

A STRUCTURAL PERSPECTIVE OF THE *FLAVIVIRUS* LIFE CYCLE

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Abstract | Dengue, Japanese encephalitis, West Nile and yellow fever belong to the *Flavivirus* genus, which is a member of the *Flaviviridae* family. They are human pathogens that cause large epidemics and tens of thousands of deaths annually in many parts of the world. The structural organization of these viruses and their associated structural proteins has provided insight into the molecular transitions that occur during the viral life cycle, such as assembly, budding, maturation and fusion. This review focuses mainly on structural studies of dengue virus.

The *Flaviviridae* is a large family of viral pathogens responsible for causing severe disease and mortality in humans and animals. The family consists of three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. This review will focus on the largest of the three, the *Flavivirus* genus, which contains more than 70 viruses including dengue virus, Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV) and yellow fever virus (YFV). Within the genus, the viruses can be further subdivided into antigenic complexes according to serological criteria, or into clusters, clades and species on the basis of molecular phylogenetics^{1,2} (FIG. 1).

The name ‘flavivirus’ is derived from the Latin word *flavus* meaning yellow, signifying the jaundice caused by YFV. The flaviviruses are primarily transmitted by arthropods. Symptoms of flavivirus infection can range from mild fever and malaise, to fatal encephalitis and haemorrhagic fever. Dengue virus, which is transmitted by mosquitoes, is responsible for the highest rates of disease and mortality among members of the *Flavivirus* genus³. Global epidemics of dengue virus have occurred over the past few decades, in part due to decreased mosquito control efforts. More than 50 million cases of dengue virus are estimated to occur annually^{3–5}. Sequential infections by multiple serotypes of dengue virus can lead to dengue haemorrhagic fever, of which there are an estimated ~500,000 annual cases worldwide³. WNV has emerged in temperate regions of Europe and North America. In 2002, more than 4,100 people in the United States were infected with WNV⁶

and hundreds of humans and thousands of animals died. Vaccines are available that have been created from live-attenuated YFV and inactivated JEV and TBEV. However, disease resulting from these viruses is still prominent worldwide². The World Health Organization estimates that each year there are 200,000 cases of yellow fever, with approximately 30,000 deaths, worldwide⁷. In addition, more than 50,000 cases of Japanese encephalitis were reported in 2001 (REF. 8).

Flavivirus virions are approximately 500 Å in diameter and are composed of a single, positive-strand RNA genome that is packaged by virus capsid protein in a host-derived lipid bilayer and surrounded by 180 copies of two glycoproteins². The ~10.8-kb genome has one open reading frame encoding a single polyprotein. The amino terminus of the genome encodes three structural proteins — capsid, membrane (M, which is expressed as prM, the precursor to M) and envelope (E) — that constitute the virus particle. Seven non-structural proteins that are essential for viral replication are encoded by the remainder of the genome². The capsid protein consists of ~120 amino acids and is involved with packaging of the viral genome and forming the nucleocapsid (NC) core². prM (~165 amino acids) and E (~495 amino acids) are glycoproteins, each of which contains two transmembrane helices. Before it is cleaved during particle maturation to yield the pr peptide and the M protein (~75 amino acids), the prM protein might function as a chaperone for folding and assembly of the E protein⁹. The E protein contains a cellular receptor-binding site(s) and a fusion peptide^{9–11}.

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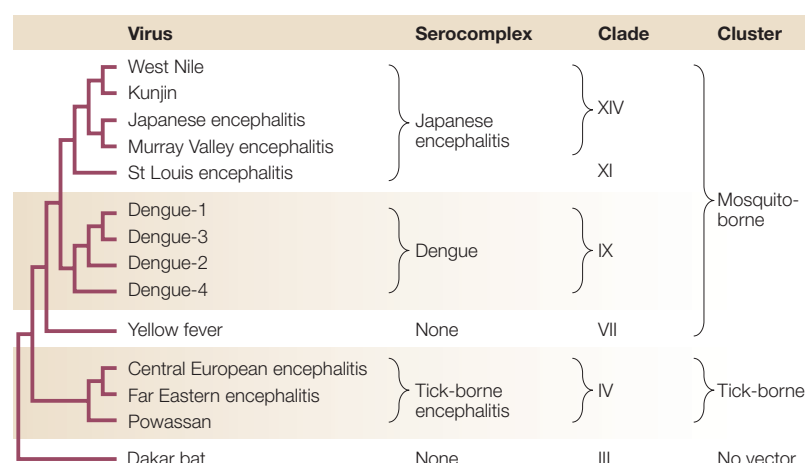
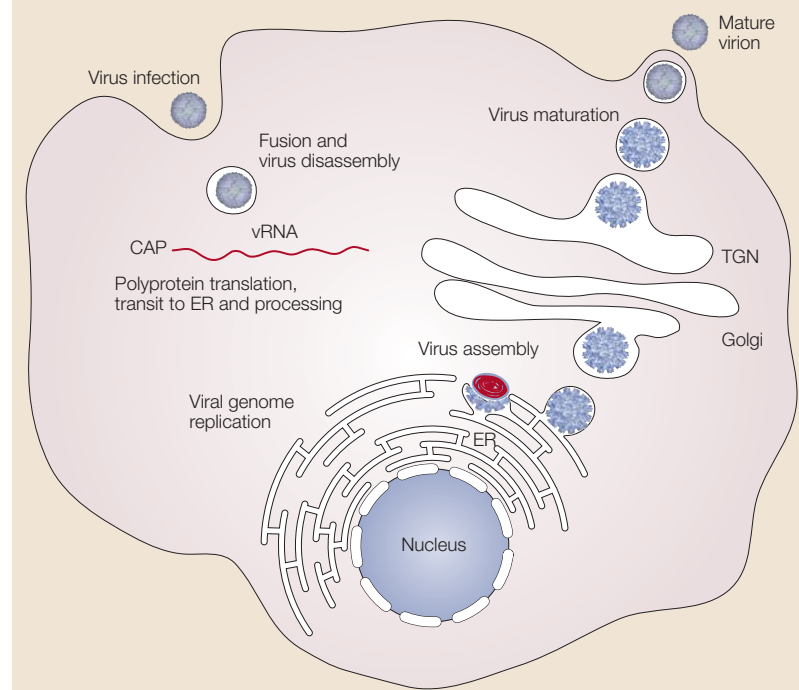


Figure 1 | **Flavivirus classification.** The relationships between selected flaviviruses are shown in the dendrogram on the left. Evolutionary distance is not represented in this figure. The serological (serocomplex) and phylogenetical (clade and cluster) classifications of these flaviviruses are shown on the right. Reproduced with permission from REF. 2 © (2002) Lippincott Williams and Wilkins.

Box 1 | Flavivirus life cycle

Virions attach to the surface of a host cell and subsequently enter the cell by receptor-mediated endocytosis (see the figure). Several primary receptors and low-affinity co-receptors for flaviviruses have been identified (as described in this review). Acidification of the endosomal vesicle triggers conformational changes in the virion, fusion of the viral and cell membranes, and particle disassembly. Once the genome is released into the cytoplasm, the positive-sense RNA is translated into a single polyprotein that is processed co- and post-translationally by viral and host proteases. Genome replication occurs on intracellular membranes. Virus assembly occurs on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA buds into the lumen of the ER. The resultant non-infectious, immature viral and subviral particles are transported through the trans-Golgi network (TGN). The immature virion particles are cleaved by the host protease furin, resulting in mature, infectious particles. Subviral particles are also cleaved by furin. Mature virions and subviral particles are subsequently released by exocytosis.



Flaviviruses enter host cells by receptor-mediated endocytosis (BOX 1). The acidic environment of the endosome triggers an irreversible trimerization of the E protein that results in fusion of the viral and cell membranes¹². Fusion has not been observed at the plasma membrane¹³. After fusion has occurred, the NC is released into the cytoplasm, the capsid protein and RNA dissociate, and replication of the RNA genome and particle assembly is initiated^{2,14,15}. Initially, immature particles are formed in the lumen of the endoplasmic reticulum (ER). These particles, which contain E and prM proteins, lipid membrane and NC, cannot induce host-cell fusion, making them non-infectious^{16,17}. Subsequently, cleavage of prM occurs in the trans-Golgi network, which creates mature, infectious particles^{10,18}. Subviral particles are also produced in the ER, but only contain the glycoproteins and membrane, and lack capsid protein and genomic RNA, making these particles non-infectious¹⁹. Mature virus and subviral particles are released from the host cell by exocytosis.

Advances in cryo-electron microscopy (cryoEM) have provided important insights into the structures of enveloped viruses^{20–22}. In addition, many of the structural proteins of these viruses have been examined at atomic resolution^{23–29}. This has allowed atomic resolution structures to be fitted into cryoEM density maps, resulting in ‘pseudo-atomic’ structures of enveloped viruses. By analysing different intermediates in the virus assembly and entry pathways, these dynamic processes can be understood at a molecular level. This review will focus on the structural aspects of flavivirus assembly, maturation and fusion.

Processing of structural proteins

During translation of the polyprotein, the structural proteins are translocated and anchored in the ER by various signal sequences and membrane anchor domains (FIG. 2). The capsid protein contains a hydrophobic signal sequence at its carboxyl terminus that translocates prM into the lumen of the ER from its site of synthesis on the surface of the ER. The prM protein has two transmembrane-spanning domains, which contain a stop transfer sequence and a signal sequence. As a result, the E protein is also translocated into the lumen of the ER (FIG. 2). After the appropriate proteolytic cleavages, the capsid protein and viral RNA are localized in the cytoplasm and the capsid protein remains associated with the ER membrane. On the luminal side of the ER, the prM and E proteins form a stable heterodimer within a few minutes of translation^{9,30,31}.

Envelope protein

Structure of E. The X-ray crystallographic structures of the ECTODOMAINS (residues 1–395) of TBEV E and dengue-2 E proteins (FIG. 3) have been determined^{24–26}. Both structures are similar, consisting of a dimer in which each monomer has three β -barrel domains. The central structural domain (domain I) contains the N-terminus and is flanked on one side by an elongated dimerization domain (domain II), which contains the fusion peptide at its distal end. On the other side of

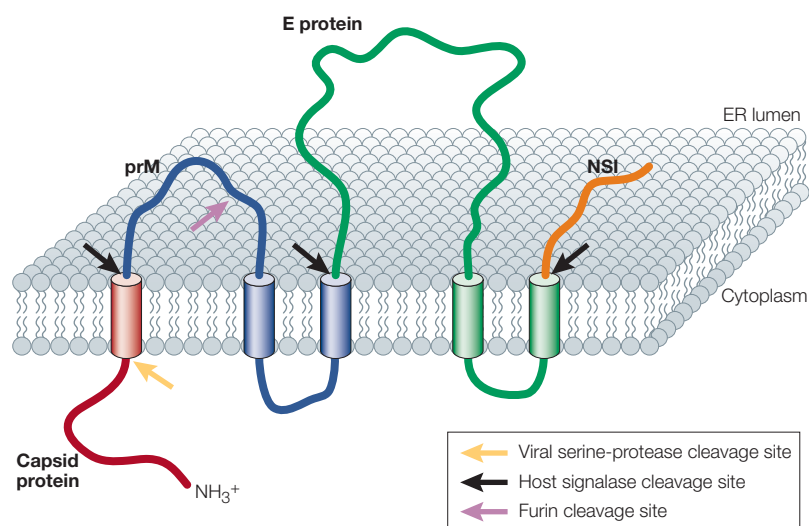


Figure 2 | Membrane topology of the flavivirus structural proteins. The predicted orientation of the structural proteins across the endoplasmic reticulum (ER) membrane is shown. Transmembrane helices are indicated by cylinders, arrows indicate the sites of post-translational cleavage and the cleavage sites of specific enzymes are indicated by different colours. E, envelope; NSI, non-structural protein 1; prM, precursor to membrane protein.

domain I is domain III, which is an immunoglobulin (Ig)-like domain that is thought to contain the putative receptor-binding sites^{32–37}. Domains I and II are connected by four polypeptide chains, whereas domains I and III are connected by a single polypeptide linker. The fusion peptide of one E monomer is buried between domains I and III of the adjacent monomer within a dimer. The angle between domains I and II differs in the three E crystal structures^{24–26} (FIG. 3). Furthermore, one of the crystal structures of dengue-2 E protein revealed a molecule of *N*-octyl- β -D-glucoside to be positioned at the domain I–II interface²⁴. This flexibility might be required to facilitate the large conformational changes the virus undergoes during maturation and fusion (see below). The NMR structure of domain III of the **JEV E protein** has also been determined³⁸ and is similar to the crystallographic structures of the TBEV and dengue-2 E proteins.

Entry and the E protein. There is ~40% amino acid identity among the flavivirus E proteins. The number and position of potentially glycosylated residues is not conserved among different strains of the same virus and it has been suggested that carbohydrate moieties on the virus surface might modulate specificity of receptor binding³⁹. The glycosylated amino acids map onto the E dimer structure spatially near domain III^{21,24–26,40}. Furthermore, viruses belonging to the Japanese encephalitis serocomplex (FIG. 1) have five extra amino acids in domain I that form a surface loop near the domain I–III interface⁴⁰. The proximity of these structural changes might indicate that this surface region regulates the specificity of flavivirus binding to their different cellular receptors.

DC-SIGN (dendritic-cell-specific ICAM-grabbing non-integrin), GRP78/BiP (glucose-regulating protein

78), and CD14-associated molecules have been suggested as primary receptors for dengue virus^{39,41–43}. DC-SIGN is a mannose-specific lectin that is proposed to specifically interact with the carbohydrate residues on the dengue E protein³⁹. Several other cell surface proteins have been proposed to be receptors for dengue^{44–53}, WNV⁵⁴, JEV⁵⁵ and TBEV^{56,57}. In addition, heparin and other glycosaminoglycans are low-affinity co-receptors for several flaviviruses^{32,33,36,58–64}. Unlike other viruses⁶⁵, there is very little structural information about the mechanisms by which flaviviruses bind to their receptors. In mosquito-borne flaviviruses (such as YFV, JEV and WNV), domain III contains an RGD/RGE sequence, which is the recognition motif for integrin binding. However, alteration of this motif in YFV did not abolish virus–cell binding, which indicates that a non-integrin receptor might also be involved in cell recognition⁶⁶.

Fusion and the E protein. The E protein has two conserved helices in the ‘stem’ region (residues 398–420 and 426–448 in dengue-2), between the ectodomain and the transmembrane region^{21,67}. *In vitro* studies with TBEV have shown that either full-length E protein or the ectodomain of E, which contains the stem region, form dimers at neutral pH. On acidification, the dimers dissociate into monomers and then reassociate irreversibly to form trimers^{12,68,69}. However, a truncated E protein that does not contain the stem region requires both acidification and the presence of lipids to form trimers⁷⁰.

X-ray crystallographic structures of the E proteins at low pH (representing the post-fusion structure) showed a trimeric arrangement, with the long dimensions of the E proteins positioned approximately parallel to each other and with their fusion peptides exposed at the tip of the trimer^{71,72} (FIG. 3). The structures showed that the fusion peptides would penetrate only into the outer lipid leaflet of the host cell membrane. There is a major conformational change in the positioning of domain III, which folds back towards domain II by ~30 Å and rotates ~20°, so that it is no longer extended linearly with domains I and II. The C-terminus of the ectodomain of the E protein (residue 395) was found to be positioned at the end of a hydrophobic groove that extends along domain II and towards the fusion peptide. The stem helices were predicted to move into this groove, bringing the transmembrane region into close proximity with the host membrane.

The E1 protein in alphaviruses (examples include Sindbis virus, Semliki Forest virus and Venezuelan equine encephalitis virus) is structurally homologous to the E protein of flaviviruses in their pre- and post-fusion conformations^{27,73}, despite no significant sequence similarity between the E1 and E proteins. The distinctive three domain arrangement with an internal fusion peptide that is present in flavivirus and alphavirus fusion proteins constitutes a new class of membrane fusion — the class II fusion process. The similarities between the class I and class II fusion processes are discussed below.

ECTODOMAIN

The part of the protein that is exterior to the lipid membrane.

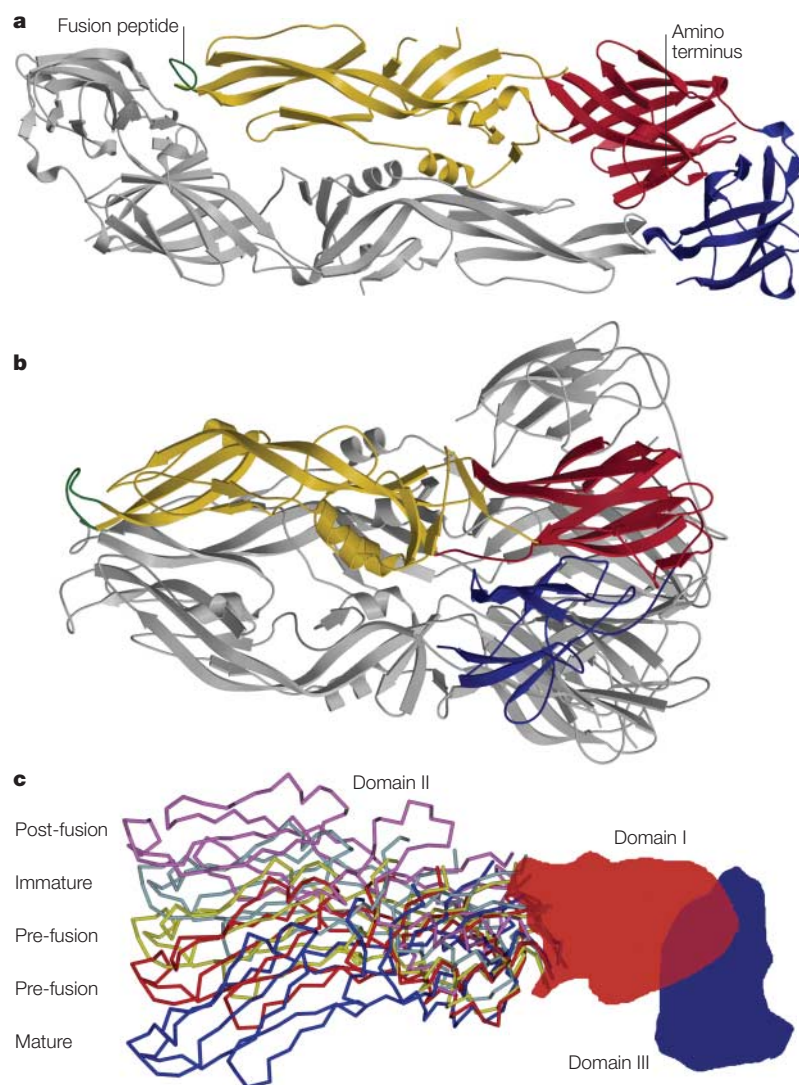


Figure 3 | Structure of the ectodomain of the E protein in pre- and post-fusion conformations. **a** | Dimeric, pre-fusion conformation of the dengue-2 E protein, residues 1–395 (REF. 26). In one monomer, domains I, II and III are coloured red, yellow and blue, respectively, and the fusion peptide is shown in green. The other monomer is coloured grey. **b** | Trimeric, post-fusion conformation of the dengue-2 E protein⁷². One monomer has the individual domains and fusion peptide coloured as in part **a**, whereas the other two monomers are coloured grey. **c** | E protein X-ray crystal structures showing the variation in the hinge angle between domains I and II. Domain II (C α backbone trace) of the post-fusion E protein structure is in purple⁷², the immature virus in grey²⁶, the dimeric, pre-fusion form in yellow²⁶ and red²⁴, and the protein in the mature virus in blue²¹. The common domain I and III structures are shown diagrammatically in red and blue. Part **c** is reproduced with permission from REF. 26 © (2004) Cell Press.

Capsid protein

Capsid protein structure. The amino acid identity between different flavivirus capsid proteins ranges from 15 to 90%. The NMR structure of the **dengue-2 capsid protein** and the X-ray crystal structure of **Kunjin capsid protein** show that both proteins form dimers. Dengue-2 and YFV capsid proteins readily form dimers in solution⁷⁴ and a dimer has been suggested to be the building block for NC assembly^{23,75}. The monomer in both structures has four helices (α 1– α 4) that are connected by short loops^{23,29}. Helices α 2 and α 4 of one monomer are anti-parallel to helices α 2 and α 4 of the neighbouring monomer, respectively^{23,29}.

Comparison of the structures of the dengue-2 and Kunjin capsid proteins shows some variability in the position of α 1 (residues 27–35 in dengue-2)^{23,29}. The X-ray crystal structure of the Kunjin capsid protein shows the surface to be mostly positively charged and consisting of helices α 1 and α 4. The cryoEM reconstructions of both mature and immature dengue virus show that in both structures the nucleocapsid lacks a well-formed protein shell. So, it is possible that the rather basic capsid protein functions like a histone²⁹. The N-terminal domains of many of the capsid proteins of other positive-strand RNA viruses have been shown to function in the same way^{76,77}. However, in the NMR structure of dengue-2 capsid protein, the position of α 1 has moved, exposing a hydrophobic surface created by α 2. It is possible, as suggested by Ma *et al.*, that the capsid protein facilitates binding of the NC to the lipid membrane^{23,78}.

Capsid protein and virus assembly. One of the earliest events in flavivirus assembly is the formation of the NC, which consists of one copy of the genomic RNA and multiple copies of the capsid protein. Unlike other enveloped viruses, NCs are rarely found in flavivirus-infected cells (although they can be assembled *in vitro*⁷⁵), which indicates that particle formation is a coordinated process between the membrane-associated capsid protein and the prM–E heterodimers in the ER.

Flavivirus capsid proteins contain a conserved hydrophobic region spanning helices α 2 and α 3. NCs isolated from TBEV particles tend to aggregate even in the presence of detergent, which indicates that the conserved hydrophobic region is exposed on the surface of the NC⁷⁵. When deletions in the hydrophobic regions in helices α 1 and α 2 were constructed, viable virus could still be recovered by second site revertants that mapped onto the dimerizing helix α 4. Virus could not be rescued when these deletions were extended into helix α 3 (REFS 78,79). These results indicate that the residues in the hydrophobic region of the capsid protein are not an absolute requirement for assembly^{78,79} and that the dimerization might be maintained by the α 4 helix.

Subviral particles

Subviral particles are routinely observed in flavivirus infections⁸⁰ and were first observed in virus-infected cell cultures. Subviral particles have a smooth exterior and most particles have an average diameter of 315 Å, although larger particles (~500 Å) have been observed^{81,82}. These particles are assembled in the ER and undergo the same post-translational modifications as infectious particles before being released by the host cell. The released particles only contain the E and M proteins and a lipid membrane. Recombinant subviral particles, also containing only E and M proteins and lipid, can be produced by co-expressing E and prM in mammalian cells^{31,81,82}, indicating that flavivirus particle formation is driven by the interactions between prM–E heterodimers. Although the organization of the E protein homodimer is different in subviral particles compared

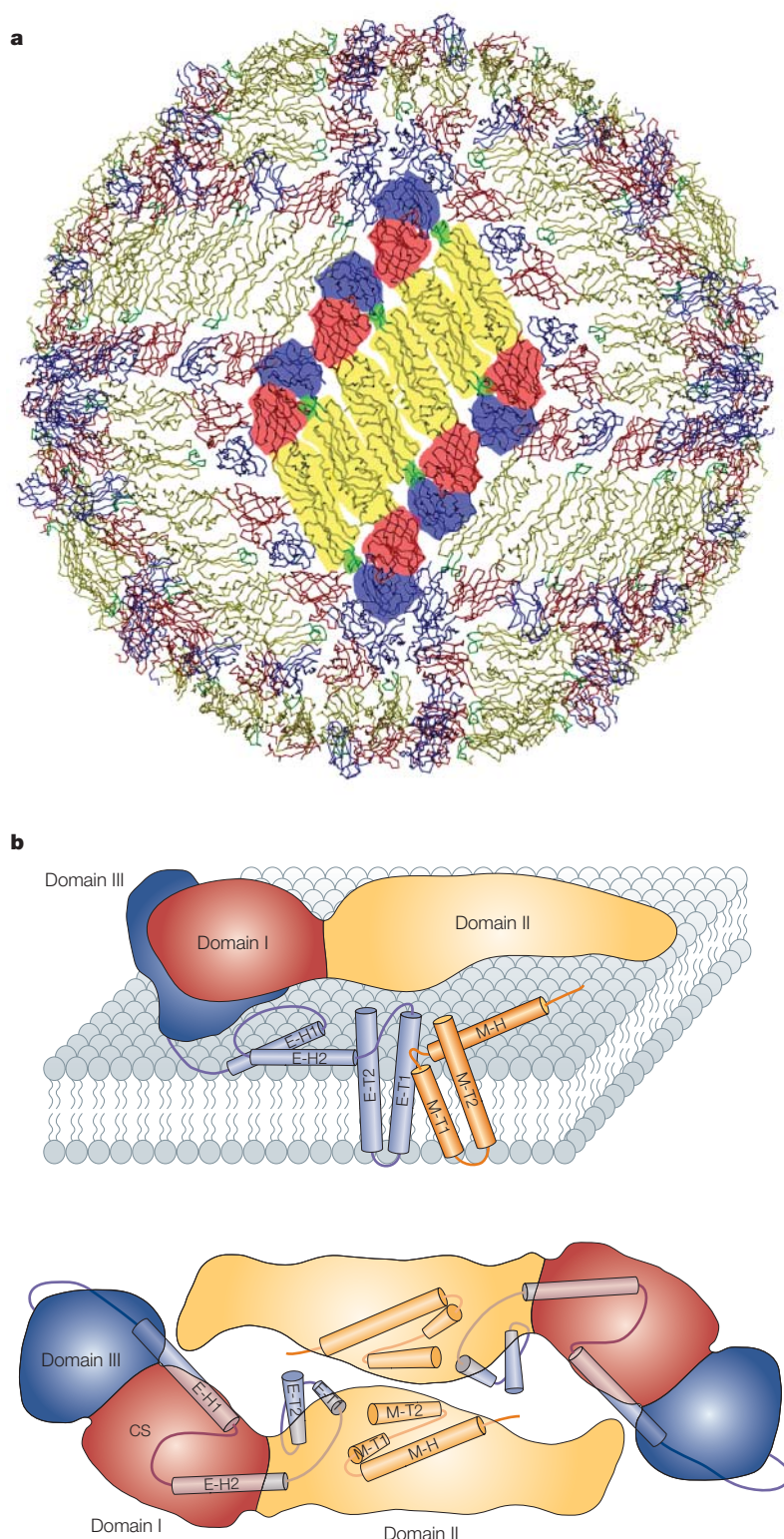


Figure 4 | Packing of the E proteins in mature flavivirus virions. **a** | One raft, consisting of three parallel dimers, is highlighted. Domains I, II and III are coloured red, yellow and blue, respectively. The fusion peptide is coloured green. Reproduced with permission from REF. 26 © (2004) Cell Press. **b** | Diagrams of the dengue virus E protein ectodomain and transmembrane domain, side and top views. The stem and transmembrane helices of the E (E-H1, E-H2, E-T1 and E-T2) and M (M-H, M-T1 and M-T2) proteins are shown in blue and orange, respectively. The conserved amino acid sequence of the region between the two E protein stem helices is marked CS. Reproduced with permission from REF. 21 © (2003) Nature Structural and Molecular Biology Macmillan Magazines Ltd.

with the mature virus (see below), these particles have similar haemagglutination and fusion activities to mature particles and can be used as an immunogen with protective capability^{13,83}.

Subviral particles containing prM and E proteins — as opposed to M and E proteins — can be produced by treating cells that are co-expressing both these glycoproteins with ammonium chloride, or by mutating the prM furin cleavage site so that it is not cleaved⁸². In either case, the ratio of larger diameter particles (500 Å) to smaller particles (315 Å) is increased^{81,82} compared with the ratio of particles that are formed after prM cleavage. The endoglycosidase digestion patterns of the smaller subviral particles and whole virions are similar, but differ from those of the larger subviral particles. So, the environment of the E protein in the larger subviral particles might be different to that of the E protein in the smaller subviral particles, making the glycoproteins inaccessible to cellular glycan-processing enzymes^{81,82}.

The first insights into whole flavivirus structures were using TBEV recombinant subviral particles⁸¹. Using cryoEM, these particles were shown to contain 30 copies of the E dimer arranged with icosahedral symmetry on the virion surface. Extrapolation of this $T=1$ (T denotes the TRIANGULATION NUMBER) structure to a larger $T=3$ structure produced a particle that is consistent with the size of mature virions, and was predicted to represent the structure of the mature particle. However, this structure is not that of the mature virus but possibly of an intermediate in the fusion process (see below).

Mature virion

In contrast with other enveloped viruses, such as influenza, HIV and measles, mature flavivirus particles have a spikeless and smooth surface^{21,40,84}. The outer layer of the particle (radii of 220–245 Å) corresponds to the E and M proteins. The radii of the outer and inner leaflets of the viral membrane are between 165 and 205 Å. The bilayer is roughly polygonal in shape and is exterior to the NC. In both mature and immature particles (see below), there is ~30-Å gap between the inner lipid leaflet and the NC, and little or no connections between them^{21,40,84,85}.

The ectodomain of the E protein comprises sets of three parallel dimers that form 30 rafts over the viral surface (FIG. 4). The arrangement of 180 monomers lacks the conventional $T=3$ symmetry that was initially predicted⁸⁶. Furthermore, this arrangement makes the lipid bilayer largely inaccessible from the viral exterior. Most of the contacts between the individual E monomers are hydrophobic²⁶. The hinge angle between domains I and II in dengue-2 decreases by 10–20° relative to the angle that is observed in the dengue-2 E X-ray crystal structures^{24,26} (FIG. 3). This conformational change adapts the structure of the E protein to the viral surface²⁶. Domain III, the putative receptor-binding domain, protrudes from the otherwise smooth viral surface, possibly facilitating its binding to cellular receptors.

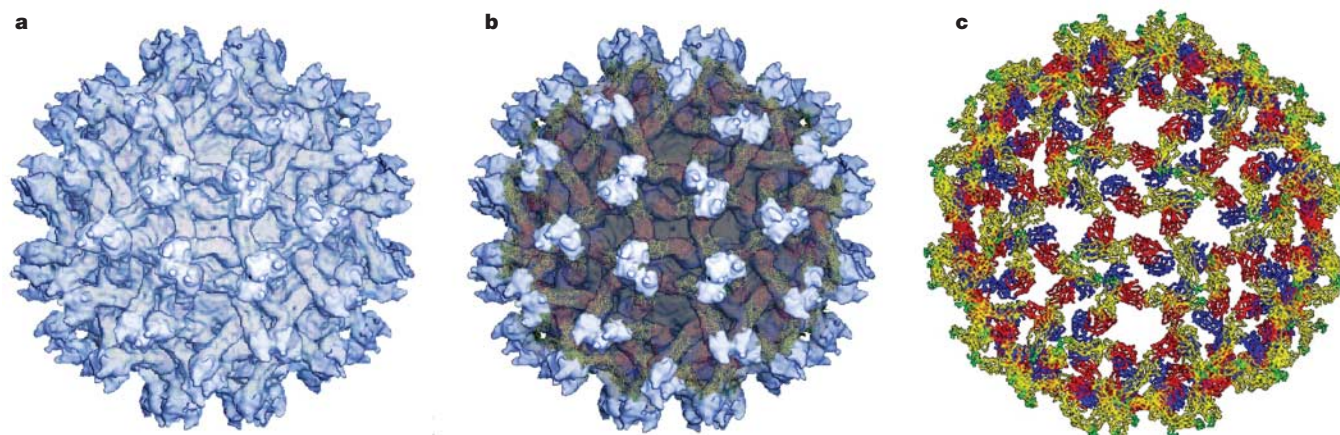


Figure 5 | **Immature flavivirus particles.** **a** | Surface-shaded cryoEM reconstruction of immature dengue-2 at 16-Å resolution. **b** | Superpositioning of the E protein onto the cryoEM density. The prM protein that caps the trimeric spike is visible. **c** | The arrangement of the E protein in the immature particle, which differs markedly from the arrangement in the mature particle, shown in FIG. 4a. The E protein is coloured as in FIGS 3,4.

Stiasny *et al.*⁶⁹ have shown that the post-fusion E homotrimers are more stable than the pre-fusion dimeric form. They further suggest that the mature virions represent a kinetically trapped **METASTABLE** assembly that undergoes structural rearrangement under acidic pH conditions, by a mechanism similar to that of influenza virus⁸⁷. The dimer to trimer transitions could not be induced by heating and specific protonation is essential for initiating dimer dissociation⁶⁹, as suggested by the putative $T=3$ structural transition.

The two transmembrane domains of the E and M proteins (residues 452–467 and 473–491 in the E protein and residues 40–54 and 58–70 in the M protein of dengue-2) form anti-parallel, approximately coiled-coil helices²¹ (FIG. 4). There are no contacts between the E and M transmembrane helices. Furthermore, neither set of transmembrane helices penetrates past the lipid bilayer to make contacts with the NC. The first helix of the stem region of the E protein (residues 398–420 in dengue-2) is angled in the outer lipid leaflet, whereas the second helix of the stem region (residues 426–448 in dengue-2) lies flat in this leaflet²¹. The stem region makes contact with the side of the E protein closest to the lipid, possibly neutralizing electrostatic repulsion between the outer phospholipid bilayer head group and the membrane-facing surface of the E protein²⁶ (FIG. 4). The stem region of the M protein (residues 22–37 in dengue-2) is also helical and is partially buried in the outer lipid leaflet. This is one of only a few membrane proteins for which structures within cellular membranes have been determined *in situ*^{88,89}.

CryoEM density indicates that the maximum height of the NC in both the mature and immature virion (see below) is ~60% of that observed for the outer glycoprotein shell^{21,40,84,85}. In addition, the structure of NC appears **DISORDERED** in both structures, which could be because the NC is inherently disordered, the icosahedral symmetry of the core is not synchronized with the glycoprotein layer, or the core is ordered but has non-icosahedral symmetry^{21,85}.

Immature virion

The structure of immature flaviviruses particles, as revealed by cryoEM, is markedly different to that of mature particles⁸⁵. The immature particles have 60 prominent, irregular, trimeric surface spikes (FIG. 5), making the external diameter 600 Å, which is somewhat larger than mature virions. Like the mature virions, these particles do not have classical $T=3$ **QUASI-SYMMETRY**⁸⁵. The E protein comprises most of the spike and the remaining density has been attributed to the pr peptide^{26,85} (FIG. 5). CryoEM has shown the hinge angle between domains I and II of each of the three symmetry-independent E proteins to differ by ~5–15° from the crystal structures^{24,26} and about 30° from the cryoEM structure of the mature particle^{21,26} (FIG. 3).

Each spike comprises a trimer of prM–E heterodimers. The three prM proteins in the spike cap the fusion peptides, which are positioned at the external tip of the E protein (FIG. 5). Each of the three prM proteins extends along the side of an E protein and enters the membrane at the point at which each prM protein forms two anti-parallel helices. The three pr peptides at the top of each spike are the main contacts between the prM and E protein heterodimers. Once cleavage of the pr peptide from prM occurs during particle maturation in the trans-Golgi network, the trimer is presumably disrupted, which allows rearrangement of the E proteins.

The distance between the E and M transmembrane regions is similar in both the immature and mature particles, although their positions, with respect to the **ICOSAHEDRAL REFERENCE AXES**, are different^{26,85}. Furthermore, the stem region of the E protein in the immature particle is in a different conformation compared with the mature particle. In the mature particles, the two stem helices are approximately co-linear, whereas in the immature particles, they are approximately anti-parallel. Nevertheless, the first stem helix is in the same position relative to domains I and III of the E protein in both the mature and immature particles. In the immature particle, the second stem helix does

TRIANGULATION NUMBER

The triangulation (T) number of an isometric virus designates the quasi-symmetry. In an icosahedron there are 60 asymmetric subunits. In an icosahedral particle, there are 60 T protein subunits that comprise the structure.

METASTABLE

A system that is above its minimum-energy state, but which requires an energy input before it can reach a lower-energy state. As a result, a metastable system functions like a stable system provided the energy input is below a certain threshold.

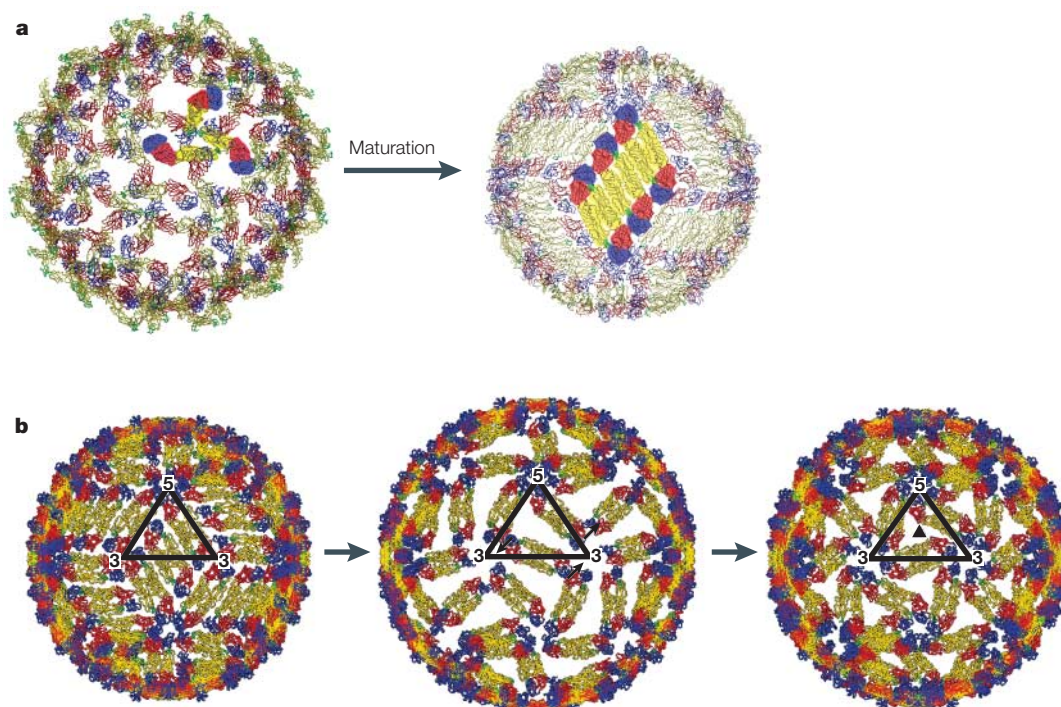


Figure 6 | Proposed rearrangement of the E proteins during maturation and fusion. a | The E proteins in the immature virus (left) rearrange to form the mature virus particle (right). **b** | The E protein dimers in the mature virus (left) are shown undergoing a rearrangement to form the putative $T=3$ fusogenic intermediate structure (right) with a possible intermediate (centre). The arrows indicate the direction of the E rotation. The solid triangle indicates the position of a quasi three-fold axis. This suggested rearrangement would require a $\sim 10\%$ radial expansion of the particle between the intermediate (centre) and fusogenic form (right). The right-hand panel of part **a** is reproduced with permission from REF. 26 © (2004) Cell Press. Part **b** is reproduced with permission from REF. 84 © (2002) Cell Press.

not contact domain II as it does in the mature particle and its position relative to the first stem helix and the transmembrane helices has changed²⁶. Insertion of alanine residues and the replacement of charged residues in the transmembrane region of prM and E by site-directed mutagenesis does not reduce the stability of the prM–E heterodimer, which indicates that few interactions occur between the transmembrane regions of the two proteins⁹⁰. This lack of transmembrane interaction between the M and E proteins is also observed in both the mature and immature structures.

Rearrangements during maturation and fusion

Flavivirus particles undergo major conformational changes during the life cycle. Some of these changes can be inferred from structural information available for the immature and mature virions as well as the pre-fusion dimer and the post-fusion trimer^{21,24–26,40,71,72,84,85}.

Virus maturation is a two-step process. First, a pH-induced irreversible conformational change involving the prM and E proteins occurs in the trans-Golgi network, which is followed by cleavage of prM by furin. During maturation, 60 trimers of prM–E heterodimers that project from the virus surface dissociate and form 90 E homodimers, which lie flat on the virus surface (FIG. 6). In addition, the E protein moves $\sim 30^\circ$ at the hinge between domains I and II. The furin cleavage does not occur unless the virus has been

exposed to low-pH conditions, which indicates that the conformational change in prM might be necessary to expose the furin cleavage site^{10,18}.

Second, a major rearrangement occurs when the virus undergoes fusion with the host cell. The anti-parallel E homodimers dissociate into monomers, which then reassociate into parallel homotrimers^{12,68,69} (FIG. 6). One hypothesis for the mechanism that underlies this reorganization is that the mature virion forms via an intermediate that is similar to the extrapolated $T=3$ structure (see above), either upon receptor binding or in the endosome before formation of the E trimers. This $T=3$ structure is composed of trimeric units and has extensive regions of exposed membranes, similar to mature alphavirus structures^{22,27,91}. Furthermore, the proposed intermediate structure has $T=3$ symmetry, as would be expected if the affinity between monomers is decreased by lowering the pH value. During this transition, the E protein moves at the domain I–II hinge region by $\sim 35^\circ$ (REF. 26).

Many virus families, such as the *Paramyxoviridae*, *Coronaviridae* and *Retroviridae*, have trimeric, α -helical, coiled-coil fusion proteins in which the fusion peptide is situated at, or near to, the N-terminus of the protein. These viruses have been defined as using the class I fusion process⁹², with the N-terminus of the fusion protein penetrating the host cell membrane. Despite major structural differences between the class I and II fusion

DISORDERED

A molecule, or part of a molecule, that has no unique structure. Every molecule has a different structure in the disordered region

QUASI-SYMMETRY

The symmetry relationship between proteins in the asymmetric unit of an icosahedron, which is determined by the ability of each protein to have almost the same local environment.

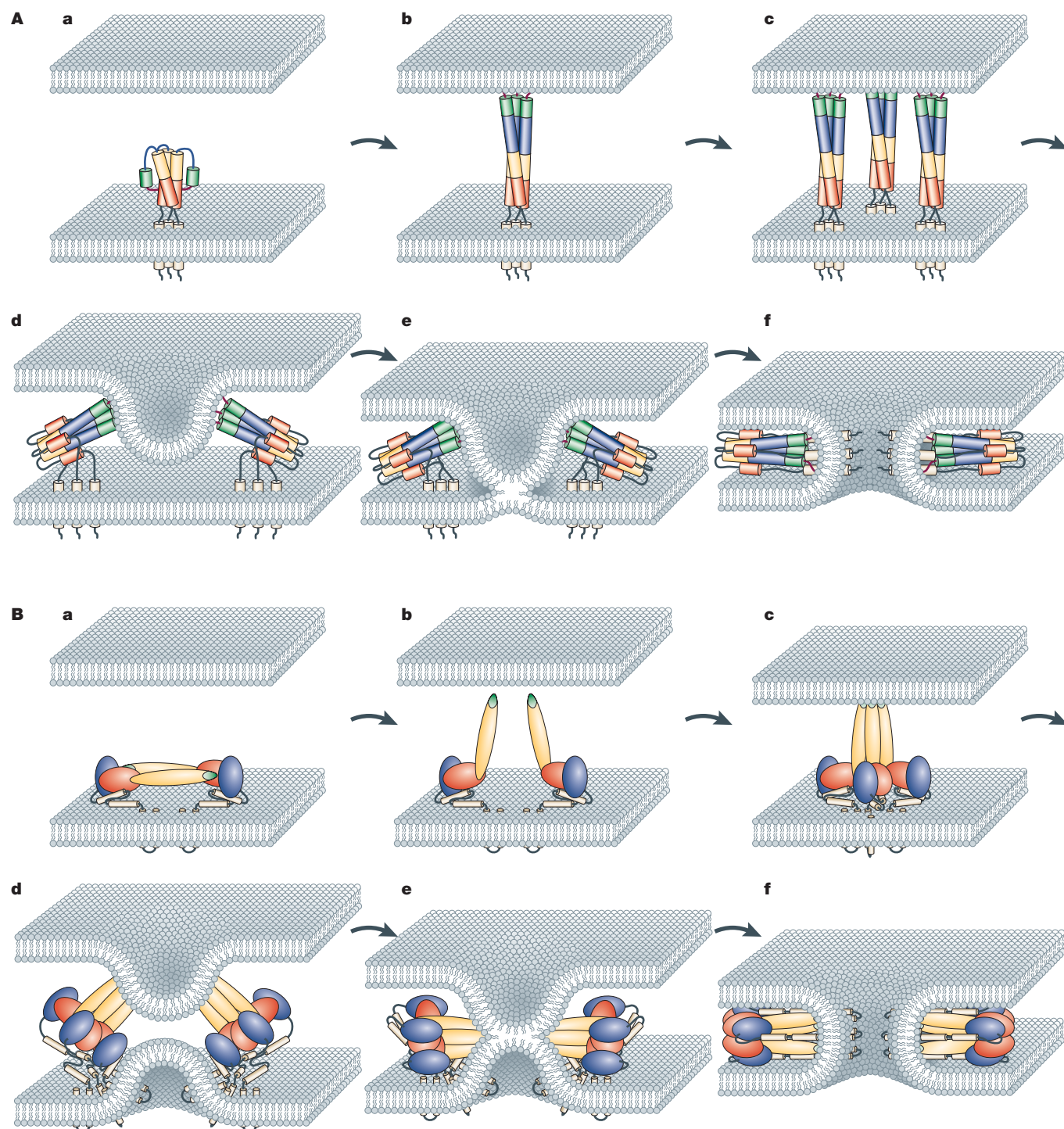


Figure 7 | Proposed mechanisms for the membrane fusion process. A | Class I fusion. The trimeric fusion protein with the fusion peptide in red (**a**). After binding to a receptor, or exposure to the low-pH conditions of the endosome, the fusion protein forms an extended conformation and the fusion peptide extends into the host membrane (**b**). Trimerization of the protein (**c**) is followed by a conformational change of the fusion protein when the host cell and viral membranes fold towards each other (**d**). A restricted hemifusion stalk is formed in which the outer leaflets of the lipid membranes mix (**e**), and finally the fusion peptide rearranges completely so that the fusion peptide and transmembrane domains are anti-parallel to each other, but in the same membrane (**f**). **B | Class II fusion.** The dimeric E protein is on the virus surface with the fusion peptide (shown in green) buried in the dimer (**a**). The protein binds to a receptor and is internalized in the endosome. Under low-pH conditions, domain II swings outward towards the host cell membrane, presumably at the domain I–II hinge region (**b**). This conformational change allows the E proteins to rearrange laterally. The fusion peptide inserts itself into the outer leaflet of the host cell membrane, enabling trimerization of the E protein (**c**). Domain III of the E protein folds back onto itself, and in the process brings the viral membrane towards the fusion peptide and host cell membrane (**d**). As domain III moves towards domain II, hemifusion of the lipid membranes occurs (**e**), and finally a trimer forms where the transmembrane regions and the fusion peptide are in close proximity (**f**). For both the class I and II processes, only the first and last steps have been observed by X-ray crystallography, but biochemical analyses provide evidence for the other steps. Reproduced with permission from Judith M. White's laboratory web site (<http://www.people.virginia.edu/~7Ejw7g/>).

ICOSAHEDRAL REFERENCE AXES

The specific symmetry axes in an icosahedron that are used to define the position of any point or atom in the icosahedron.

proteins, the proposed fusion mechanisms are similar^{71,72,93,94} (FIG. 7). The class I fusion process is initiated by the virus binding to a receptor or by exposure to low-pH conditions. The previously buried fusion peptide then undergoes a conformational change and inserts itself into the host cell membrane, thereby anchoring the fusion protein in both the cellular and viral membranes. Subsequently, the fusion protein folds back onto itself, bringing the fusion peptide (at the N-terminus) and transmembrane domain of the fusion protein (at the C-terminus) into close proximity and, as a result, bringing the viral and host membranes towards each other. In the class II process, a trimer of E proteins is formed, each domain II of the E proteins extends towards the host cell and the internal fusion peptide inserts itself into the membrane, probably by bending at the hinge between domains I and II. Similar to the class I process, the fusion protein is again anchored to both the cellular and viral membranes. When the two lipid

membranes have fused, the stem region is bound into the channel along the side of domain II and, as a result, the fusion peptide and transmembrane domains of the E protein are positioned near each other, as is the case for the class I fusion process.

Concluding remarks

There have been significant advances in understanding the structure of icosahedral enveloped viruses. Progress in combining cryoEM data with atomic structures of virion components has produced 'pseudo-atomic' structures of mature, immature and subviral particles. These viruses undergo major conformational changes during their life cycle, and further advances should be expected in the analysis of the molecular mechanisms that are required for particle assembly and disassembly. Comparisons of different viruses should provide insights into the evolution of some of these viruses from a common precursor.

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Competing interests statement
The authors declare no competing financial interests.

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