b), a crystal structure of the DENV pr peptide has been reported in complex with the soluble ectodomain of E (Li et al., 2008) (see Figs 1b and 3). The pr peptide forms a tightly folded protein domain consisting of two small β-sheets linked by disulfide bridges, with the entire structure positioned at the distal end of E domain II (DII) within the heterodimer where it obscures the fusion loop and facilitates pr glycan presentation at the tip of trimeric spikes (Li et al., 2008; Yu et al., 2008; Zhang et al., 2003). The C-terminal region of the M protein ectodomain is also predicted to form a helical domain (prM-H) that may assist in prM/E heterodimer formation (Hsieh et al., 2011; Peng & Wu, 2014; Zhang et al., 2012).

Together with the E protein, prM forms an integral part of the flavivirus envelope. Proposed roles for prM include assisting in the chaperone-mediated folding of the E protein (Konishi & Mason, 1993; Lorenz et al., 2002), contributing to the pH-dependent conformational rearrangement of trimeric prM/E heterodimer spikes in immature virions via the influence of the helical stem domain of M (Zhang et al., 2012), and in the concealment of the fusion loop of E by the pr peptide to prevent premature fusion during virion egress (Yu et al., 2009).

**E protein**

The E protein of flaviviruses is a large (approx. 53 kDa) membrane-bound glycoprotein that is responsible for virus fusion and acts as the main antigenic target for the develop-
ment of neutralizing humoral immunity. Several structures of the E protein ectodomain have been resolved for DENV, TBEV, JEV and WNV in a mature conformation (Kanai et al., 2006; Li et al., 2008; Luca et al., 2012; Modis et al., 2003; Nybakken et al., 2006; Rey et al., 1995). The E protein consists of three β-barrel structural domains. The central domain (domain I; DI) contains the N terminus and appears to act as an anchor for the hinge point of the large distal domain (domain II; DII) (Zhang et al., 2004). This second domain contains the fusion-loop peptide and is involved in virus fusion during entry (Mukhopadhyay et al., 2005), and in interactions with prM, which occludes the fusion loop in immature particles during cellular transit (Zhang et al., 2003, 2012). The third, immunoglobulin-like domain (domain III; DIII) on the opposite side of DI is hypothesized to interact with cellular receptors and is anchored at the C terminus to the two ‘stem’ helices and the two transmembrane helices (Fig. 1b) (Mukhopadhyay et al., 2005). All three domains appear to be involved in the dimerization of the E protein through hydrophilic interactions in mature virions (Zhang et al., 2004). The E protein is liberated from the genomic polyprotein at its N and C termini via cleavage with host signal peptidase in the ER lumen.

The E protein is synthesized immediately downstream of prM with which it rapidly heterodimerizes, an important interaction associated with the correct folding of E (Lorenz et al., 2002). These heterodimers coalesce on the luminal face of the ER membrane forming trimeric spikes and ultimately bud off to give rise to either empty prM/E particles, or nucleocapsid-containing immature virions (Roby et al., 2012). The observed formation and secretion of prM/E particles upon ectopic prM and E expression as part of a recombinant gene cassette (Allison et al., 1995; Goto et al., 2005; Konishi & Mason, 1993; Ohtaki et al., 2010; Zhang et al., 2011) suggest that this process is intrinsic to the prM and E proteins. This hypothesis is supported by a recent analysis of WNV and DENV membrane curvature induced by the arrangement of prM and E within the virus particle, suggesting that the overall spherical shape of the particles may be attributable to viral protein interaction with lipids without the need of assistance from host cellular factors (Zhang et al., 2013a).

Low-pH-induced conformational rearrangement of the prM/E spikes leads to the formation of a ‘herringbone’ pattern of three adjacent prM/E complex homodimers lying flat on the virion surface, with the pr peptide obscuring the fusion loop at the DII terminus (Figs 2 and 4) (Kuhn et al., 2002). The pr peptide is subsequently excised via furin cleavage during virion egress leaving a smooth, spherical mature particle in which only the E protein is exposed to solvent (Yu et al., 2008). Polyprotein processing

Co-ordinated cleavage at the C–prM junction

Of crucial importance to the life cycle of flaviviruses is the regulated sequence of proteolytic cleavages that occur at either side of the prM signal sequence in the virus polyprotein (Fig. 3). The currently accepted paradigm is that liberation of mature C by viral NS2B/3 cleavage at the cytosolic face of the ER membrane induces a change at the ER luminal face that promotes efficient signalase cleavage of prM. This temporal separation of cleavage events is believed to promote efficient nucleocapsid incorporation into budding virions (Lobigs & Lee, 2004; Lobigs et al., 2010).

Early investigations using ectopically expressed flavivirus structural cassettes in vitro determined that two independent cleavage sites were recognized on either side of a signal sequence separating C and prM and that signalase cleavage of prM appeared dependent on the addition of NS2B/3 protease (Amberg et al., 1994; Lobigs, 1993; Yamashchikov & Compans, 1993). The parameters controlling this sequential cleavage of C–prM were demonstrated via a series of mutations and chimeras in recombinantly expressed Murray Valley encephalitis virus (MVEV) proteins (Stocks & Lobigs, 1998). Mutation of various residues within C or replacement of C with ubiquitin in a structural gene cassette did not alter the dependence of signalase cleavage of prM upon the prior excision of the cytosolic portion of the polyprotein. Mutation of the prM signal sequence from GFAAA to PQAQA and expression in the absence of NS2B/3 allowed efficient prM cleavage despite the C protein remaining anchored to the signal sequence. Conversely, however, replacement of prM downstream of the signal sequence with E or the mouse H-2Kd MHC antigen showed only partial or no dependency on NS2B/3 activity for signalase cleavage. Thus, the regulation of prM cleavage is dependent primarily upon elements within the C-terminal region of the signal sequence, with a minor involvement of the prM protein itself.

This model of sequential cleavage (Fig. 3a) was finally confirmed to be important in the context of live flavivirus replication via the mutation of the NS2B/3 cleavage site in the YFV C–prM junction (Amberg & Rice, 1999). Mutations that prevented efficient processing of C–prM quickly reverted to large plaque-forming isolates with efficient polyprotein cleavages. The converse of these mutations was investigated via the introduction of the foot-and-mouth-disease virus (FMDV) 2A sequence to the C terminus of WNV and TBEV C inducing an immediate separation of C–2A from the rest of the polyprotein upon translation (Schrauf et al., 2009). These mutations were generally tolerated, as WNV-2A replicated well in mosquito and mammalian cells (albeit with smaller plaques) and TBEV-2A was shown to replicate efficiently in mammalian cells, although it was unable to replicate efficiently in tick cells. These experiments were able to demonstrate that C interaction with NS2B/3 was not important for virion formation per se; rather that it is important for C to be cleaved from the remainder of the polyprotein.

A purpose for the co-ordinated C–prM cleavages was finally proposed based upon experiments in which the PQAQA mutation was introduced into the signal sequences...
of YFV and MVEV, effectively removing the requirement for sequential processing of prM (Fig. 3b) (Lee et al., 2000; Lobigs & Lee, 2004). Efficient signalase cleavage of prM independent of prior C excision proved fatal for the replication of YFV, with only revertant viruses containing mutations in the N terminus of the signal sequence able to productively replicate (Lee et al., 2000). In contrast, the PQAQA mutation was tolerated to some degree in MVEV, culminating in a drastic reduction in mature C expression and nucleocapsid incorporation whilst maintaining prM/E particle secretion (Lobigs & Lee, 2004). Thus, two key points were revealed: (i) a delay in prM cleavage by signalase promotes incorporation of the nucleocapsid into budding virus particles, and (ii) efficient cleavage of C by NS2B/3 requires prM to remain attached to the signal sequence at its N terminus.

The unexpected revelation of a requirement for an intact signal sequence of prM to facilitate efficient C cleavage was investigated in PQAQA mutant strains of MVEV and by utilizing a packaging cell line expressing different C-terminally extended forms of C (Lobigs et al., 2010). Mutations that enhanced the hydrophobicity of the C-terminal region of the signal sequence, anchored the signal sequence in membranes with tryptophans or mimicked the viral protease cleavage site in NS5 failed to enhance the growth of PQAQA MVEV. However, the introduction of a GS dipeptide to the C-terminal region of the prM signal sequence allowed the selection of an isolate with an S111Y mutation (two residues downstream of the GS dipeptide within the signal sequence) that allowed more efficient C maturation and thus better replication (Fig. 3c). Despite the mutant virus replicating more efficiently than PQAQA MVEV, it still displayed a packaging defect compared with WT MVEV. Analysis of the packaging efficiency of cell lines expressing various forms of C demonstrated that extension of C to include the prM sequence up to the furin cleavage site (pr) improved genomic RNA incorporation compared with expression of the mature C alone. Thus, it appears that a cleavable transmembrane anchor at the C terminus of C promotes efficient packaging of viral RNA.

A recent investigation by our group sought to confirm this hypothesis by comparing the capacity of ectopically expressed soluble mature C, extended C (containing the signal sequence and the first 10 aa of prM), and Cpr (containing the prM sequence up to the furin cleavage site) to package C-deleted KUNV genomic RNA in trans (Roby et al., 2014). Both the extC and Cpr constructs were demonstrated to be able to be cleaved to generate mature C by the viral protease (albeit incompletely, as uncleaved forms of the proteins were detectable in cell lysates at 2 days post-transfection) and facilitate encapsidation of C-deleted KUNV genomic RNA. Thus, the presence of only 10 luminal residues of prM at the C terminus of the signal sequence allowed the selection of an isolate with an S111Y mutation (two residues downstream of the GS dipeptide within the signal sequence) that allowed more efficient C maturation and thus better replication (Fig. 3c). Despite the mutant virus replicating more efficiently than PQAQA MVEV, it still displayed a packaging defect compared with WT MVEV. Analysis of the packaging efficiency of cell lines expressing various forms of C demonstrated that extension of C to include the prM sequence up to the furin cleavage site (pr) improved genomic RNA incorporation compared with expression of the mature C alone. Thus, it appears that a cleavable transmembrane anchor at the C terminus of C promotes efficient packaging of viral RNA.

**Fig. 4.** Low-pH-induced conformational change in prM/E heterodimer organization during virus maturation and secretion. As immature flavivirus particles are transported from the ER to the Golgi apparatus, they encounter a lower-pH environment. This induces the rearrangement of prM/E heterodimers from 60 trimeric spikes to an anti-parallel ‘herringbone’ arrangement of 90 dimers that lie flat along the virion surface. This rearrangement exposes the furin cleavage site in prM, facilitating cleavage within the trans-Golgi network. The liberated pr peptide (shown here as a golden surface model) is then secreted along with the mature M/E-containing virion. Figure modified from Li et al. (2008).
sequence is sufficient to allow NS2B/3 cleavage of C. However, this investigation did not confirm a greater packaging efficiency for anchored forms of C, as the production of infectious particles was determined to be at efficiencies comparable to soluble C. It is possible that the use of the stronger EF1α promoter to drive trans-C expression in these experiments (Roby et al., 2014) saturated the capacity for nucleocapsid formation, thus overcoming any inherent inefficiencies in mature C. Further investigations utilizing a limited supply of C protein may better be able to resolve this phenotype.

Maturation of prM by furin cleavage

An integral process in the maturation of flavivirus particles is the cleavage of prM within the trans-Golgi network by the host enzyme furin, liberating the pr peptide and ultimately facilitating the exposure of the fusion loop within E protein DII (Fig. 2) (Stadler et al., 1997). Interestingly, in vitro furin cleavage assays show that maturation of prM only occurs at low pH, even though the enzymic activity of furin is optimal at neutral pH (Stadler et al., 1997; Yu et al., 2008). This suggests that the low-pH-induced structural rearrangement of the particle is required to expose the furin cleavage site on prM. Indeed, the currently accepted model of flavivirus maturation within the trans-Golgi involves the stem region of the prM protein interacting with the associated E protein DII in a manner that pulls E down from a trimeric spike into a flat dimeric conformation parallel to the virion surface (Zhang et al., 2012, 2013b), with this interaction dependent upon decreasing pH and originating from one or more discrete nucleation centres (Plevka et al., 2011, 2014). In addition, the low-pH environment in the trans-Golgi compartment allows the pr peptide to remain associated with E (Yu et al., 2009), effectively preventing premature fusion within the cell.

The release of prM-containing immature particles is consistently observed along with release of mature particles (Zhang et al., 2007a), as well as partially mature particles (Junjhon et al., 2010; Plevka et al., 2011), suggesting that either furin cleavage is not 100% efficient or the speed of prM/E conformational rearrangement on the particle is slower than the rate of cell-driven particle secretion through the trans-Golgi compartment. This phenomenon appears to be dependent upon the identity of the virus, as high rates of immature/partially mature virions are most consistently observed for DENV (Rodenhuis-Zybert et al., 2011), and this phenotype has been linked to a DENV-conserved acidic residue at position 89 in prM (P3 within the furin cleavage site) (Junjhon et al., 2008; Keelapang et al., 2004). WNV and TBEV grown in BHK-21 cells have similarly been identified as partially incomplete in maturity, with faint staining of uncleaved prM in immunoblots prepared with purified virus (Elshuber & Mandl, 2005; Moesker et al., 2010), although complete prM cleavage has also been observed with TBEV in this same cell line (Stadler et al., 1997).

Although immature flavivirus particles have been demonstrated to be non-infectious in vitro (Elshuber et al., 2003; Elshuber & Mandl, 2005; Moesker et al., 2010; Stadler et al., 1997; Yu et al., 2008, 2009), opsonization of immature DENV virions with anti-prM antibodies has been demonstrated to enhance the infection of Fc-receptor-positive cells (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010). Bound virions are endocytosed, with acidification of the endosome allowing furin-mediated cleavage of prM and subsequent virus fusion. The identification of this prM antibody-enhanced infection process has led researchers to postulate that this phenomenon may allow a degree of escape from the neutralizing host anti-E antibody response (reviewed by Rodenhuis-Zybert et al., 2011) and may inform the design of better vaccines (see below). The role that immature/partially mature DENV virions play during infection of Aedes spp. mosquito hosts has yet to be fully resolved.

Initial stages of virion assembly

Formation of the nucleocapsid

Assembly of progeny virions proceeds following initial replication of positive-strand flavivirus genomic RNA within replication complex (RC)-containing vesicle packets (VPs) derived from the ER (Fig. 2) (Roby et al., 2012). The exact nature of nucleocapsid assembly remains elusive, as the nucleocapsids themselves display no inherent order to their structure (Kiernayr et al., 2004; Kuhn et al., 2002; López et al., 2009; Zhang et al., 2007b) and a physical interaction of the nucleocapsid with the envelope is not observed. In accordance, the production of empty prM/E particles during virus infection (Lindenbach et al., 2007; Murray et al., 2008) and when these genes are expressed in the absence of C (Allison et al., 1995, 2003; Konishi & Mason, 1993; Lorenz et al., 2003; Wang et al., 2009) suggests that prM/E formation and nucleocapsid assembly are independent processes. However, the efficiency of C dimerization impacts on the overall integrity of the assembled particle in a thermal stability assay (Patkar et al., 2007), suggesting that some interaction between C and prM/E does still occur in the virion. Recent evidence describing the ability of DENV C to act as a histone mimic and cause nucleic acid condensation (Colpitts et al., 2011) supports the hypothesis that the flavivirus nucleocapsid is not an ordered icosahedral shell surrounding the genome but rather the RNA condenses around C dimers.

Determination of the precise structure of the flavivirus nucleocapsid remains elusive despite documentation of assembly of spherical nucleocapsid-like particles in vitro following incubation of recombinant C with nucleic acids (Kiernayr et al., 2004; López et al., 2009; Teoh et al., 2014), as well as the visualization of purported nucleocapsids adjacent to VP pores in electron micrographs of DENV-infected cells (Wesch et al., 2009). Regardless of the nature of flavivirus nucleocapsids, their assembly appears to be dependent on the association of C with the ER membrane and lipid droplets, presumably to optimally position the protein between the VP pore where nascent genomic RNA
exists and the ER sites of prM/E envelope generation (Samsa et al., 2009; Welsch et al., 2009; Westaway et al., 1997).

Heterodimerization of prM and E

Heterodimerization between the flavivirus prM and E proteins is an important first step in the generation of the envelope, with prM purported to play a role in the chaperone-mediated folding of E (Konishi & Mason, 1993; Lorenz et al., 2002). Heterodimerization may facilitate the arrangement of prM/E at the ER membrane forming heterotrimeric spikes (Zhang et al., 2007a). In turn, the ordered arrangement of these prM/E spikes may induce curvature of the ER membrane, resulting in budding of nascent virions into the ER lumen (Zhang et al., 2013a).

The prM protein is believed to primarily mediate this heterodimerization, as it folds more rapidly than E and is required to be present for E to achieve complete antigenic conformation (Lorenz et al., 2002). As such, detailed investigations have been undertaken to determine specific residues or domains in prM that may be responsible for efficient assembly and secretion of virus particles. A conserved 9 aa region was identified within the prM protein corresponding to residues 62–70 of TBEV and 61–69 of JEV prM (Yoshii et al., 2012). This region (which is located adjacent to the fusion loop of E in heterotrimeric spikes) was deemed critical for the assembly and secretion of prM/E particles from both TBEV and JEV (Yoshii et al., 2012). The GXXXG motif within the prM protein of JEV (corresponding to residues 142–146 within the first transmembrane domain) was also deemed to be important for prM and E interaction, as alanine insertion mutants displayed reduced heterodimerization and prM/E particle secretion (Lin et al., 2010).

The role of the DENV prM-H domain in particle formation has also been revealed in two thorough proline/alanine mutation studies (Hsieh et al., 2011, 2014). These investigations identified residues S112, E114, W117, A120, V123, E124, W126, I127, L128 and R129 as strongly influencing the assembly of virus particles, with substitutions at M111, E114, G115, W117, V123, E124, W126, I127, L128 and R129 also impairing particle infectivity. Interestingly, only E114 in DENV-1 was also demonstrated to be crucial for heterodimeric interactions between prM and E (Hsieh et al., 2014), indicating that the other residues may play a greater role in influencing the conformational rearrangement of structural proteins during virion maturation (Zhang et al., 2012). This hypothesis is particularly supported by recent evidence linking residue E125 in the JEV prM-H domain (corresponding to DENV E124) in electrostatic interactions with the K93 and H246 residues in E protein DII (Peng & Wu, 2014). The flexible linker between prM-H and the first transmembrane domain in DENV also appears to play a similar role in the secretion of virus particles, as mutation of H130 to a non-polar amino acid greatly reduces the recovery of either infectious virions or prM/E particles from culture supernatants (Pryor et al., 2004).

ER retention signals have been identified by mutation analysis of the transmembrane segments of YFV prM protein and were proposed to be important for prM/E interaction, particle formation and secretion (Ciczora et al., 2010). Thus, as with the 9 aa motif, the makeup of the transmembrane regions of prM may influence heterodimerization and hence virion formation in a manner broadly applicable to diverse flaviviruses. Additionally, a series of recent investigations (Calvert et al., 2012; Setoh et al., 2012; Tan et al., 2009) have identified two regions in WNV prM (residues 20–31 and 72–78) that have been proposed to be important for the overall structural stability and function of prM, and thus are likely to influence its interactions with E.

Heterodimers of prM and E are believed to coalesce at ER membranes directly opposed to the sites of RNA replication within the induced membranes of the VP (Roby et al., 2012). Although this theory has been supported by immunofluorescence assays (Lorenz et al., 2003; Mackenzie & Westaway, 2001), the exact conformation of the prM/E heterodimers (i.e. trimeric spikes, as in immature virions) at this site has yet to be ascertained.

Virion budding into the ER lumen

The ER membrane of infected cells is believed to support the translation of the flavivirus polyprotein, heterodimerization of newly formed prM and E, and formation of virions and prM/E particles by budding into the lumen (Roby et al., 2012; Welsch et al., 2009). Although this is the classical model of flavivirus assembly, it may not be universal, as at least one report concerning WNV-Sarafend has demonstrated unusual virion budding at the plasma membrane (Ng et al., 1994). Although visualization of these discrete events on the ER is often limited by their rapid kinetics, co-localization experiments with ER markers have demonstrated prM and E residing at this organelle by immunofluorescence microscopy (Lorenz et al., 2003; Mackenzie & Westaway, 2001) and virion accumulation in ER cisternae by immunogold electron microscopy (Mackenzie & Westaway, 2001; Welsch et al., 2009). Recent investigations utilizing electron tomography have even purported to demonstrate direct visualization of flavivirus budding into the ER lumen at membrane sites directly opposite the pores of VPs (Junhon et al., 2014; Welsch et al., 2009).

Particle trafficking and secretion

After completion of the viral budding process, an immature particle pf about 60 Å in diameter is formed in the lumen of the ER and consists of 60 prM/E heterotrimeric spikes arranged with icosahedral symmetry (Zhang et al., 2004, 2007a) (Fig. 4). Virions that have accumulated in the ER cisternae are transported to the Golgi apparatus in individual vesicles for glycan maturation (Mackenzie & Westaway, 2001; Welsch et al., 2009). Transition from the
ER through to the trans-Golgi compartment involves a drop in pH from 7.2 to 6.0, which induces a structural rearrangement from 60 prM/E heterotrimeric spikes to 90 prM/E heterodimers arranged in a herringbone pattern that is flattened parallel to the virion (Li et al., 2008; Zhang et al., 2003), a process that is reversible prior to furin cleavage (Yu et al., 2008) (Fig. 4). As detailed previously, host furin-mediated cleavage of prM (to a greater or lesser extent) occurs in acidified compartments of the trans-Golgi complex, with liberated pr peptide remaining associated with the transiting virion or prM/E particle (Yu et al., 2009). Upon release of virus particles from endosomes, the neutral pH triggers the dissociation of the pr peptide, forming an infectious mature virus particle of roughly 500 Å in diameter, with 90 E homodimers on the surface of the particle (Yu et al., 2008) (Fig. 4).

Glycan modification

Flaviviruses secrete three discrete glycoproteins from infected cells: prM (Calvert et al., 2012; Goto et al., 2005; Kim et al., 2008), E (Beasley et al., 2005; Goto et al., 2005; Hanna et al., 2005; Lee et al., 2010; Li et al., 2006; Mondotte et al., 2007; Scherret et al., 2001; Setoh et al., 2012; Winkelk et al., 1987) and NS1 (Muller & Young, 2012; Mylaira et al., 1996; Somnuk et al., 2011). Asn-linked carbohydrate modifications are always present and are relatively conserved in prM and NS1 with one to three modifications in the pr portion of prM (believed to be important in protecting the fusion peptide in E during virion egress) (Goto et al., 2005; Hanna et al., 2005; Kim et al., 2008; Li et al., 2008; Mandl et al., 1988; von Lindern et al., 2006), and modification at two to three residues in NS1 (important for multimerization of soluble NS1) (Muller & Young, 2012; Mylaira et al., 1996; Somnuk et al., 2011). Glycosylation of the E protein is more variable, usually occurring at residues 153/154 and at residue 67 in the four serotypes of DENV (Bryant et al., 2007; Lee et al., 2010). However, some strains of WNV (Beasley et al., 2005; Botha et al., 2008; Shirato et al., 2004b; Wengler et al., 1985), including KUNV (Adams et al., 1995), as well as strains of St Louis encephalitis virus (SLEV) (Vorndam et al., 1993), Alfiv virus (May et al., 2006) and YFV-17D (Post et al., 1992) lack any active carbohydrate modification in E. Therefore, such glycosylation is not absolutely required for flavivirus replication.

The significance of prM glycosylation in flaviviruses appears to be twofold, impacting on both particle assembly and release. Glycosylation of the prM protein (at residue N15 in WNV) appears to be absolutely required for authentic virion assembly. Removal of the N-linked glycosylation site from the prM protein may result in a complete misfolding of the E protein and a failure to form prM/E particles efficiently (Goto et al., 2005; Hanna et al., 2005; Kim et al., 2008; Zai et al., 2013). A T20D mutation in WNV prM was also shown to reduce (but not completely prevent) glycosylation of prM, and secretion of prM/E particles via ectopic expression of a mutant prME gene cassette was severely reduced (Calvert et al., 2012). However, the specific influence conferred on this phenotype by either the incomplete loss of glycosylation or the nature of the amino acid substitution remains to be resolved.

In the context of live virus infection, the loss of prM glycosylation in JEV led to an approximately 20-fold reduction in the production of extracellular virions, which had protein compositions and infectivities nearly identical to those of WT virions. This reduction appears to have occurred at the stage of virus release, rather than assembly per se (Kim et al., 2008). Removal of the prM glycosylation site in a lineage I or II strain of WNV also significantly decreased prM/E particle release (97–99% reduction compared with WT) (Hanna et al., 2005). In a prM glycosylation-deficient TBEV mutant, the level of secreted E protein was reduced to 60% of the WT level. On the other hand, in E or prM/E protein glycosylation-deficient mutants, the level of secreted E protein was reduced to 10% of the WT level (Goto et al., 2003). Thus, it appears that prM glycosylation may be more significant in regard to particle secretion than glycosylation of the E protein.

Glycosylation at the E protein of flaviviruses is of particular interest due to the variety of roles such a post-translational modification appears to play in the flavivirus life cycle, and due to the repeated isolation of viruses lacking such glycosylation at all. E protein glycosylation has been linked to increased virulence in mammalian and avian models of infection (Beasley et al., 2005; Brault et al., 2011; Kariwa et al., 2013; Murata et al., 2010; Shirato et al., 2004b; Totani et al., 2011) and efficient transmission by mosquitoes (Moudy et al., 2009). Glycosylation at the E protein is proposed to potentiate these virulence phenotypes by facilitating receptor-mediated virus entry (Davis et al., 2006a, b; Martina et al., 2008; Mondotte et al., 2007; Pokidysheva et al., 2006; Tassaneithrip et al., 2003), a greater efficiency of particle assembly and egress (Goto et al., 2005; Hanna et al., 2005; Lee et al., 2010; Li et al., 2006; Scherret et al., 2001), increased pH stability (Beasley et al., 2005; Guirakhoo et al., 1993; Lee et al., 1997) and possibly the concealment of immunogenic epitopes (Zhang et al., 2011).

Two of the best-characterized receptors for flaviviruses rely almost solely on glycosylation of the E protein for interaction (and prM in immature/partially mature virions): the type II tetrameric transmembrane proteins containing Ca²⁺-dependent carbohydrate recognition domains DC-SIGN and DC-SIGNR (Davis et al., 2006a, b; Fernandez-Garcia et al., 2009; Martina et al., 2008; Mondotte et al., 2007; Pokidysheva et al., 2006; Tassaneithrip et al., 2003). Dendritic cells and macrophages express DC-SIGN in humans, with homologous Ca²⁺-dependent (C-type) lectin receptors (CLRs) in mice displaying distinct patterns of expression (Koppel et al., 2005; Kwan et al., 2008; Navarro-Sanchez et al., 2003; Tassaneithrip et al., 2003). This lectin receptor recognizes high-mannose glycans and
fructose-containing Lewis antigens (Koppel et al., 2005) and has been demonstrated as a primary cellular attachment moiety for DENV and WNV (Davis et al., 2006a, b; Kwan et al., 2008; Lozach et al., 2005; Martina et al., 2008; Navarro-Sanchez et al., 2003; Tassaneentithep et al., 2003; Wang et al., 2011). Polymorphisms within the promoter encoding DC-SIGN have also been associated with severe TBEV infection in humans (Barkhash et al., 2012). Cryo-electron microscopy reconstructions of DENV E bound to the dependent carbohydrate recognition domain of DC-SIGN have demonstrated that the glycans attached to N67 in E protein rather than those on the flavivirus-conserved N153/4 residue are required for virus binding (Pokidysheva et al., 2006). This is supported by experiments that demonstrated increased WNV binding to DC-SIGN when the E protein was mutated to introduce glycosylation at residue 67 (Davis et al., 2006a). However, this may not represent the sole means for glycosylated flaviviruses to interact with DC-SIGN as it does not account for the observed impact of polymorphisms on TBEV infection (Barkhash et al., 2012) or the ability of WT glycosylated WNV to use it as a receptor (Davis et al., 2006b; Martina et al., 2008), as both viruses only contain the N154 glycan. It may be that high-mannose glycosylation of arthropod-derived flavivirus virions facilitates better DC-SIGN interaction with the N153/4 residue (Davis et al., 2006b; Navarro-Sanchez et al., 2003).

DC-SIGNR is a close homologue of DC-SIGN (77 % amino acid sequence identity) and is expressed on microvascular endothelial cells in the human liver, lymph nodes and placenta (Davis et al., 2006b; Koppel et al., 2005; Pöhılmışmann et al., 2001). Evidence from experimental WNV incubation with K562 cells overexpressing CLRs demonstrated a preferential usage of DC-SIGNR over DC-SIGN for attachment and infection (Davis et al., 2006b). Curiously, whilst the infection enhancement was strongest with mosquito cell-derived virions, mammalian cell-derived WNV still displayed a markedly increased infection of K562-DC-SIGNR cells compared with K562-DC-SIGN and control K562 cells. Thus, it seems that DC-SIGNR is able to bind vertebrate host-generated complex carbohydrates in addition to the high-mannose pathogen-associated molecular patterns (PAMPs) recognized by DC-SIGN. In addition, the observed preference for DC-SIGNR was maintained when infecting with a lineage II WNV deficient in E glycosylation, indicating that carbohydrates in the residual prM remaining uncleaved by furin are likely to take an active part in this interaction.

It should be noted that experiments with DENV have demonstrated a heterologous degree of glycan maturation that may be dependent on the cell type used to grow virus stocks, as well as, possibly, the degree of prM protein cleavage (Dejnirattisai et al., 2011). The authors demonstrated that DENV grown in insect (C6/36), immortalized mammalian (Vero) and primary mammalian cells (monocyte-derived dendritic cells) contained E glycans with different degrees of complexity (insect-derived virus was entirely high mannose, DC-derived virus contained only complex glycans, and Vero-derived virus was intermediate between the two states). Viruses derived from insect and immortalized mammalian cells were able to utilize DC-SIGN as a receptor; however, DENV grown in primary cells and containing solely complex glycans could only bind to the liver/lymph node-associated related CLR, L-SIGN. This phenotype may, however, only be DENV-specific, as WNV grown in Vero cells has been demonstrated solely to contain complex glycosylation of the E protein as demonstrated by a lack of sensitivity to endoglycosidase H (endoH), which specifically recognizes glycans containing three or more terminal mannose residues (Hanna et al., 2005). Ectopic expression of prM/E particles of WNV in HEK-293T cells and DENV-2 in COS cells has also demonstrated insensitivity to endoH digestion and thus complete maturation to complex glycans (Hanna et al., 2005; Pryor et al., 2004).

Interestingly, the work of Dejnirattisai et al. (2011) also revealed that utilization of both of the glycosylation sites in DENV-2 E only occurs in approximately 50 % of cases, as demonstrated by a faster-migrating band in immunoblots of untreated WT samples corresponding to E protein with glycosylation at only a single residue. A previous study investigating all four serotypes of DENV failed to show the same heterogeneous population of singly and doubly glycosylated E in untreated DENV-2 samples (Hacker et al., 2009). This report did, however, reveal that, for viruses grown in Vero cells, endoH digestion of DENV-1,-3 and -4 E protein removed glycosylation at one site only, whereas endoH digestion of DENV-2 resulted in a mixed population of E protein with no glycosylation or glycosylation at one site. Thus, it appears that the N67 and N153 glycosylation sites in DENV E are utilized to differing degrees, and the glycans positioned at each site are differentially matured. Considering that structural studies of DENV prM and E have demonstrated partial occlusion of one or both E glycans by the pr peptide (Li et al., 2008; Yu et al., 2008), and that DENV has a demonstrated tendency towards incomplete prM cleavage by furin (Rodenhuis-Zybert et al., 2011), these results raise the interesting possibility that the degree of prM processing controls the maturation status of one or both glycans. Such a hypothesis remains to be properly explored, although it would be intriguing to investigate whether partially matured DENV displays a greater capacity to infect DC-SIGN-expressing cells.

Evidence is also emerging that CLRs recognizing glycosylated flaviviruses may play a role in the infection of vector mosquito species (Arjona et al., 2011; Cheng et al., 2010). In both Aedes and Culex mosquitoes, the soluble galactose-specific lectin mosGCTL-1 binds to glycosylated WNV (but apparently not to DENV) in the haemolymph and then coordinates with a cell-surface CD45 phosphatase homologue mosPTP-1 to facilitate virus entry (Cheng et al., 2010). As an evolutionary strategy, the use of CLRs as receptors for glycosylated flaviviruses appears to be a trade-off. Although the viruses gain the use of a subset of otherwise unavailable attachment molecules, they risk potentially triggering host lectin-based innate immunity such as activation of the com-
plemem cascade through mannos-binding lectin (Fuchs et al., 2010, 2011) or inflammasome activation and cytokine stimulation through CLEC5A (Chen et al., 2008, 2012; Fernandez-Garcia et al., 2009; Watson et al., 2011; Wu et al., 2013).

In contrast to the benefit to CLR-mediated infection conferred by E glycosylation, several groups have reported that virion or replicon-containing virus-like particle (VLP) infectivity is reduced in vertebrate and especially insect cell culture when glycosylation is present. Infection of mammalian, avian and mosquito cells with lineages I and II WNV replicon VLPs or lineage II WNV either containing glycosylation motifs or deficient in these sites on prM and E demonstrated that loss of glycosylation in E was beneficial for particle infectivity, and that this phenotype was most pronounced in mosquito C6/36 cells (Hanna et al., 2005). This observation has since been extended to KUNV, MVEV and DENV, with E protein glycosylation-null mutants of each of these viruses displaying less than a twofold difference in infectivity compared with the glycosylated E viruses in most mammalian cells, whereas infectivity in C6/36 cells was greatly enhanced by the lack of E glycosylation (Allen et al., 2012; Lee et al., 2010). It should be noted, however, that one study has presented conflicting results demonstrating that DENV-2 with an N153Q mutation removing E glycosylation has reduced infectivity in mammalian BHK cells (Mondotte et al., 2007), although this may in fact be due to the specific mutation (N→Q) rather than the loss of glycosylation per se. Indeed, a similar N→Q mutation that eliminated glycosylation of TBEV E (Yoshii et al., 2013) appeared to demonstrate misfolding of E protein secreted from mammalian cells. Although specific infectivity was not measured, the authors attributed this misfolding of E to the attenuated phenotype observed in mammalian cells (but not observed in tick cells). Additionally, an N→D mutation within a selected DENV-2 mutant led to less than a twofold difference in infectivity of BHK cells but demonstrated a severe reduction in infectivity of DC-SIGN- and L-SIGN-expressing Raji cells (Allen et al., 2012). This general loss of glycosylation/enhanced infectivity phenotype in cells that are not positive for CLR expression may be due in part to the observation that non-glycosylated flaviviruses are more labile at higher pH. DENV-2 and -3 lacking E glycosylation at N153 are able to fuse with C6/36 cells at a higher pH than glycosylated forms (Guirakhoo et al., 1993; Lee et al., 1997), and WNV with an N154S mutation in E is more sensitive to low-pH inactivation (presumably through fusogenic E rearrangement) than WT virus (Beasley et al., 2005).

Another important biological outcome of E protein glycosylation is an increase in the rate of flavivirus particle assembly and secretion, and the resulting enhancement of virus replication. Investigations utilizing ectopic expression of WNV and TBEV prM and E proteins have ascertained that glycosylation of the E protein leads to a more rapid and efficient secretion of prM/E particles (Goto et al., 2005; Hanna et al., 2005). This enhanced secretion is observed with live DENV (Bryant et al., 2007; Lee et al., 2010) and WNV (Beasley et al., 2005; Li et al., 2006; Scherret et al., 2001) and has recently been demonstrated by our group using non-infectious C-deleted WNV replicons (Roby et al., 2013). However, an investigation utilizing live TBEV failed to demonstrate a difference in the degree of E secretion between glycosylation-mutant and WT viruses in both mammalian and tick cells (Yoshii et al., 2013).

Glycosylated flaviviruses have in some cases been demonstrated to display enhanced virulence in animal models. With regard to mammalian virulence, E glycosylation has been demonstrated to be important for WNV (Beasley et al., 2005; Shirato et al., 2004b) and MVEV (Prow et al., 2011) peripheral replication and neuroinvasion in mice. WNV strains lacking glycosylation have also been demonstrated to be attenuated for replication in birds (Murata et al., 2010; Totani et al., 2011) and in Culex mosquitoes (Moudy et al., 2009). This last point at first appears counterintuitive, considering the apparent severe detriment to mosquito cell infectivity conferred by WNV E glycosylation (Hanna et al., 2005). Perhaps this is merely a reflection of differential selective pressures imposed by the mosquito midgut infection barrier (ability for initial infection to occur, probably reliant on virion infectivity) and midgut escape barrier (ability for virus to disseminate from the midgut, possibly reliant on degree of virion secretion) ( Olson & Blair, 2012). This is supported by the observation that there was no apparent change in the replication of a DENV-2 N67Q mutant compared with the WT virus when the viruses were inoculated intrathoracically into Aedes mosquitoes (Bryant et al., 2007). Glycosylation of the E protein, however, was shown to be non-essential for virulence, as strains of SLEV (Month et al., 1980) and WNV (Shirato et al., 2004a) lacking E glycosylation are not severely attenuated in mice.

It is plausible that E glycosylation may represent an evolutionary strategy that assists viruses in expanding into new territories. Increased virion secretion leads to higher viraemias in vertebrate hosts (Beasley et al., 2005; Murata et al., 2010; Shirato et al., 2004b; Totani et al., 2011) and thus may improve the likelihood that novel vector species become exposed to and infected with the virus. This may explain why North American (emergent) WNV is predominantly glycosylated (Beasley et al., 2005; Charrel et al., 2003), whereas isolates of WNV (including KUNV) that circulate in the Old World (with long-established vector–host relationships) have been demonstrated to lack E glycosylation (Adams et al., 1995; Botha et al., 2008).

It is important, therefore, to note that, as the emergent and most virulent strains of WNV display glycosylation at N156 of E, vaccines that are produced to target these viruses should probably also be glycosylated to ensure an appropriate immune response. This statement does not at first appear justified by the literature. Reports have demonstrated that aspects of the antigenic profile of TBEV E
protein are quite stable when Asn-linked carbohydrates are removed (Winkler et al., 1987) and that removal of glycosylation may enhance JEV E immunogenicity (Zhang et al., 2004). However, E glycosylation still appears to be important to generate an authentic immune response against virulent WNV in horses, as the carbohydrate modification has been demonstrated to be crucial for the correct presentation of an antibody-inducing epitope (Hobson-Peters et al., 2008).

### Post-translational modification of structural proteins in vaccine design

A greater understanding of the degree of post-translational modification enacted upon flavivirus structural proteins during the course of an infection has led to increasing exploitation of such events in the development of novel vaccines (Table 1). Three primary aspects of post-translational modification have been targeted: (i) the co-ordinated cleavage at the C–prM junction; (ii) furin cleavage maturation of prM; and (iii) glycosylation of the E protein.

C-deleted flavivirus genomes have emerged as an attractive approach for the design of vaccines against flaviviruses (Mandl, 2004; Roby et al., 2011). They rely on the effective production and secretion of prM/E particles from self-replicating RNA in the absence of genome packaging from functional C. Thus, in essence, each of these constructs relies on co-ordinated C–prM cleavage, engineering the C-deletion to maintain efficient liberation of the truncated C by NS2B/3 and thus proper prM processing (although these events have not been examined directly). The latest iterations of these vaccines developed in our group also provide a functional C gene in trans in order to allow packaging of nucleocapsids into particles for a single round of infection (Roby et al., 2011). Rationally, extension of the trans-C gene to include the prM signal sequence should target its translation to the ER (the proposed site of polyprotein translation and virus particle formation). Thus, recent investigations by our and other groups have built on the discoveries of Lobigs et al. (2010) to create constructs in which an anchored form of C should still be cleavable by NS2B/3 in the absence of downstream prM. Constructs with trans-C genes extended to either include the signal sequence and the first 10 codons of prM (Roby et al., 2014), or the entire sequence of the pr peptide (Pang et al., 2014), were generated and shown to be highly efficient at both infectious and non-infectious particle secretion, thus representing attractive candidates for future vaccine development.

Exploitation of the maturation state of prM has also been investigated in the development of better flavivirus vaccines. By purifying their prM/E particle vaccine using sucrose gradients, Ohtaki et al. (2010) found that they could separate fully mature, M-containing particles [fast-sedimenting VLPs (F-VLPs)] from immature, prM-containing particles [slow-sedimenting VLPs (S-VLPs)]. Subsequently, it was discovered that F-VLPs with fully matured M elicited a more robust immune response than S-VLPs with uncleaved prM. Thus, this simple purification step was able to improve the efficacy of their vaccine candidate. In addition to purification to remove uncleaved prM, furin cleavage has also been improved in a vaccine through the use of mutation (Keelapang et al., 2013). By introducing the E203A mutation into prM in their chimaeric DENV-1/2 live-attenuated vaccine (DENV-1 prM and E in a DENV-2 genomic background), Keelapang et al. (2013) were able to enhance furin cleavage and improve the immune response in vaccinated rhesus macaques. Such enhanced furin cleavage mutations may be particularly beneficial for DENV vaccines, as antibodies to prM have been demonstrated to lead to antibody-dependent enhancement of infection (Dejnirattisai

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<td>WNV SRIP-producing vaccine</td>
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et al., 2010; Luo et al., 2013; Rodenhuis-Zybert et al., 2010), and indeed the presence of prM as an antigen may prevent the development of an efficient neutralizing antibody response against DENV E (Rodenhuis-Zybert et al., 2011).

Glycosylation of the E protein has been demonstrated to be an effective means of enhancing particle secretion in flavivirus vaccines, thus increasing their potential to stimulate an immune response. This has been applied by our group to C-deleted KUNV-based vaccines that normally lack glycosylation at N154 (Roby et al., 2013, 2014) and by others to add DENV-like N66 glycosylation to a TBEV prM/E particle vaccine (Goto et al., 2005). Conversely, however, elimination of E protein glycosylation in a DNA-based JEV prM/E particle-producing vaccine led to the development of a more robust immune response in vaccinated mice (Zhang et al., 2011) and addition of E glycosylation to C-deleted KUNV-based vaccines did not appear to significantly enhance its immunogenicity (J. A. Roby, R. A. Hall & A. A. Khromykh, unpublished data). It appears that the glycosylation state of the E protein is a parameter worth investigating in the construction of all particle-secreting flavivirus vaccines due to these potentially opposing outcomes.

Conclusions

Flaviviruses employ various post-translational modifications on their structural proteins in order to regulate the formation and export of virus particles. Proteolytic cleavage of structural proteins is regulated temporally (as at the C-prM junction) and spatially (furin maturation of prM) in order to ensure efficient nucleocapsid incorporation into virus particles and to prevent fusion of the nascent virion during export. Conformationally fluid and pH-dependent interactions between the prM and E proteins may assist particle budding into the ER lumen and ensure the regulated cleavage activity of furin. The addition of branched glycans to prM and E proteins may regulate the kinetics of virion secretion, as well as allowing the utilization of lectin receptors on host cells. Mounting evidence also suggests that glycosylation of the E protein may also cause modulation of the host immune response, enhancing detection by lectin receptors, yet reducing host antibody production via an unknown mechanism.

Exploitation of these post-translational regulation events is beginning to be adopted in the development of new vaccines. Although these approaches have yet to reach the market, strategies to enhance the efficacy of trans-packaging C (applying lessons from co-ordinated C–prM cleavage), stimulate a greater antibody response (via reducing the proportion of uncleaved prM) and increase the secretion of immunogenic particles (by enhancing the glycosylation of E) are each beginning to show promise in their ability to boost the efficacy of vaccine candidates. In the design of future vaccine preparations, the application of these strategies should be greatly encouraged. The addition of a minor set of mutations, specifically aiming to exploit post-translational regulation of structural proteins, has the potential to vastly enhance vaccine efficacy.

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