

Structural biology of old world and new world alphaviruses

A. Paredes^{1,*}, S. Weaver², S. Watowich^{3,4}, and W. Chiu¹

¹National Center for Macromolecular Imaging, Verna and Marrs McLean
Department of Biochemistry and Molecular Biology,
Baylor College of Medicine, Houston, TX, U.S.A.

²Department of Pathology, University of Texas Medical Branch,
Galveston, TX, U.S.A.

³Department of Human Biological Chemistry and Genetics,
University of Texas Medical Branch, Galveston, TX, U.S.A.

⁴Department of Sealy Center for Structural Biology, University of Texas
Medical Branch, Galveston, TX, U.S.A.

Introduction

Alphaviruses (family *Togaviridae*, genus *Alphavirus*) have been classified as belonging to either Old World or New World types depending on where they occur [4]. Some Old World alphaviruses such as Sindbis virus are found in Africa, Europe, Asia and Australia, whereas New World alphaviruses such as Venezuelan equine encephalomyelitis virus (VEEV) are found in the Americas. Alphaviruses are typically transmitted to humans and animals through the bite of an infected mosquito. In general, Old World alphaviruses cause less severe disease and have lower mortality rates in humans relative to New World alphaviruses [7]. Old World alphavirus infections are typically characterized by rash and arthritis, while New World alphavirus infections are typically characterized by debilitating febrile disease and, sometimes, encephalitis. Because of their disease severity, and their ease of growth and transmission, many New World alphaviruses are classified as category B bioagents. New World alphaviruses can also cause severe economic impacts, since they cause disease in livestock as well as in humans.

Alphaviruses are positive-sense ssRNA enveloped viruses that measure ~700 Å in diameter. They are composed of two membrane associated glycoproteins, a host cell derived lipid bilayer, and a nucleocapsid composed of capsid proteins and a 49S RNA molecule. The outer envelope is believed to be made up of 80 E1/E2 heterotrimers arranged as 120 dimers of the E1 protein around 80 homotrimers of E2 in a T = 4 icosahedral lattice [7, 8, 13]. The 400 Å diameter nucleocapsid is made up of 240 copies of the capsid protein organized in a T = 4 icosahedral arrangement [11]. The envelope proteins and the nucleocapsid are believed to interact through C-terminal residues of E2 that are exposed on the inner surface of the lipid bilayer and the capsid protein C-terminal domain. The

E1–E1 glycoprotein interactions form the scaffolding lattice, which help stabilize the virus icosahedral structure. The E2 glycoprotein is the primary component of the morphological spikes and is responsible for host cell receptor recognition [1].

The mechanism by which alphaviruses penetrate host cells has been studied for many years. It has been widely accepted that many enveloped viruses employ a fusion-type mechanism to pass through the host cell membrane and initiate infection [16, 17]. This conclusion was based in part on early observations that cell-to-cell fusion of infected cells occurred upon exposure of the virus to low pH [14]. Although many enveloped viruses do not rely on low pH to trigger fusion, for those that do the endosome was believed to be the site of virus-host cell penetration because it was the only site within the cell where the virus could be expected to encounter a low pH environment. A notable exception to the endosomal route in a system shown to demonstrate low pH triggered fusion, was the alphaviruses. Although alphavirus-mediated fusion has been observed, this phenomenon has not been demonstrated to occur at the endosomal pH. Rather, for alphavirus-mediated fusion to be observed, alphaviruses attached to cells must be briefly exposed to low pH, and returned to neutral pH for fusion to occur [3]. This is a situation never expected to occur within an endosome. Alphavirus penetration may occur instead through the action of a pore formed from the interaction between the virus and the host-cell receptor. It is through this pore that virus RNA may be injected into the cell cytoplasm [12].

Structural studies of alphaviruses and components

Because of their impact on human health and the livestock industry, alphaviruses are an important group of viruses to study. Their RNA and protein sequences are highly conserved among species, suggesting that their three-dimensional structures may be similar [4]. The low resolution structures of Sindbis, Semliki Forest (SFV), Ross River, VEEV and Aura viruses support this generalization. However, since alphavirus lineages display significant differences in tissue tropism and pathobiology [7], it is possible that local conformational differences between the viruses and their molecular components are responsible for their different phenotypes.

High-resolution structural studies of alphaviruses are complicated by their enveloped nature and their pathogenicities. Three-dimensional alphavirus crystals, although relatively easy to produce, typically have not diffracted beyond 30 Å resolution [5, 18]. X-ray structural studies have been limited to the individual viral proteins [2, 8]. While the structures of stable domains of the E1 and capsid protein have been determined by X-ray crystallography, the structure of E2 is unknown. The difficulty in producing diffraction-quality E2 crystals may lie in the strong affinity between E2 and E1.

Isolated alphaviruses are icosahedral, highly homogenous, and stable. These properties facilitate electron cryomicroscopy (cryo-EM) structural studies of these viruses. Cryo-EM has succeeded in generating intermediate resolution structures of SFV, Sindbis virus and VEEV [10, 11, 20]. Combining high-resolution X-ray

structures with cryo-EM structures has allowed us to generate pseudo-atomic models of the virus and its intermolecular interactions. This approach has been used to investigate intermolecular contacts among and between the nucleocapsid and E1 proteins [8, 20].

Structural studies of VEEV

VEEV is one of the more virulent alphaviruses and poses a significant health threat within the Americas [15]. Understanding its structure is vital for developing effective strategies to combat VEEV infections. The structure of TC-83 VEEV has been determined by cryo-EM to 15 Å resolution (Figs. 1–3). The 680 Å diameter structure is composed of 80 E2 trimers arranged on a $T = 4$ lattice. This envelope structure appears similar to the 11 Å resolution structure of SFV [10]. Both structures show that the trimers are flattened at the extended tips, possibly to display the receptor recognition site on the virus surface. In VEEV virus, the trimers rise ~ 84 Å above the outer leaflet of the virus membrane (Fig. 3). As was described for SFV, the outer spike layer is divided into two regions, the exposed projecting domains and the skirt region [10]. In our reconstruction, the projecting domains measure 48 Å and sit on top of the skirt region which is 36 Å deep. There is evidence that the projecting domains are primarily E2 while the skirt region is composed of E1 [1, 8]. Interactions between the E2 proteins in the center of the timer seal the cavity within the skirt region in both SFV and VEEV [10].

The cryo-EM structures from different alphaviruses are sufficiently resolved to detect subtle structural differences. For instance, the 400 Å diameter nucleocapsid of VEEV (Fig. 2) is structurally different from those of the Old World alpha-

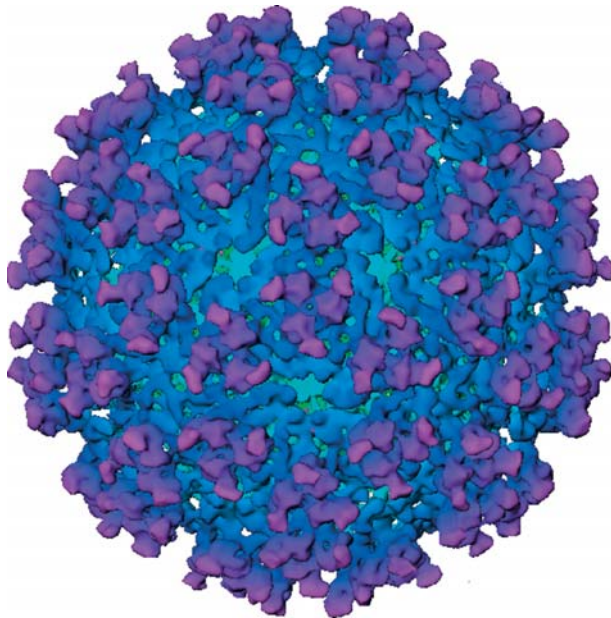


Fig. 1. 15 Å structure of Venezuelan equine encephalomyelitis virus (680 Å in diameter) generated from approximately 900 particle images recorded in a JEOL4000 electron cryomicroscope operated at 400 kV and -170 °C specimen temperature. The map has been bilaterally filtered [6]. The surface representation of the map is colored according to radius

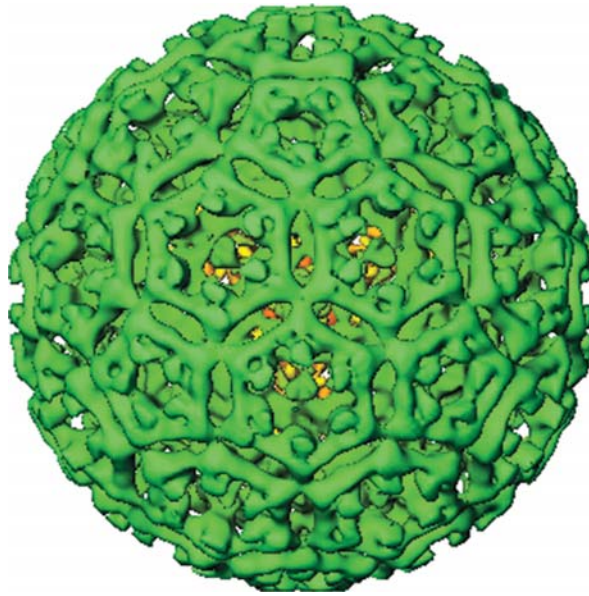


Fig. 2. VEEV Nucleocapsid ($\sim 400 \text{ \AA}$ in diameter) viewed along the 3-fold axis

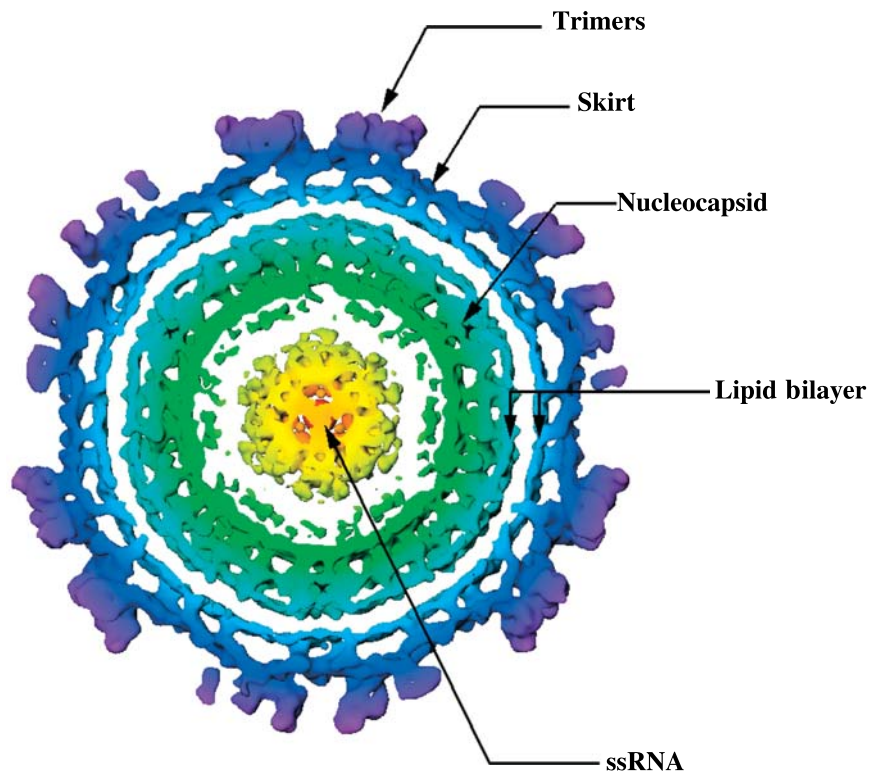


Fig. 3. Slice (53 \AA in thickness) of density of the VEEV normal to the 3-fold axis. The slice shows the outer most trimers which are composed primarily of E2 and sit on the skirt region. The skirt is composed of E1–E1 interactions forming the icosahedral structure of the virus through which the transmembranal domains of E2 pass to associate and bind to the virus nucleocapsid. The slice also includes regions attributed to the nucleocapsid, the lipid bilayer and the ssRNA

viruses. In all alphavirus structures determined so far, the 240 individual capsid proteins are arranged into 12 pentamers and 30 hexamers in a $T = 4$ arrangement. In structures of Old World alphaviruses, the nucleocapsids appear to have a slight clockwise twist of the pentamer relative to the hexamer. This twist of nucleocapsid pentamers and hexamers is not seen in the VEEV nucleocapsid, providing the first evidence that the structures of New and Old World alphaviruses differ. In VEEV, the vertices of each pentamer point towards the two fold axis of symmetry at the vertices of two neighboring hexamers, forming a strong local three fold axis of symmetry (Fig. 2). A reexamination of the New World Aura virus has shown that its nucleocapsid is arranged similar to that of VEEV [19]. This difference in nucleocapsid structure can be traced to the different capsid–capsid interactions that likely exist within the VEEV nucleocapsid. Based on examination of 15 Å resolution structures, these differences do not appear to be transmitted through the viral membrane to the envelope proteins because the VEEV envelope appears to adopt a conformation that is structurally similar to that of the Old World alphaviruses. Given the observation that New World alphaviruses typically cause a more severe disease in humans and livestock than do Old World alphaviruses, these structural differences may be related to the pathobiology of the virus. In addition, conformational differences in the nucleocapsid may influence virus entry, disassembly, assembly, and/or budding.

Future cryo-EM studies of hazardous viral pathogens

The ability to visualize virus structures has been extremely important in understanding virus replication, assembly and pathobiology. Cryo-EM has provided numerous intermediate resolution structures of icosahedral viruses that are generally considered low health risk pathogens (see review: [21]). However, most microbes classified by the Centers for Disease Control and Prevention as category A, B and C pathogens have not been pursued structurally due in part to the difficulty in examining these pathogens. In elucidating the structures of pathogenic viruses by cryo-EM, we will need to confront several challenges.

The first challenge is to produce highly purified and concentrated virus samples. Generally speaking, 10^{10} – 10^{11} particles per ml yield sufficient particle density per micrograph to enable an intermediate resolution structure to be efficiently determined by cryo-EM. Less concentrated virus samples require a significantly longer time to record the 5,000–10,000 particle images needed for an icosahedral particle reconstruction below 10 Å resolution.

The second challenge is the lack of icosahedral symmetry in many category A–C viruses, thus requiring reconstruction algorithms that are not dependent on the symmetry of the object for structural determination. Without particle symmetry to improve the signal-to-noise ratio of the reconstruction, ~ 60 times more particle images are required to produce equivalent resolutions from asymmetric virus particles. Robust algorithms to perform such reconstructions have recently been developed and used successfully to determine the structure of non-viral particles to subnanometer resolution [9].

The third challenge is possible structural heterogeneity of the particles. It is conceivable that viral particles are dynamic and exist as an ensemble of structural conformations. This structural heterogeneity will limit the resolution of any three-dimensional reconstruction. However, it is plausible that one could develop new software to computationally “purify” particles that occur as discrete states within conformational ensembles. Because of the relatively large size of the virus particles and the possible continuum sampling of structural conformers, such an approach will present new challenges in the computational approach both at the level of algorithm development and implementation.

The fourth challenge in working with highly infectious particles is operator safety, since the particles maybe biologically active when placed inside the electron cryomicroscope. In a typical sample preparation, each cryo-EM grid would contain over a million of virus particles which are frozen, hydrated and can become aerosolized simply by allowing the grid to thaw inside a laboratory environment. Therefore, special specimen handling procedures for cryo-specimen preparation and transfer into the microscope column need to be developed to fulfill stringent safety guidelines. Furthermore, the maintenance of the instrument by microscope engineers also requires new protocols to ensure safety during instrument maintenance. Active research in robotic methods to handle specimens and microscope operation may yield a practical solution to safety issues within biosafety laboratories.

It is anticipated that in the near future active research programs investigating structures of highly pathogenic organisms will yield technical solutions to the above challenges. For instance, safety issues can be addressed by placing electron cryomicroscopes in BSL3 and 4 facilities so that biologically active viral particles can be examined at the highest possible resolution while remaining preserved in a completely contained environment. By determining the three-dimensional structures of important viral pathogens, we can build a structural encyclopedia of important pathogens. Such information can have a number of useful applications, including provisional viral identification during an outbreak, structure-based design of novel therapeutics and vaccines, and understanding molecular mechanisms of virus assembly and disassembly. The utility of such a research program will undoubtedly contribute to the overall effort in biodefense research in this country.

Acknowledgement

We thank the support of the grants from NIH (P41RR02250, R01AI38469), the Robert Welch Foundation and the Sealy Center for Structural Biology (UTMB). We thank Dr. Wen Jiang for assistance in data processing.

References

1. Anthony RP, Brown DT (1991) Protein–protein interactions in an alphavirus membrane. *J Virol* 65: 1187–1194
2. Choi HK, Tong L, Minor W, Dumas P, Boege U, Rossmann MG, Wengler G (1991) Structure of the Sindbis virus core protein reveals a chymotrypsin-like serine protease and the organization of the virion. *Nature* 354: 37–43

3. Edwards J, Brown DT (1986) Sindbis virus-mediated cell fusion from without is a two-step event. *J Gen Virol* 67: 377–380
4. Griffin DE (2001) Alphaviruses. In: Knipe DM, Howley PM (eds) *Fields virology*. Lippincott-Raven, Philadelphia, PA, vol 1, pp 917–962
5. Harrison SC, Strong RK, Schlesinger S, Schlesinger MJ (1992) Crystallization of Sindbis virus and its nucleocapsid. *J Mol Biol* 226: 277–280
6. Jiang W, Baker ML, Wu Q, Bajaj C, Chiu W (2003) Applications of a bilateral deionising filter in biological electron microscopy. *J Struct Biol* 144: 114–122
7. Johnston RE, Peters CJ (1996) Alphaviruses. In: Fields BN, Knipe DM, Howley PM (eds) *Fields virology*. Lippincott-Raven, Philadelphia, PA, pp 843–898
8. Lescar J, Roussel A, Wien MW, Navaza J, Fuller SD, Wengler G, Rey FA (2001) The Fusion glycoprotein shell of Semliki Forest virus: an icosahedral assembly primed for fusogenic activation at endosomal pH [comment]. *Cell* 105: 137–148
9. Ludtke SJ, Chen DH, Song JL, Chuang DT, Chiu W (2004) Seeing GroEL at 6 Å resolution by single particle electron cryomicroscopy. *Structure (Camb)* 12: 1129–1136
10. Mancini EJ, Clarke M, Gowen BE, Rutten T, Fuller SD (2000) Cryo-electron microscopy reveals the functional organization of an enveloped virus, Semliki Forest virus. *Mol Cell* 5: 255–266
11. Paredes AM, Brown DT, Rothnagel R, Chiu W, Schoepp RJ, Johnston RE, Prasad BV (1993) Three-dimensional structure of a membrane-containing virus. *Proc Natl Acad Sci USA* 90: 9095–9099
12. Paredes AM, Ferreira D, Horton M, Saad A, Tsuruta H, Johnston RE, Klimstra WB, Ryman KD, Hernandez R, Chiu W, Brown DT (2004) Conformational changes in Sindbis virions resulting from exposure to low pH and interactions with cells suggest that cell penetration may occur at the cell surface in the absence of membrane fusion. *Virology* 324: 373–386
13. Pletnev SV, Zhang W, Mukhopadhyay S, Fisher BR, Hernandez R, Brown DT, Baker TS, Rossmann MG, Kuhn RJ (2001) Locations of carbohydrate sites on alphavirus glycoproteins show that E1 forms an icosahedral scaffold. *Cell* 105: 127–136
14. Stegmann T, Booy FP, Wilschut J (1987) Effects of low pH on influenza virus. Activation and inactivation of the membrane fusion capacity of the hemagglutinin. *J Biol Chem* 262: 17744–17749
15. Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC (2004) Venezuelan equine encephalitis. *Annu Rev Entomol* 49: 141–174
16. Weissenhorn W, Dessen A, Calder LJ, Harrison SC, Skehel JJ, Wiley DC (1999) Structural basis for membrane fusion by enveloped viruses. *Mol Membr Biol* 16: 3–9
17. White JM, Hoffman LR, Arevalo JH, Wilson IA (1997) Attachment and entry of influenza virus into host cells: pivotal roles of the hemagglutinin. In: Chiu W, Burnett RM, Garcea RL (eds) *Structural biology of viruses*. Oxford Press, New York, pp 80–104
18. Wiley DC, von Bonsdorff CH (1978) Three-dimensional crystals of the lipid-enveloped Semliki Forest virus. *J Mol Biol* 120: 375–379
19. Zhang W, Fisher BR, Olson NH, Strauss JH, Kuhn RJ, Baker TS (2002) Aura virus structure suggests that the T = 4 organization is a fundamental property of viral structural proteins. *J Virol* 76: 7239–7246
20. Zhang W, Mukhopadhyay S, Pletnev SV, Baker TS, Kuhn RJ, Rossmann MG (2002) Placement of the structural proteins in sindbis virus. *J Virol* 76: 11645–11658
21. Zhou ZH, Chiu W (2003) Structural determination of icosahedral viruses by electron cryomicroscopy at sub-nanometer resolution. *Adv Protein Chem* 64: 93–130

Author's address: Wah Chiu, National Center for Macromolecular Imaging, Baylor College of Medicine, Houston, TX 77030, U.S.A.; e-mail: wah@bcm.tmc.edu