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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 pleiomorphic 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.

Keywords

flavivirus; dengue; maturation; symmetry; icosahedron; mismatch

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.

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Flavivirus virions are enveloped with a diameter of $\sim 500\text{\AA}$. 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 (Crick and Watson, 1956; Crick and Watson, 1957) as well as Caspar and Klug (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; Zhang et al., 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.

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 10nm colloidal gold particles. The solution (3.5 μ l) 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 300keV. The Krios microscope was equipped with a Gatan (Pleasanton, CA) energy filter operated in zero-energy-loss mode with a slit width of 30e⁻V. Images were recorded on a 2048 $\times$ 2048-pixel CCD camera at a nominal magnification of 19500. First, the single-particle images were collected at $\sim 5\mu$ m defocus with a total dose of 20 e⁻/ $\text{\AA}^2$. Then tomographic tilt series were obtained with $\sim 7\mu$ m defocus using a 2 $^\circ$ angular increment and ranged from approximately -64° to 64° . The cumulative electron dose was ~ 80 e⁻/ $\text{\AA}^2$ for the whole tilt series. Thus, ultimately, the sample was exposed to a cumulative dose of ~ 100 e⁻/ $\text{\AA}^2$.

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 $\times$ binned tomograms with a voxel size of 15.6 $\text{\AA}$.

Sub-tomograms of $50 \times 50 \times 50$ 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 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 $\phi = (-20.9^\circ; 20.9^\circ)$ $\psi = (0^\circ; 31.7^\circ)$ $\kappa = (0^\circ; 360^\circ)$ (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 matrices, therefore, describes the rotation between the immature structure in standard orientation and the mature structure. However, the relative rotations between the two domains for different particles would not necessarily end within one icosahedral asymmetric unit $\phi = (-20.9^\circ; 20.9^\circ)$ $\psi = (0^\circ; 31.7^\circ)$ $\kappa = (0^\circ; 360^\circ)$ (Fig. 2). Therefore, such a combination of the 60 possible I matrices with a specific M matrix was selected to be within a specified icosahedral asymmetric unit (Fig. 2). This demonstrated that there was no unique combination of the icosahedral symmetries in the mosaic particles.

2.8. Assessing the correlation of the mosaic particles with immature and mature dengue virus structures

As the orientations of the immature and mature domains had been established, the correlation coefficients could be calculated for volumes corresponding to individual icosahedral asymmetric units with the immature and with the mature structures. These correlation coefficients compared the electron density maps of the sub-tomograms and appropriately oriented models affected by a simulated missing wedge.

3. Results and Discussion

3.1. Structure of dengue virus maturation intermediates

Cryo-electron microscopy (cryo-EM) and cryo-electron tomography (cryo-ET) analyses had shown that maturation intermediates of dengue virus 2 are pleomorphic with different ratios of the envelope glycoproteins within one particle organized partially as in the immature and partially as in the mature virus (Fig. 3) (Cherrier et al., 2009; Nelson et al., 2008; Plevka et al., 2011). Therefore, the three-dimensional structure of these particles cannot be obtained by single-particle methods that rely on averaging of particles with the same structure.

Maturation intermediates of dengue virus 2 were prepared *in vitro* by growing the virus in the presence of ammonium chloride, which leads to release of immature particles from infected cells (Yu et al., 2008). When the immature particles were transferred into a low pH buffer, some of them changed conformation to the mature herringbone structure while others displayed regions of the glycoprotein envelope with both immature and mature conformations (Fig. 3C and D). Similarly, some such “mosaic” particles can be observed in mature virus preparations (Cherrier et al., 2009; Nelson et al., 2008). There is nearly complete prM cleavage at low pH in the presence of furin (Yu et al., 2008). However, there are still some domains with the immature conformation at low pH when there is no furin present. Thus, apparently, the furin cleavage may further promote the conformational change of the glycoproteins. However, in our *in vitro* experiments there was no furin. This may account for the fact that we observe many mosaic particles.

We used a combination of two-dimensional single particle and three-dimensional tomographic analyses to study the maturation mechanism of dengue virus 2 (Fig. 3C–F). First a “high” dose ($\sim 20\text{e}^-/\text{\AA}^2$) image was taken of the virions in a particular hole in the holey carbon, and then a tomographic tilt series (total dose of $\sim 80\text{e}^-/\text{\AA}^2$) was collected of the same region in the EM grid. The orientations of the icosahedral symmetry axes of the immature and mature regions of each mosaic virus were determined from the “high” dose images by calculating the correlation coefficients between the “high” dose images and a survey of projections of the previously determined immature dengue virus structure and also of the mature dengue virus structure covering all possible orientations (at 2.5° increments) (Kuhn et al., 2002; Yu et al., 2008; Zhang et al., 2013a). This established the orientation of the mature and immature regions of each mosaic particle (Fig. 1). It was necessary to use the two-dimensional “high” dose images as opposed to the three-dimensional tomographic reconstructions for determining the orientations as it was not possible to determine the orientation of the mature region at the limiting $\sim 50\text{\AA}$ resolution of the tomograms because of the lack of prominent features in the mature structure at this resolution (Plevka et al., 2011). The icosahedral symmetry axes of the immature and mature regions within any one particle were found to be mismatched (Fig. 1B and C).

The tomographic tilt series were used to calculate a three-dimensional reconstruction of each chimeric particle. The previously determined three-dimensional structure of the immature dengue virus particle was used to find the orientation of the immature region within each mosaic particle in the tomographic reconstructions (Fig. 1A). In 105 out of the 129 particles

analyzed, the orientation searches for the immature region using the two-dimensional “high” dose images and the three-dimensional tomographic reconstructions were within 5° of each other (Fig. 1A and B). However, the mature structure does not have enough features to be recognized in the tomograms to permit determination of the orientation and position of the mature region.

Knowledge of the orientations of the immature and mature regions within each mosaic particle (as determined from the two-dimensional “high” dose images) made it possible to compare the three-dimensional tomographic reconstructions with appropriately oriented immature and mature dengue virus structures and thus determine which regions of each mosaic particle corresponded to the mature and immature structures (please see Materials and methods section for details). The immature and mature structures formed compact regions on opposite sides of the particles (Fig. 4). The different orientations of the icosahedral symmetry axes of the immature and mature regions within one particle, together with the localization of the mature and immature components on opposite sides of each particle, indicate that the nucleation center of the mature structure did not form within the icosahedral framework of the immature virus.

The relative orientations of the immature and mature domains in different mosaic particles were compared by bringing the immature regions into a standard orientation and then displaying orientations of the icosahedral symmetry axes of the mature regions (Fig. 2). The relative orientations of the immature and mature structures were found to differ among particles (Fig. 2). Therefore, the orientation of the nucleation center of the mature structure had to be random with respect to the icosahedral symmetry of the immature domain. Thus, there cannot be a single maturation pathway that is followed by all dengue virus virions.

3.2. Maturation process and structural dynamics of flavivirus virions

The following proposed mechanism (Fig. 5) for dengue virus maturation is based on the above analysis of mosaic, partly immature and partly mature virions. When the virions encounter low pH in the trans-Golgi compartments some of the trimeric spikes on immature particles fall apart. The prM-E heterodimers can then sample different orientations. However, the heterodimers are anchored in the virion surface by their transmembrane helices and are limited in their lateral movements by neighboring subunits. Eventually, a few of the prM-E heterodimers will form a stable “nucleation center” with a mature, herringbone arrangement. As the nucleation center is formed independently of the original immature structure, in most cases the orientations of the icosahedral symmetries of the immature and mature regions will be different (Fig. 2). Subsequently, the mature region spreads whenever the prM-E heterodimers at the boundary of the region achieve a suitable orientation and conformation to add to the already existing herringbone structure. This maturation mechanism is possible because the envelope glycoproteins are attached to the virion membrane and their movement is restricted to the two-dimensional surface of the particle. It is possible that some of the virions might contain more than one nucleation center. However, no particles were detected that contained more than one mature region with mismatched icosahedral symmetries.

The present results show that the icosahedral symmetry of flavivirus virions is disrupted during the maturation process. Additional observations also indicate that flavivirus virions can transiently lose ordered structure of the envelope glycoproteins without losing infectivity. Incubation of virions at pH 10 induced detachment of E protein ectodomains from the virion membrane. However, after back neutralization to pH 7, the virions were infectious (Stiasny et al., 2007). Dengue virions produced at 28°C that were subsequently heated to 37°C reorganized into “bumpy” particles and, as a group, became rather heterogeneous in structures (Fibriansah et al., 2013; Zhang et al., 2013b). The

transformation of the particles into the bumpy conformation did not affect their infectivity. Thus, the transient disruption of icosahedral organization of flavivirus particles is probably crucial not only for assembly and maturation processes but also for the initiation of membrane fusion. Therefore, compounds that could limit the freedom of movement of the glycoproteins in the virion membrane might be used as inhibitors targeting both maturation and fusion. Such compounds may include agents affecting the fluidity of the viral membrane.

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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.

Fig. 2.

Comparison of relative orientations of icosahedral symmetries of immature and mature regions in mosaic dengue virus particles. The icosahedral symmetry of the immature region of each mosaic particle was brought to a standard orientation. Borders of the icosahedral asymmetric unit in the standard orientation are demarcated by blue lines and the positions of the twofold, threefold, and fivefold axes are shown as semi-transparent ovals, triangles, and pentagons, respectively. The position of the fivefold axis of the mature region relative to the immature region of each particle is shown as a black “t”. The vertical line of the letter “t” points towards the twofold axis. The red “t” shows the situation when the mature symmetry is coincident with the immature symmetry. However, such a particle did not exist (Materials and methods)

Fig. 3.

Cryo-electron microscopy images of dengue virus virions in different maturation stages. (A and B) Immature particles. (C and D) Mosaic particles displaying immature and mature regions on their surface. (E and F) Projections of reconstructed tomograms of the same particles as in C and D. (G and H) Mature virions.

Fig. 4. Structural analysis of a mosaic particle. The colors of the icosahedral faces represent the correlation coefficient of a particular icosahedral asymmetric unit compared with appropriately oriented immature (upper row) and mature (bottom row) structures. The particle is shown in three orientations to demonstrate that the immature and mature structures occupy opposing regions of the particle.

Fig. 5.

Overview of flavivirus maturation. Immature particles form by budding into the lumen of the ER. The virions encounter a decrease in pH from 7 to 5.5 as they are transported from the ER to the Golgi and trans-Golgi network. The pH change induces disruption of the immature structure leading to formation of the nucleation center of the mature structure. The orientation of the mature structure is random with respect to the immature structure. The mature structure gradually spreads over the particle surface. Once the capsid is in the mature conformation cellular protease furin can cleave the prM protein into pr and M fragments. When the particles are released from cells into neutral pH, the pr fragments dissociate from the particles leaving mature infectious virions. The three quasi-equivalent E proteins are colored in blue, green, and red. The pr domain is shown in yellow. The E proteins in the immature structure are shown in light colors whereas the proteins in the mature structure are shown in bright colors.