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Degrees of maturity: the complex structure and biology of flaviviruses

Theodore C Pierson[1] and Michael S Diamond[2]

Flaviviruses are small enveloped virions that enter target cells in a pH-dependent fashion. Virus attachment, entry, and membrane fusion are orchestrated by the envelope (E) and pre-membrane (prM) proteins, the two structural proteins displayed on the surface of virions. Flaviviruses assemble as an immature non-infectious form onto which prM and E form trimeric spikes. During egress from infected cells, flaviviruses undergo dramatic structural changes characterized by the formation of a herringbone arrangement of E proteins that lie flat against the surface of the virion and cleavage of the prM protein by the cellular protease furin. The result is a relatively smooth, infectious mature virion. This dynamic process is now understood in structural detail at the atomic level. However, recent studies indicate that many of the virions released from cells share structural features of both immature and mature virus particles. These mosaic partially mature virions are infectious and interact uniquely with target cells and the host immune response. Here, we will discuss recent advances in our understanding of the biology and significance of partially mature flaviviruses.

Addresses
[1] Viral Pathogenesis Section, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, United States
[2] Departments of Medicine, Molecular Microbiology, and Pathology & Immunology, Washington University School of Medicine, St. Louis, MO 63110, United States

Corresponding author: Pierson, Theodore C (piersontc@mail.nih.gov)

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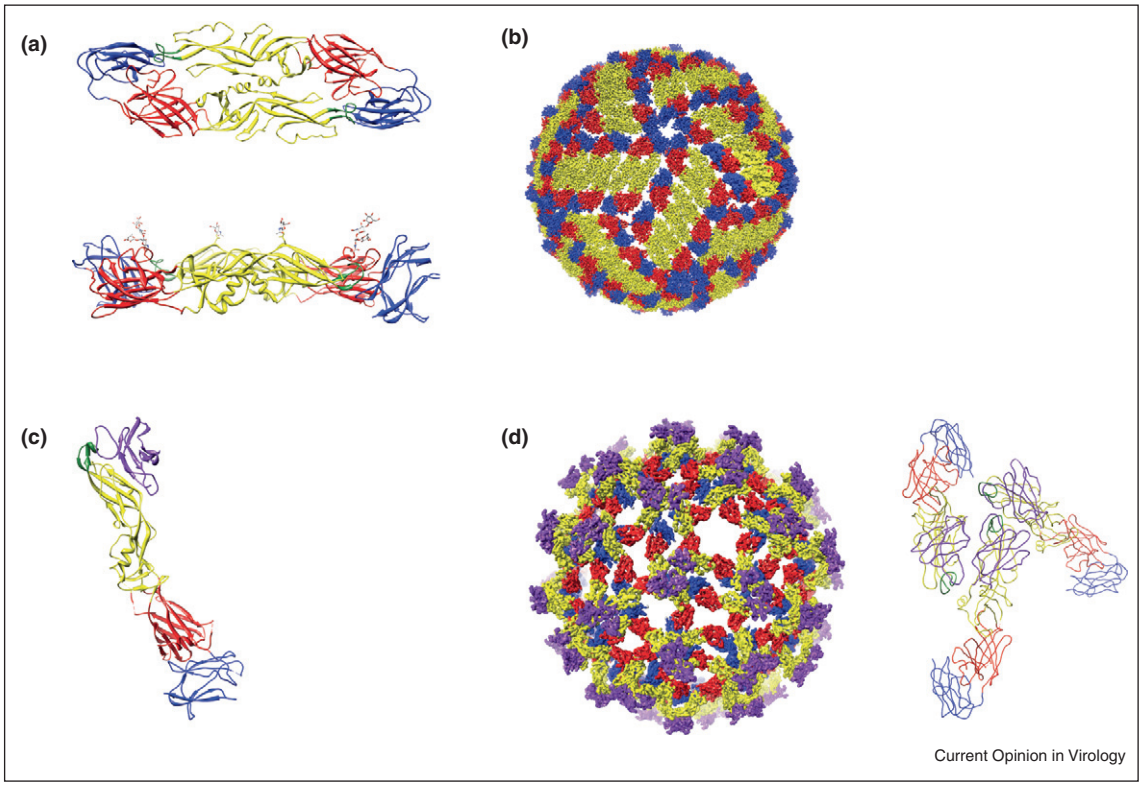
Flaviviruses are a group of enveloped positive-stranded RNA viruses responsible for considerable morbidity and mortality throughout the world. Members of this genus with a significant impact on public health include dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) and West Nile virus (WNV). These viruses are typically transmitted to humans through the bite of mosquitoes or ticks, and cause a spectrum of severe illnesses that include encephalitis and hemorrhagic disease. While vaccines have been effective at reducing the burden of several flaviviruses when available (YFV, JEV, and TBEV) [1–3], an urgent need exists for additional vaccines and therapeutics against this genus of viruses. Antiviral antibodies contribute significantly to protection against flavivirus infection [4,5], and have proven to be a good correlate of protection for existing flavivirus vaccines [2,6]. An understanding of the structural and immunological basis for antibody-mediated protection against flavivirus infection has evolved rapidly [5]. However, recent insights into the composition, structure, and dynamics of flavivirus virions identify previously unappreciated complexities that may impact the potency of anti-flavivirus antibodies and, in the case of DENV, their potential to exacerbate disease [7,8]. This review will discuss new insights into the structural heterogeneity of flaviviruses, and how this advances our current understanding of the biology of the virus particle and its interaction with the humoral immune response.

The envelope proteins

The ~11 kb positive-stranded genomic RNA of flaviviruses encodes a single polyprotein that is cleaved into ten functionally distinct proteins, including three structural proteins incorporated into the virus particle. High-resolution structures of portions of all three structural proteins have been reported [9]. The envelope protein (E) is a ~53 kDa elongated protein that orchestrates the processes of viral entry and virion budding [10]. It is composed of three distinct domains and may be modified by the addition of one or two asparagine-linked (N-linked) carbohydrates, depending on the flavivirus strain (Figure 1a). E proteins are arranged on mature virions as 90 anti-parallel dimers [11]. E domain III (E-DIII) is an immunoglobulin-like domain that forms small protrusions on the surface of an otherwise smooth spherical mature virus particle (Figure 1b); this structure is thought to interact with cellular receptors on target cells [12–14]. Domain II (E-DII) is composed of two 'finger-like' structures involved in E protein dimerization and contains a highly conserved 13 amino acid hydrophobic fusion loop at its distal end [15]. These two structures are linked through a third central domain I (E-DI) via short flexible loops. The complex structural changes in E that occur during the course of virion maturation and fusion involve rotation between these three domains [16–19]. The E protein is anchored to the viral membrane through the stem anchor helical domain and two anti-parallel transmembrane domains

Figure 1



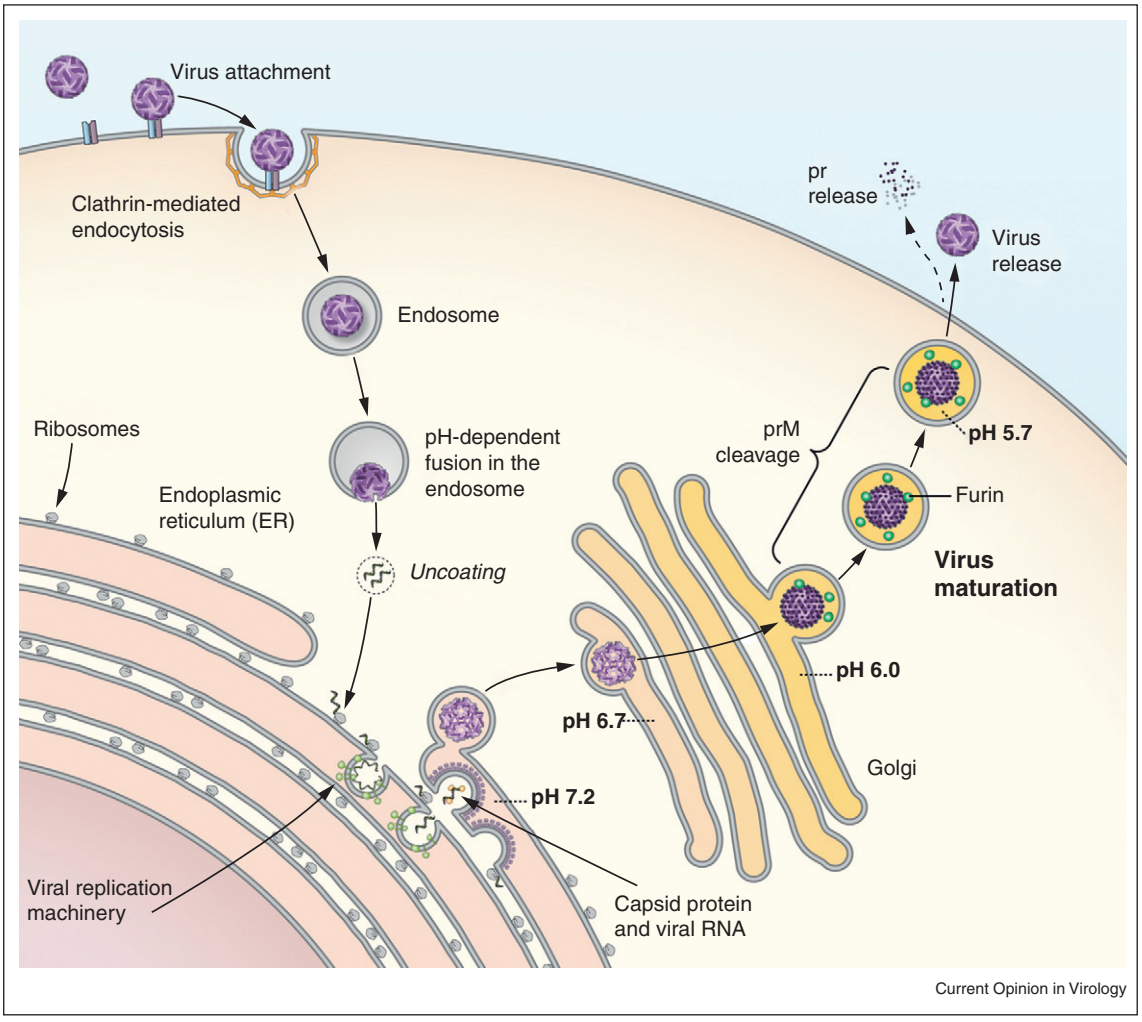
Structure of the flavivirus envelope proteins and their organization on the virus particle. Flaviviruses are small spherical virions that incorporate a dense array of prM and E proteins that function to promote virus assembly, budding, and entry. **(a)** The E protein is composed of three structurally distinct domains and is present on mature virions as anti-parallel homodimers. The dimeric arrangement of DENV E proteins is shown from the top and side. Domain III (E-DIII, shown in blue) is thought to interact with receptors on target cells. The conserved 13 amino-acid fusion loop (shown in green) is located at the distal end of domain II (E-DII, shown in yellow). E-DIII and E-DII are connected by the central domain I (E-DI, shown in red). The carbohydrate modifications of E-DI and E-DII are shown in the side view using a ball and stick representation and vary in number among different flaviviruses. The stem anchor that anchors the E protein to the viral membrane is not shown. **(b)** The arrangement of E proteins on the mature DENV virion is depicted. Each virus particle is composed of 30 rafts of three antiparallel dimers in a herringbone pattern. **(c)** The structure of the 'pr' portion of DENV prM is shown in complex with the E protein. prM is shown in purple, whereas the domain organization of the E protein is represented as described in panel A. **(d)** The arrangement of prM and E proteins on the fully immature DENV virion is displayed. Each immature virion is composed of 60 prM-E heterotrimeric spikes arranged with icosahedral symmetry. The arrangement of the prM and E protein within each spike is shown on the right.

[20,21]. The pre-membrane protein (prM) is a seven β-stranded glycoprotein that facilitates E protein folding and regulates the oligomeric state of E proteins to prevent adventitious fusion during the egress of virus particles from infected cells, as detailed below [22,23].

Flavivirus biogenesis and structure

Flavivirus infection results in a marked proliferation and reorganization of membranes in the cytoplasm of cells [24]. While the cell biology of flavivirus assembly is incompletely understood, virions are thought to assemble on membranes derived from the endoplasmic reticulum (ER) at locations proximal to the site of viral RNA replication [25–27] (Figure 2). Nascently synthesized virions bud into the lumen of the ER as immature virus particles that incorporate 60 heterotrimeric spikes of prM and E arranged with icosahedral symmetry [19,28] (Figure 1d). On immature virions, prM is located at the distal end of these spikes adjacent to the E-DII fusion loop; a recent structure of the DENV prM-E heterodimer details the molecular basis for this interaction [18] (Figure 1c). Assembled virions exit the cell via the secretory pathway (Figure 2). Transit of virions through acidic compartments of the trans-Golgi network (TGN) results in a reorganization of the surface of the virus particle. The E proteins of immature virions at low pH lie flat against the surface of the virus in a herringbone pattern; prM remains attached on the surface of the immature virion positioned atop the fusion loop [29••]. This pH-dependent change in virion structure exposes on prM a cleavage site recognized by the cellular serine protease furin [30]. Mutagenesis studies with TBEV demonstrate that cleavage of prM is a required event in the infectious viral life cycle [31], presumably because the interactions between prM and E prevent the conformational changes in the E

Figure 2



The maturation of flaviviruses. Flaviviruses enter cells via a clathrin-dependent endocytic pathway and fuse with endosomal membranes of the target cell in a pH-dependent fashion. Flaviviruses assemble at and bud into membranes derived from the endoplasmic reticulum (ER) as immature virions on which prM and E proteins interact as heterotrimeric spikes. Assembling virions are shown on the ER membrane; the viral capsid protein is depicted as yellow spheres in complex with viral RNA. Flavivirus assembly sites are thought to be located proximal to the site of viral RNA replication (shown schematically as invaginations of membrane decorated with components of the viral replication complex (green spheres)). Transit of immature virions through the mildly acidic compartments of the secretory pathway results in a pH-dependent change in the arrangement of E proteins on the surface of the virus particles. E proteins on immature virions at an acidic pH are positioned flat against the surface of the virion in a herringbone pattern. The prM protein (represented as dark purple spheres) remains associated with the virus particle. In this conformation, prM may be cleaved by the cellular serine protease furin. Release of the virion from cells into the more neutral extracellular milieu results in the disassociation of the 'pr' portion of prM and the formation of a mature virion.

proteins required to drive pH-dependent membrane fusion after entry [23,32]. Secretion of the virion from cells into the neutral pH of the extracellular space triggers the release of the ~91 amino acid 'pr' portion of prM [29••]. The resulting mature flavivirus has a relatively smooth spherical structure on which antiparallel dimers of E are arranged with T = 3 pseudo-icosahedral symmetry [11,33] (Figure 1b). In the event cleavage of prM does not occur during egress through the TGN, immature virions decorated by prM-E heterotrimeric spikes are released from cells [19,28].

Partially mature virions

Biochemical analysis of the protein composition of flaviviruses reveals the presence of uncleaved prM protein, suggesting that the process of virion maturation may be inefficient under some circumstances (see [31] and references within). As a majority of virions released from DENV-infected cells can be immunoprecipitated using monoclonal antibodies (mAbs) specific for prM, virions containing significant amounts of prM may be quite prevalent [34••,35]. Factors that control the extent of virion maturation within cells remain poorly understood.

The prM content of flaviviruses produced in mosquito cells appears greater than that of virus propagated in mammalian cells [36,37]. An ability to increase the efficiency of prM cleavage through the ectopic expression of furin suggests that exposure to a furin-like protease in the TGN may be limiting in some cellular contexts [38,39•]. Mutations in both the prM [35] and E proteins (S. Nelson and T. Pierson, unpublished data; [40]) also have been shown to impact the efficiency of prM cleavage.

Genetic studies with TBEV demonstrate that cleavage of prM is required for the production of infectious virions [31]. In support, the production of virus under conditions that reduce the efficiency of virion maturation (e.g. treatment of cells with ammonium chloride (NH_4Cl) or in cell lines that do not express furin) markedly decreases the specific infectivity of virus preparations ([31,41,42] and references within). However, the residual (albeit significantly reduced) infectivity of virions produced under these conditions suggests that virions that retain uncleaved prM protein might be infectious. Three lines of experimental evidence indicate that uncleaved prM are present on infectious virions: first, the prM content of flaviviruses modulates the conditions required to trigger the conformational changes that drive membrane fusion and promote infection. Murray Valley encephalitis virus grown in the presence NH_4Cl was more resistant to inactivation by acidic conditions [43]; second, uncleaved prM on virions may participate in the attachment and entry of infectious virus particles. Interactions between the C-type lectin DC-SIGNR and the carbohydrate present on uncleaved prM were sufficient to promote the infectious entry of WNV into cells [38]; and third, the prM content of flaviviruses markedly impacts the outcome of antibody–virion interactions, as discussed in detail below.

Partially mature virus particles share structural characteristics with both mature (complete prM cleavage) and immature (no prM cleavage) virus particles. Because partially mature virions are heterogeneous and lack icosahedral symmetry, structural information is limited. Examination of electron micrographs of WNV and DENV identify individual virions characterized by a mixture of smooth and spiky surfaces [28,34••,44•]. A recent cryo-electron tomographic analysis of the structure of partially mature DENV suggested that partially mature virions are mosaics of two regions corresponding to E proteins in mature and immature arrangements; the relative size of these two regions varied among the virus particles analyzed [45••]. Viral proteins at the junction of these two regions appear to be arranged in a unique fashion that was not resolved by the tomographic model.

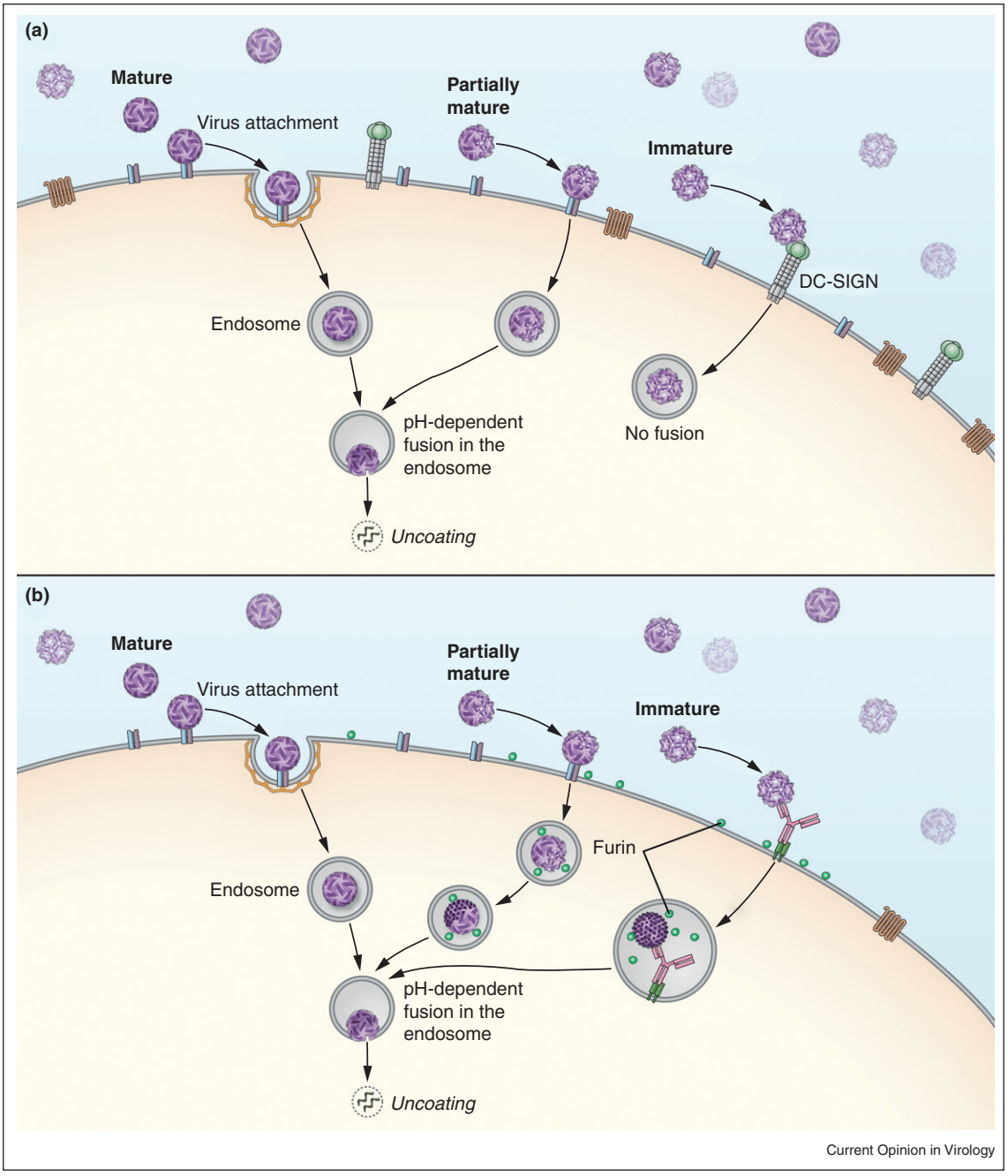
Impact of virion heterogeneity on the interaction of flaviviruses with antibody

The presence of uncleaved prM on flaviviruses impacts the interaction of virions and antibodies in several functionally significant ways. Antibody-mediated neutralization of flavivirus infection can be modeled as a 'multiple-hit' process requiring engagement of the virion with a stoichiometry that exceeds a required threshold number of antibody molecules [5,46]. From this perspective, the number of antibodies that simultaneously bind the virion is the critical parameter determining neutralizing activity. Conversely, the engagement of virions with a stoichiometry that does not exceed the neutralization threshold has the potential to promote more efficient infection of cells that express Fcγ-receptors. This antibody-dependent enhancement (ADE) of infection is thought to contribute mechanistically to the severe clinical manifestations associated with secondary DENV infections [5,47].

Epitope accessibility governs the activity of neutralizing antibodies [7]. Many of the epitopes recognized by mAbs are not predicted to be accessible on the surface of the mature virion, yet display considerable neutralizing activity [48–51]. The molecular basis for this neutralizing activity may be explained in part by the complex structure of partially mature virions. Differences in arrangement and oligomeric state of E proteins on mature and immature virions translate into changes in antibody reactivity [43,52]. Antibodies may differentially interact with the mature and immature portions of individual partially mature virions, or the envelope proteins located at the poorly resolved junction between these ordered regions. For example, the WNV E-DII fusion loop-reactive mAb E53 efficiently binds virions that contain uncleaved prM [44•]. In support of this, the neutralizing activity of many (perhaps most) WNV mAbs are strongly influenced by the maturation state of virion [39•]. WNV produced under conditions that promote more efficient cleavage of prM are markedly less sensitive to neutralization by several classes of mAbs (including E53), presumably due to a reduction in epitope accessibility.

While the majority of neutralizing mAbs are directed against the E protein, antibodies specific for prM have been reported in mice [53–57] and humans [58•,59•]. Two recent studies of the repertoire of antibodies present in DENV-immune individuals suggest that anti-prM antibodies may be prevalent [58•,59•]. Anti-prM antibodies can contribute to protection from flavivirus infection through effector functions mediated by the Fc-portion of the antibody molecule [60], after interaction with uncleaved prM on virions. Studies with anti-prM mAbs reveal that they possess limited neutralizing activity. It is presently unclear whether these molecules inefficiently neutralize infection because they fail to bind virions with a required stoichiometry (too few prM molecules on infectious partially mature virions) or because they are unable to interfere with a required step in the virus entry process, such as virion attachment or fusion. In contrast, antibodies against prM readily promote ADE *in vitro* [42,56,58•,59•,61••] and *in vivo* [62]. These antibodies

Figure 3



The heterogeneity of flaviviruses complicates an understanding of the interactions of virions with antibodies and the cell surface. **(a)** Populations of flaviviruses released from cells are heterogeneous with respect to the extent of prM cleavage. Mature virions are defined as those virus particles on which prM has been completely cleaved and are infectious. Conversely, immature virions retain 180 copies of uncleaved prM. Genetic studies indicate that these virus particles are non-infectious. Partially mature virions describe a heterogeneous population of virions that retain varying amounts of uncleaved prM. Recent structural studies indicate that the partially mature virions contain distinct regions of mature and immature character [45]. At least some partially mature virions are infectious, although the relationship between the specific infectivity of virions and prM content has not yet been investigated. The significant differences in the arrangement of the E proteins on mature, immature, and partially mature viruses raises the interesting (yet unexplored) possibility that they differentially interact with cellular factors on the surface of target cells. **(b)** A role for furin-like proteases during flavivirus entry? The presence of the furin protease in endocytic compartments raises the possibility that the prM protein on partially mature and immature virions may be processed during entry [61••]. This phenomenon may contribute to the marked increase in the specific infectivity of largely immature populations of DENV in the presence of enhancing concentrations of antibody [42,61••].

can markedly increase the specific infectivity of largely immature preparations of DENV [42,61••], and have been shown to enhance the infectivity of WNV *in vivo* [63]. Because of the significant cross-reactivity of many prM-reactive antibodies and their propensity to promote ADE, antibodies of this specificity have been proposed to be a primary contributor to the more severe clinical manifestations of secondary DENV infection [59•].

Maturation delayed: cleavage of prM during virus entry

A hallmark of virion maturation is the cleavage of prM by a cellular furin-like protease. While furin is enriched in the TGN, it also traffics to the cell surface and is recycled in endosomes [45••]. Flaviviruses enter cells via clathrin-dependent endocytosis and fuse in acidic compartments of the late endosome [64] (Figure 2). Thus, incoming flaviviruses may colocalize with furin during the virus entry process in the low pH-environment required for both exposure of the furin cleavage site and E protein-mediated viral membrane fusion [30,65]. Recently, Rodenhuis-Zybert and colleagues raised the possibility that furin may act on incoming immature virions to increase infectivity [61••] (Figure 3a). Treatment of DENV produced in furin-deficient Lovo cells with prM-specific or E-specific antibodies markedly enhanced the infection of Fcγ-receptor-expressing cells in a furin-dependent fashion [61••,66]. Cleavage of prM during virus entry provides a mechanistic explanation for the observation that prM antibodies increase the specific infectivity of prM-containing virions discussed above [42,61••,66]. Nonetheless, additional studies are required to determine the cellular contexts in which furin-mediated cleavage of prM during the entry of flaviviruses is important. For example, the infectivity of WNV was not significantly reduced by inhibitors of furin-like proteases, even when virions were produced in the presence of NH_4Cl or infections were performed in the presence of enhancing concentrations of antibody [67]. In contrast, the replication and release of Lovo cell-derived WNV was enhanced under conditions that support ADE [66]. Because the stoichiometric threshold for prM cleavage for virus infectivity is unknown, and whether this differs in the presence or absence of enhancing antibody, it is possible that a requirement for furin-like proteases exists only for a subset of virions that retain exceptionally large numbers of uncleaved prM molecules. The size of the population of prM-containing virions released from cells for which infectivity can be enhanced through the activities of furin may be virus strain and producer cell-type dependent.

Conclusions and future directions

While recent progress has been considerable, the biology of partially mature virions and their contribution to pathogenesis remains incompletely understood. Partially mature virions are defined by the presence of uncleaved prM protein; an unknown proportion of these virions are infectious. The recent model of the structure of partially mature virions suggests that their immature character is restricted to a single region on the virion surface, presumably the size of which will vary as a function of the prM content of the individual virus particle [45••]. However, the relationship between the number of prM molecules on the virion (the size of the immature region) and its specific infectivity has not yet been studied. Understanding the stoichiometric requirements for prM cleavage will provide insight into the number of E protein molecules required for fusion, and how virions interact with target cells. Because prM controls the oligomeric state of E proteins and regulates fusion [23,32], presumably E protein molecules in immature regions cannot contribute to fusion. If E-DIII indeed binds cellular receptors on target cells [12–14], the orientation of the E protein could modulate access to this receptor binding structure; E-DIII at the bottom of a trimeric spike may render it less able to attach to its cognate receptor. Is it possible that the cellular factor used by a virion during entry into cells varies as a function of the prM content of the virus particle? Does the retention of uncleaved prM modulate the efficiency of interactions with other host factors, such as mannose binding lectin, that may be important for immune control or pathogenesis [68]? Studies with TBEV suggest that cleavage of prM is significantly more efficient than that observed for mosquito-borne flaviviruses [30]. What viral and cellular factors control the extent of prM cleavage and the proportion of mature, immature, and partially mature virions released from cells? What is the efficiency of prM cleavage *in vivo*? Resolution of many of these issues will require new approaches that enable the analysis of the functional and biochemical properties of individual virus particles with controlled levels of uncleaved prM.

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