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Revisiting an old friend: new findings in alphavirus structure and assembly

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Abstract

Alphaviruses are transmitted by an arthropod vector to a vertebrate host. The disease pathologies, cellular environments, immune responses, and host factors are very different in these organisms. Yet, the virus is able to infect, replicate, and assemble into new particles in these two animals using one set of genetic instructions. The balance between conserved mechanisms and unique strategies during virus assembly is critical for fitness of the virus. In this review, we discuss new findings in receptor binding, polyprotein topology, nucleocapsid core formation, and particle budding that have emerged in the last five years and share opinions on how these new findings might answer some questions regarding alphavirus structure and assembly.

Introduction

Alphaviruses are enveloped viruses that are transmitted by arthropod vectors to vertebrate hosts [1–3]. Alphaviruses can be divided into two groups based on disease outcome. The arthralgia-causing viruses, including Chikungunya virus (CHIKV) and Ross River virus (RRV), which cause fever, rash, and polyarthritis, are often endemic to certain areas, and can trigger large scale epidemics [4,5]. The second group of alphaviruses is neuroinvasive, includes Venezuelan equine encephalitis virus (VEEV) and Eastern equine encephalitis virus (EEEV), and frequently causes encephalitis and death in a range of mammalian species [6]. Despite having different disease outcomes, the two groups of alphaviruses are very similar in their genome organization, replication, assembly pathway, and virion structure [7••,8,9].

The goal of this review is to highlight recent findings in alphavirus structure and assembly and to discuss some unanswered questions. As we cannot cover every aspect of this topic in

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this review due to space limitations, we will refer readers to several recent reviews that provide details on subjects that we do not address.

The virion: what is in it and what is needed to make it

The alphavirus genome is a positive-sense, single-stranded RNA that encodes four nonstructural proteins and six structural proteins. The structural proteins are produced from a subgenomic mRNA or second open-reading frame. The proteins produced are capsid (CP), E3, E2, 6K, transframe (TF), and E1, and are involved in assembly of an infectious virion; however, not all of these proteins need to be produced or incorporated into a virion for it to be infectious. The alphavirus virion consists of three concentric layers: the viral genome surrounded by the CP (the nucleocapsid core), a host-derived lipid bilayer, and 80 trimeric glycoprotein spikes embedded in the lipid bilayer (Figure 1a). The spikes are trimers of E2 and E1 heterodimers that bind to the cellular receptor and mediate fusion between the viral and endosomal membranes. Multiple receptors for alphaviruses have been identified [2,3,10].

Alphaviruses are unique enveloped viruses because the nucleocapsid core and glycoprotein spikes are symmetrically aligned in a $T = 4$ arrangement [7••,8,11]. The E2 protein has a 33-amino acid domain that is embedded in the lipid bilayer and interacts with the hydrophobic pocket of CP (Figure 1b). Initial structures suggested that each of the 240 E2 proteins interact with each of the 240 CPs and that these interactions facilitate the symmetrical matching between the two protein layers [12–16]. One-to-one interactions are energetically favorable, however during virion assembly, the nucleocapsid core consists of hexamers and pentamers and the spikes are trimers. From an assembly perspective, this suggests that the interacting components are not symmetrically matched and that the virus must undergo additional steps to ensure E2 and CP do interact. From a functional perspective, the advantages of a symmetrical match, such as transmission or an internal checkpoint during particle assembly and disassembly, remain unclear.

The structural proteins E3, 6K, and TF have roles in particle assembly, but do not need to be present in a virus particle for it to be infectious. E3 aids in the heterodimerization and transport of the E2–E1 glycoprotein spikes from the endoplasmic reticulum (ER) to the plasma membrane. E3 presumably acts as a clamp that holds the E2–E1 dimer together to prevent premature disassembly as the dimer travels through the host secretory pathway and is exposed to low pH [17–19]. TF is produced from a programmed ribosomal frameshifting event during translation of 6K [20], resulting in a different amino acid sequence after the frameshifting slip site. The N-termini of 6K and TF are identical but the C-termini are unique [20,21]. 6K and TF are dispensable in tissue culture, but are required for robust infection in animals. Studies with Sindbis, CHIKV, RRV, and VEEV [21–25] showed that TF is a virulence factor. Although the mechanistic roles of 6K and TF have not been fully determined, palmitoylation of TF has been shown to be important for virus assembly [21] and TF has been shown to antagonize interferon production during early infection [26]. 6K has been proposed to function as a viroporin [27,28]. A schematic of alphavirus replication and assembly and the cellular locations of the different viral proteins are shown in Figure 2; we refer you to Jose *et al.* [29•] for more details.

Mxra8: first atomic structure of a receptor-alphavirus particle complex

Many receptors for alphaviruses have been identified in both mammalian and arthropod hosts [2,3,10] and neutralizing antibodies have been mapped to alphavirus particles [7••,8,30], but the atomic level interactions between receptor and particle were unknown. In 2018, Zhang *et al.* [31] identified the cell adhesion molecule Mxra8 as a receptor specific for the entry of arthritogenic alphaviruses, including CHIKV and RRV; this receptor was not used by the encephalitic viruses. Zhang *et al.* [32••] showed attenuated infectivity in mice upon infection with a recombinant CHIKV with reduced binding to Mxra8 and enhanced CHIKV infectivity in transgenic flies expressing Mxra8.

The crystal structures of the Mxra8 receptor, which is a novel two-domain immunoglobulin-like receptor, alone and in complex with the virion were solved independently by two groups and provided the first atomic structure of a receptor with an alphavirus particle (Figure 3) [32••,33••]. Although the two groups had similar findings, they used different nomenclatures to describe the Mxra8 domains and the regions on E2–E1 where the receptor bound. In this review, we follow the nomenclature of Basore *et al.* [32••] because this group published several additional papers using this nomenclature. Mxra8 forms a unique structure than previously described two-domain immunoglobulin-like receptors in that the full domain 1 (D1) is inserted between two fragments of domain 2 (D2) forming two hinge loops between these domains which are held together by an interdomain disulfide bond.

Previously, it was thought that only E2 was involved in receptor binding [34], but both E1 and E2 interact with the two domains of Mxra8. Mxra8 is deeply embedded in a cleft or ‘canyon’ between the two E1–E2 heterodimers of a single CHIKV spike trimer, and Mxra8 makes most of its contacts at the distal end of one E1–E2 heterodimer, which is referred to as ‘wrapped’. Contacts are also made at the adjacent ‘intraspike’ heterodimer on the same spike trimer and an adjacent ‘interspike’ heterodimer of the neighboring spike trimer [32••]. Overall, Mxra8 adopts a 3:3 binding motif with a single spike trimer in CHIKV [33••]. However, depending on the presence or absence of E3 on the trimer, two classes of binding sites have been identified. Mxra8 occupies both high-affinity and low-affinity binding sites on CHIK virus-like particles (VLPs) lacking E3, but occupies only a single high-affinity binding site when E3 is present because retention of E3 blocks the low-affinity binding sites [32••]. In addition to the two domains D1 and D2, Mxra8 has a 48-residue stalk region, a 23-residue transmembrane region, and an 81-residue cytoplasmic tail. Truncations and mutations to the stalk region of Mxra8 diminishes binding; thus, the stalk region of Mxra8 also plays a crucial role in viral binding and entry [33••].

Not all species use Mxra8 for entry. Kim *et al.* [35] identified a 15-amino acid insertion in the Mxra8 receptor of a Bovinae species that prevents alphaviruses from binding by sterically hindering interaction with E2 residues. Interestingly, a mosquito ortholog of Mxra8 does not exist, indicating that other unidentified host factors must be involved in the binding and entry of alphaviruses in mosquito cells.

This new information on Mxra8 may contribute to the development of novel vaccines, inhibitors that block virus entry, and broadly neutralizing antibodies that target arthritogenic

alphaviruses. Nevertheless, questions regarding the mechanism of alphavirus infection post-entry and post-fusion remain. Upon alphavirus binding and entry, alphaviruses are in an early endosome and the low pH mediates viral-host membrane fusion. E1 undergoes a permanent conformational change and forms a fusogenic trimer. It is also unclear how many receptors the virus needs to bind to for entry, how many E1 trimers are necessary for fusion, and what conformational changes E2 undergoes in the endosome before/during fusion. To begin to answer these questions, Cao *et al.* [36] determined the low resolution structure of the Sindbis virus in a liposome at low pH. With advances in cryo-electron microscopy (cryo-EM), the different stages of fusion and the movement and conformational changes that E2 undergoes during entry may be identified.

Two topologies: connection to frameshifting

The canonical model for the topological arrangement of the alphavirus structural polyprotein, in which E2 and 6K each have two transmembrane domains, is shown as Old Model in top panel (Figure 4). There are, however, inconsistencies between the virus structure and this topology. First, structural studies show that E2 has only one transmembrane domain and a cytoplasmic domain that interacts with CP on the interior side of the lipid bilayer [7••]. Although it has been speculated that a second TM domain within E2 is extruded from the membrane during processing [37], energetic predictions of transmembrane helices suggest that this predicted second transmembrane helix may fail to undergo trans-locon-mediated membrane integration in the first place [38,39•]. Second, the predicted hydrophobic region of 6 K is 35 amino acids in length (based on Sindbis virus numbering and similar to that of other alphaviruses [21]), which is too short to form two complete helices. Third, the palmitoylated residues on TF are present before the programmed ribosomal frameshift site; therefore, they should also be present in 6K. However, these residues are not palmitoylated in 6K, possibly because of self-regulation or because these cysteine residues are not on the cytoplasmic side of the membrane [21].

Recent work by Harrington *et al.* [39•] evaluated the topology of the structural polyprotein in relation to TF frameshifting and found that there are two topological conformations of the nascent form of the alphavirus structural polyprotein within the ER. Upon production of 6K, both E2 and 6K have one transmembrane domain each. When the cytoplasmic domain of E2 enters the membrane bilayer, this causes tension on the ribosome, promotes ribosomal frameshifting, and causes TF production. In this situation, the orientation of TF is inverted relative to 6K, and the cysteine residues can be palmitoylated (Figure 4), shown as New Model in the bottom panel. Palmitoylation of TF is important for its incorporation into particles [21] and for its role as a virulence factor [26].

Although TF is produced in low amounts relative to 6K, it is more abundant in released virions [20]. TF is present in lower stoichiometric amounts relative to CP, E2, and E1, but its location in the virion and its oligomeric state is unknown. Although several functions of 6K have been proposed [21,27], its role in virus assembly remains unclear. The function of TF as a virulence factor may depend on its role in structure and assembly and vice versa. It is important to understand the regulation of the proteins that are not incorporated into the virion to identify their roles in infectious particle assembly.

Why are cores not closed spherical capsids?

The ‘perfect’ nature of the alphavirus particle, which is an icosahedral core with spikes aligned together and no external host proteins in the glycoprotein layer, should make alphavirus particles ideal candidates for high resolution structures. However, only a handful of alphavirus structures have resolutions better than 5Å [7••,8,9,40]. Heterogeneity of the glycan structures and conformations, as well as the unknown location of TF could limit the resolution. In addition, the core and its lack of interactions with the E2 cytoplasmic domain could contribute to an imperfect virion with internal heterogeneity.

Traditionally, the nucleocapsid core of alphaviruses has been thought to be composed of a viral genome surrounded by 240 copies of CP arranged in a closed shell with $T = 4$ icosahedral symmetry [41]. However, both closed complete and open incomplete shells of RRV cores exist [42•,43], and these structural differences have been observed in virus particles purified from both vertebrate and mosquito cells. The incomplete cores were observed when only the cores were used in the reconstruction process, instead of using the glycoprotein layer or the entire particle. The interpretation was that the symmetry of the glycoprotein in the particle dominated the analysis, especially when imposing icosahedral symmetry [42•]. As of now, it is unknown whether closed cores, open cores, or both are biologically active. An incomplete core may be more sensitive to the environment and may assist in the disassembly of the virus upon entry into a new cell [43].

Reconstructions of the flavivirus Kunjin have also shown different results depending on whether the entire particle or only the core region was used for reconstruction and icosahedral averaging was imposed [44]. Therkelsen *et al.* [44] found that organization of the nucleocapsid core changes during particle maturation. In addition, in both immature and mature virions, the densities of the glycoprotein shell and inner membrane leaflet at the distal pole of the virion were perturbed or missing [44]. The authors proposed that the different interactions in the immature versus mature virions may be important for assembly and budding. It is becoming clear that selecting only the best particles and imposing symmetry to get the best structures results in a significant loss of information.

The highest resolution cryo-EM structures do not provide information regarding CP interactions with viral RNA [7••,8,9,40] or the proteins incorporated into the virions [45,46]. With higher resolution structures, structures of different classes, and structures of assembly intermediates becoming available, we can begin to determine the steps of core and virion assembly and disassembly. The complex interactions that occur between the nucleocapsid core and the cytoplasmic domain of E2 have led to the hypothesis that these interactions cause a conformational change in the core that primes it for disassembly in a newly infected cell [7••,16,47]. This disassembly likely occurs at the threefold axes because there are only weak stabilizing interactions between the subunits. It has also been proposed that the disassembly is mediated by the low pH environment of the endosome during entry because the intrasubunit-stabilizing interactions are electrostatic [7••,15].

Budding: more than E2–CP interactions?

During budding, nucleocapsid cores that form in the cytoplasm interact with the cytoplasmic domain of E2 at the plasma membrane [48]. With the discovery of partially open cores, it remains to be determined how these particles assemble and bud from the membrane. For example, it is not yet known whether these cores form core-E2 interactions or whether their assembly mechanism differs from that of closed core assembly. Although many studies have modeled the interaction of CP with the cytoplasmic domain of E2 [12–16], it is unclear how many of these interactions are necessary for budding to occur [7••,15]. Furthermore, because the most detailed alphavirus structures have been produced by imposing icosahedral symmetry, any differences in the interactions between CP and the cytoplasmic domain of E2 within a particle would not be identified. Because the core is often incomplete, it is unlikely that there would be 240 equivalent E2–CP interactions within a particle. Molecular dynamic simulations have shown that nucleocapsid core-mediated budding results in particles of uniform size and morphology [49•], an advantage of nucleocapsid core mediated budding.

Previous work can now be reconciled with new structural findings. It has been observed that CP interacts with the viral genome in the cytoplasm without forming a complete closed core [50,51]. CP likely interacts with E2 at the plasma membrane and in cytopathic vesicles II (CPV-II), intracellular, virus-induced vesicles (Figure 2) [29•,52]. In multiple Semliki Forest virus mutants, CP can be detected in the virus particle but cannot form nucleocapsid cores in the cytoplasm [50–53]. It has recently been shown in CHIKV, Semliki Forest, and Sindbis viruses that CP is not required for assembly or budding of infectious virus particles [54,55]. When the genomes of these viruses lack the CP sequence, infectious microvesicles (iMVs) containing glycoproteins and genomes are released from the plasma membranes of infected insect and mammalian cells. The iMVs are pleomorphic, have a significantly lower titer than the wild-type virus [54], and are analogous to the subviral particles produced by flaviviruses [56].

Interestingly, multiple studies have shown differences in the assembly and budding of viruses in vertebrate and invertebrate cells; for example, a viral mutant is able to form nucleocapsid cores in mosquito cells, but not in mammalian cells. It has also been demonstrated that internal budding in CPV-II is more common in mosquito cells than in mammalian cells [29•,57]. In addition, it has been shown that the organization of viral proteins and RNA in replication complexes differs between mammalian and mosquito cells and that novel replication complexes form in mosquito cells [29•].

The scission machinery that releases budding particles remains unknown for alphaviruses. Alphaviruses do not use the host endosomal sorting complexes required for transport (ESCRT) system [58], but it is unclear if another host system is used or if the virus encodes for its own viral scission machinery (e.g. the Influenza M2 protein) [59]. Analysis of mutations in 6 K and TF suggest that they could function in viral budding [60–62]. A neutralizing antibody that interferes with CHIK virus budding, but not entry, has recently been identified. This antibody reduces the number of released particles by crosslinking the glycoprotein spikes at the plasma membrane [63•]. Alphaviruses can also spread via cell-to-cell transmission [64,65], and this mechanism is reviewed in Brown *et al.* [66].

Conclusion

Structure and function go hand in hand. The structure of the released virion is the final metastable form of the particle, and to get to that conformation, many regulated events must occur. Similarly, when a virus enters a cell to initiate an infection, there is a systematic process for disassembly. With the recent advances in high-resolution structural biology, it is a great time to focus on the assembly intermediates that allow a virion to form and the disassembly intermediates that form during infection. New and much improved tools for single-particle structural biology [67,68] and advances in sample preparation and data acquisition for high resolution cryo-tomography [69] make it possible to describe the structure of assembly and disassembly intermediates. Identifying the intermediates and determining their cellular locations will guide our hypotheses for cellular studies. Structural and cellular data will connect the dots between structure and function, which has tremendous implications for vaccine development, drug design, evolutionary biology, and the use of viral particles as biological templates.

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• of special interest

•• of outstanding interest

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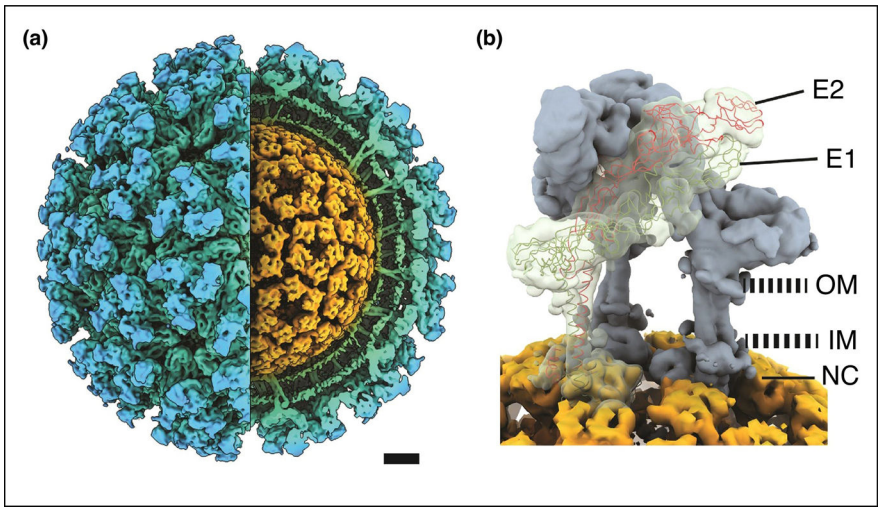

Figure 1.

Overview of alphavirus structure.

(a) A cutaway isosurface representation of the alphavirus Sindbis viewed along two-fold axis. Structure determined by cryo-EM and 3D reconstruction. The virion consists of three concentric layers: the glycoprotein spikes (*blue*) embedded in the lipid bilayer (*green*), and the nucleocapsid core (*orange*) which consists of the capsid protein and RNA genome. Both glycoprotein and the core are arranged in a $T = 4$ surface lattice. **(b)** Each spike is a trimer of E1 and E2 heterodimers. Shown are two heterodimers in gray and one with the atomic trace. The cytoplasmic domain of E2 interacts with capsid protein. OM, outer membrane. IM, inner membrane. Scale bar, 5 nm.

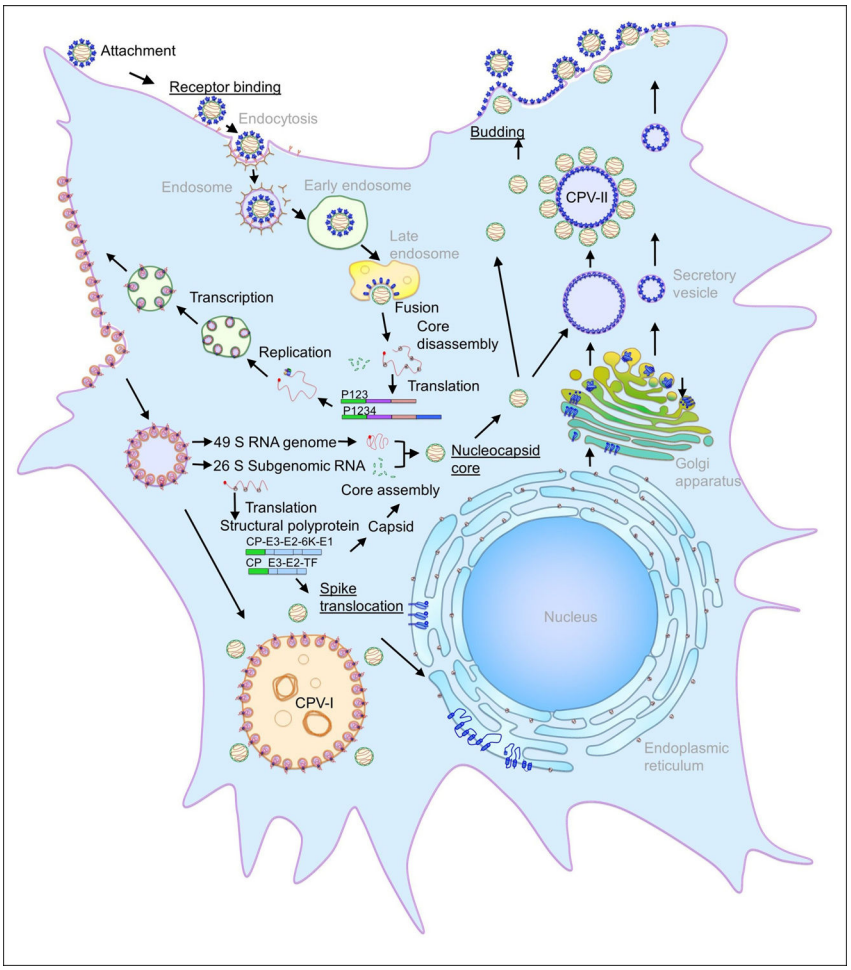

Figure 2.

Alphavirus replication and assembly in a vertebrate cell.

Alphavirus particles enter the host cell through receptor-mediated endocytosis and the low pH environment triggers viral and host membrane fusion followed by core disassembly. The viral genome is replicated and subsequently the core and the spikes are assembled in two independent pathways. Particles are released from the cell by budding from the plasma membrane. The steps of the replication and assembly pathway are labeled in *black*, the steps discussed in this review are underlined, and host processes/components are in *gray*. Figure adapted from Jose *et al.* Spatial and temporal analysis of alphavirus replication and assembly in mammalian and mosquito cells. (2017) mBio 8:e02294–16 [29•] and with permission from the authors.

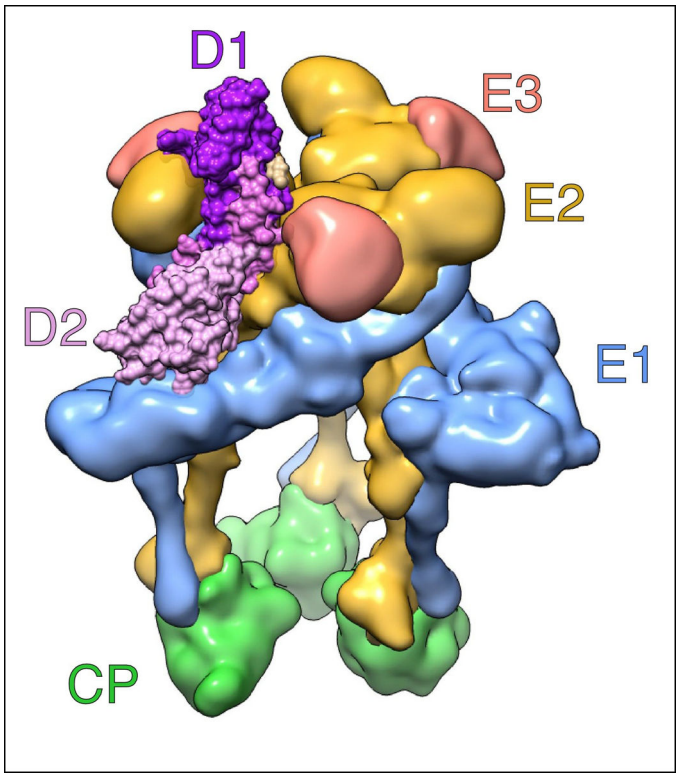

Figure 3.
Mxra8 bound to CHIKV.
Side view of a CHIKV spike complexed with its Mxra8 receptor. The viral proteins are CP (*green*), E1 (*light blue*), E2 (*yellow*), and E3 (*pink*). The two domains of Mxra8, D1 and D2, are shown in *dark purple* and *light purple*, respectively. Interestingly, the receptor binds to both E2 and E1 and intercalates itself between adjacent E2–E1 heterodimers in the spike complex. The nomenclature is based on Basore *et al.* [32••] and is consistent with findings from Song *et al.* [33••]. EMD-9395 with PDB-6NK6 were used to construct the figure in Chimera [70].

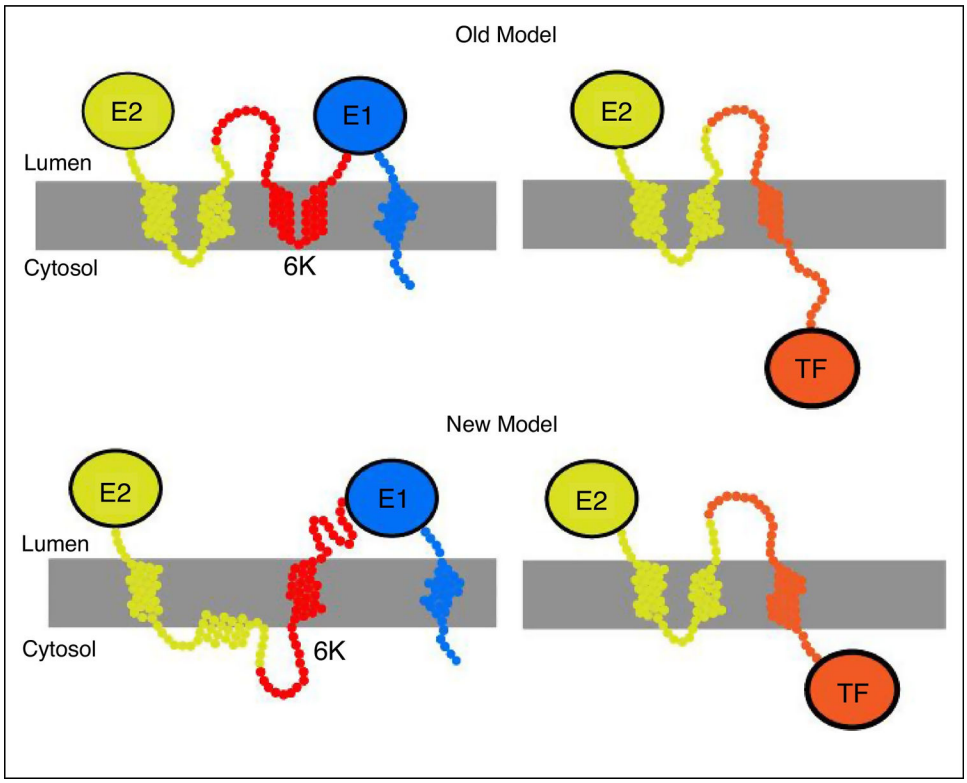

Figure 4.

Topological models of the alphavirus structural polyprotein.

The top panel shows the ‘old’ model in which E2 and 6 K each have two transmembrane helices and TF contains a single transmembrane helix. The bottom panel shows the ‘new’ model in which E2 contains a single transmembrane helix, the cytoplasmic domain of E2 is in the cytoplasm, and 6 K contains a single transmembrane helix. When the cytoplasmic domain of E2 enters the membrane, programmed ribosomal frameshifting occurs and TF is produced. This model is based on Figure 3 in Harrington *et al.* [39•].