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Mechanism for maturation-related reorganization of flavivirus glycoproteins

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ABSTRACT

Flaviviruses, such as dengue, West Nile, and yellow fever viruses, assemble as fusion-incompetent particles and subsequently undergo a large reorganization of their glycoprotein envelope resulting in formation of mature infectious virions. Here we used a combination of three-dimensional cryo-electron tomography and two-dimensional image analysis to study pleomorphic maturation intermediates of dengue virus 2. Icosahedral symmetries of immature and mature regions within one particle were mismatched relative to each other. Furthermore, the orientation of the two regions relative to each other differed among particles. Therefore, there cannot be a specific pathway determining the maturation of all particles. Instead, the region with mature structure expands when glycoproteins on its boundary acquire suitable orientation and conformation to allow them to become a stable part of the mature region. This type of maturation is possible because the envelope glycoproteins are anchored to the phospholipid bilayer that is a part of flavivirus virions and are thus restricted to movement on the two-dimensional surface of the particle. Therefore, compounds that limit movement of the glycoproteins within the virus membrane might be used as inhibitors of flavivirus maturation.

1. Introduction

Dengue, West Nile, and yellow fever viruses are members of *Flaviviridae* which includes many human pathogens. Although dengue virus infection usually induces flu-like symptoms, some of the infections may progress to life-threatening dengue hemorrhagic fever or dengue shock syndrome (Halstead, 2007). It has been estimated that 50–100 million people are infected with dengue virus every year (Whitehorn and Farrar, 2010). Furthermore, regions where mosquitoes transmit dengue are spreading because of human activity.

Flavivirus virions are enveloped with a diameter of ~500 Å. The surface glycoproteins have icosahedral symmetry with three envelope glycoproteins in one icosahedral asymmetric unit (Kuhn et al., 2002; Zhang et al., 2013a). However, contrary to the principles suggested by Crick and Watson (1956, 1957) as well as Caspar and Klug (1962) and unlike most icosahedral viruses, the three molecules have different interactions with surrounding subunits. Each glycoprotein is anchored in the viral membrane by two transmembrane helices. The core of the virus is composed of a single-stranded RNA genome and capsid proteins but lacks icosahedral symmetry.

The immature virions form by budding into the lumen of the endoplasmic reticulum (ER) as non-infectious, fusion-incompetent particles that are characterized by a "spiky" arrangement of surface glycoproteins. Each spike contains a trimer of hetero-dimers of pre-membrane (prM) and envelope (E) glycoproteins (Zhang et al., 2003, 2007). Subsequently, the virions are transported from the ER to the Golgi complex and the trans-Golgi network where they encounter a pH of ~6, a decrease from the neutral pH in the ER. The pH change induces a large conformational reorganization of the glycoproteins into the low-pH, herringbone-like arrangement (Yu et al., 2008). The maturation-related conformational changes start from a nucleation center and then spread around the particle (Plevka et al., 2011). Immediately after the conformational change the virions contain intact prM molecules that cover the E protein fusion loops (Stiasny et al., 2007; Yu et al., 2009). The prM is subsequently cleaved by host protease furin into pr and M fragments (Yu et al., 2008). For the benefit of simplicity and clarity, within the following text the term "mature structure" indicates herringbone organization of E glycoproteins regardless of the cleavage state of the prM.

Here we show that regions with mature and immature structures within one dengue virion have mismatched icosahedral symmetries. This observation has led us to formulate a mechanism for flavivirus maturation.

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2. Materials and methods

2.1. Sample preparation for cryo-EM and cryo-ET

Immature wild-type dengue virus 2 16681 was produced and purified as described previously (Junjhon et al., 2008; Yu et al., 2008). For cryo-EM and cryo-ET analysis the virus solutions at pH 6.0 were mixed with a suspension of 10 nm colloidal gold particles. The solution (3.5 μl) was applied to a holey carbon film, blotted and vitrified by plunging into liquid ethane.

2.2. Cryo-EM and cryo-ET

"High" dose cryo-EM images and cryo-ET tilt series of the same particles were obtained using an FEI (Hillsboro, OR) Titan Krios electron microscope operated at 300 keV. The Krios microscope was equipped with a Gatan (Pleasanton, CA) energy filter operated in zero-energy-loss mode with a slit width of 30 e^{-}V. Images were recorded on a 2048 × 2048-pixel CCD camera at a nominal magnification of 19,500. First, the single-particle images were collected at ~5 μm defocus with a total dose of 20 $e^{-}/Å^2$. Then tomographic tilt series were obtained with ~7 μm defocus using a 2° angular increment and ranged from approximately −64° to 64°. The cumulative electron dose was ~80 $e^{-}/Å^2$ for the whole tilt series. Thus, ultimately, the sample was exposed to a cumulative dose of ~100 $e^{-}/Å^2$.

2.3. Image processing

Tomograms were calculated from tilt series using the program IMOD (Kremer et al., 1996). The final analyses were performed on 2× binned tomograms with a voxel size of 15.6 Å. Sub-tomograms of 50 × 50 × 50 voxels, each containing an image of a virus, were excised. Particles were contrast inverted and roughly centered using the program Proc3d (Ludtke et al., 1999).

2.4. Sub-tomogram analysis

The orientation of the immature structure within each mosaic particle in a sub-tomogram was determined by finding the highest correlation coefficient between the particle in the sub-tomogram and a series of differently-oriented structures of immature dengue virus 2 derived from a single-particle cryo-EM reconstruction (Yu et al., 2008). The immature structure was superimposed onto the observed tomogram in all possible orientations to cover the icosahedral asymmetric unit. The orientations were separated by angular increments of 2.5°. The highest correlation coefficient, determined by comparing the density distribution of the model and the tomogram, was then selected from this series and plotted onto a stereographic projection. The orientation and particle center position were then refined with smaller angular and translational increments, respectively.

2.5. Determination of the orientation of the icosahedral symmetries of the immature and mature regions of one mosaic particle using the cryo-EM data

The mosaic dengue virus particles were boxed, centered, and corrected for the contrast transfer function with programs from eman and eman2 packages (Ludtke et al., 1999; Tang et al., 2007). The Spider program was used to prepare two-dimensional projections of cryo-EM reconstructions of immature and mature dengue virus particles (Shaikh et al., 2008). Projections were created covering all possible views of the icosahedral particles with angular separation of 2.5°. The cryo-EM "high" dose images of the mosaic particles were then correlated with projections of both mature and immature dengue virus structures. The correlation coefficients were plotted using program O2D from Uppsala Software Factory (Kleywegt et al., 2001).

2.6. Determination of the relative orientations of the mature and immature regions within one particle

The results of the searches for the orientations of the immature and mature regions were expressed in polar angles within one icosahedral asymmetric unit $\varphi = (-20.9°;\ 20.9°)$, $\psi = (0°;\ 31.7°)$, $\kappa = (0°;\ 360°)$ (Fig. 1). The relative orientations of the two regions could then be directly compared.

2.7. Comparison of the relative orientations of icosahedral symmetries of immature and mature regions within mosaic particles

The relative orientations of icosahedral symmetries of the immature and mature regions among different particles were compared using the following approach for each of the mosaic particles. A rotation matrix (I) was calculated to bring the immature structure to a standard icosahedral orientation based on the orientation of the immature region as determined in the "high" dose images (note that there are 60 such rotations possible). In addition, a matrix (M) was calculated that rotates the icosahedral symmetry from the standard orientation to the orientation of the mature domain (there are also 60 possibilities). The product of the

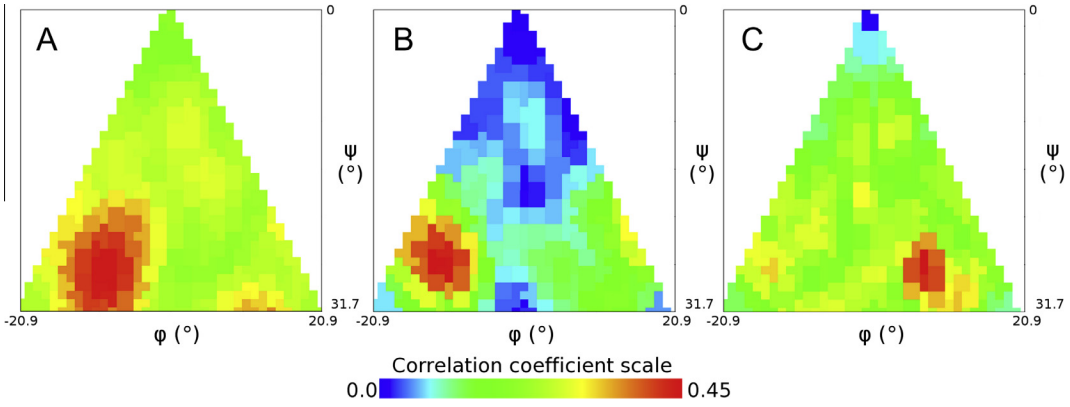

Fig.1. Plots of correlation coefficients. (A) Determination of the orientation of the immature region of a mosaic particle by correlating the sub-tomogram with an immature single-particle model. (B and C) Analysis of the two-dimensional cryo-EM image of the same particle as in A. (B) Determination of the orientation of the immature region. (C) Determination of the orientation of the mature region.