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Review

Divergence from canon: an additional opportunity for alphavirus entry

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ABSTRACT

The study of animal virus entry is a very difficult endeavor with many obscurities; thus the field of virology finds itself with vast chasms of disagreement on this topic. To further complicate this difficulty, characteristics specific to each virus system combine to make experimental interpretation difficult. For example, a virus species may be programmed to enter through differing pathways depending on the cell type and host species of cell that it infects. Further, viruses can characteristically have high particle-to-plaque-forming unit (PFU) ratios that make observations by direct means difficult to interpret. These problems have plagued the field of virology for many years and will likely persist into the foreseeable future until methods and technologies advance to allow for increased resolution of these scientific impasses. This review will attempt to highlight the history, methodologies, and new developments within the field of alphavirus biology in the context of both receptor-mediated endocytosis and direct penetration hypotheses of virus penetration. Additionally, comparisons will be made between the proposed entry mechanisms of *Alphaviruses*, and other enveloped viruses that may not follow orthodox pathways, to demonstrate the technical problems encountered by these studies.

KEYWORDS: alphaviruses, entry mechanisms, replication.

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1. Introduction

1.1. Alphaviruses, their superstructure, and genomic organization

The prototypical alphavirus (Figure 1), Sindbis virus, has an icosahedral configuration and a triangulation number of 4 [1]. The ~70 nm virion is comprised of two nested protein shells with a host-derived membrane that resides between the two shells [1]. The outer shell is composed of 240 copies each of the E1 and E2 glycoproteins which form heterodimeric E1/E2 complexes which further trimerize into the 80 glycoprotein spikes found on the surface of the virion with E1 being nearly completely covered by E2 [1, 2, 3]. The inner protein shell contains 240 copies of the capsid protein (C) and retains an identical icosahedral, T = 4 configuration [1]. Out of both of the structural glycoproteins, only E2 spans the membrane and interacts through hydrophobic interactions with the capsid protein to form a connection between the core of the protein and the surface components [4, 5]. Within the capsid of the virion resides a single-stranded, ~11 kb RNA molecule of positive polarity. From 5' to 3', this molecule contains genes for the nonstructural and structural proteins in the following order: nsP1, nsP2, nsP3, nsP4, capsid, E3, E2, 6K/TF, and E1 [6]. This RNA molecule contains a 5' methylguanosine cap and a 3' poly-A tail and can function as infectious mRNA in the host cell [6].

2. Alphavirus structural proteins

Alphaviruses have three main proteins that contribute to the structure of the mature virion:

E1, E2, and capsid. Recently, nsP2, the viral protease has been found associated with mature infectious particles and may also participate in a structural capacity (manuscript in review). Here, the current knowledge pertaining to the structure and/or function of the structural proteins will be discussed. E1 and E2 are both glycoproteins that interact on the surface of the virion to facilitate entry into the host cell [1, 2, 7, 8]. Evidence has been put forth that implicates E2 in receptor recognition and E1 as the fusogenic element involved in the fusion event between the virus and host endosomal membrane [8].

E1 (47.3 kDa) is considered a Class II virus membrane fusion protein and exhibits a β-dominated secondary structure that forms an ectodomain of elongated tertiary structure containing three domains (Figure 2). The X-ray crystal structure of the E1 ectodomain of Semliki Forest virus (SFV) has been characterized [9]; SFV-E1 bears 50% sequence identity and 68% similarity to that of Sindbis virus E1. Domain I (d1) is a β-sandwich consisting of 8 β strands (10 total β strands in the domain) that encodes high-mannose, N-linked glycosylation sites at residues 139 and 245, though these specific glycosylation sites are not conserved across the alphaviruses, or may be absent [9-12]. E1 Domain II (dII) is comprised of 13 β strands that form a finger-like projection, an α helix, and 3 one-turn 3/10 helices [9]. DII is not a continuous domain in itself, but is formed by 2 “excursions” of the polypeptide that interact to form the two opposing sides of the DII module [9]. DII also contains a notable functional feature: the putative fusion loop. The fusion loop exists at the tip of the elongated E1 structure and, as would be expected, contains ~20 hydrophobic residues [13]. This putative fusion loop is proposed to facilitate fusion by its insertion into the membrane of the host endosomal membrane during the fusion [8] event of the virion and endosomal membrane, as described by the receptor-mediated endocytosis hypothesis. Domain III (dIII) connects the ectodomain to the transmembrane domain that anchors E1 to the viral membrane. This domain contains 8 β strands that are organized into a fold that is characteristic of the immunoglobulin super family [9]. Immediately after the DIII domain is the C-terminal transmembrane region of approximately 24 residues that anchors E1 to the envelope of the virion. This region is not represented in the crystal but has been modeled into the CryoEM density of Sindbis virus [8]. Depending on the alphavirus, there may be a short E1 tail that extends past the membrane [9].

Preliminary structures of the E2 (46.9 kDa) protein are presented (Figure 2); however, the accuracy of these structures falls under question [14, 15]. Uncertainties in one study [14] regarding the biological relevance of the structure arose because the structure was characterized in the absence of phospholipids and because the E1 and E2 proteins were grown recombinantly, connected with a linker without the normal proteolytic processing or the membrane anchors. The resulting crystal structure showed all cysteine residues participating in disulfide bridges [9]. However, an analysis of a whole infectious virus preparation (particle/PFU ratio ~1) [16] by liquid chromatography-tandem mass spectrotemtry showed that two disulfide bonds (C49-C114 and C259-C271) are available for chemical reduction and the disulfide bonds are therefore not present, as proposed by a crystal structure [9] suggesting that preparations of recombinant virus proteins used for crystallization may adopt various conformations.

The capsid protein (29.4 kDa) forms a T = 4 shell around the genomic RNA of the alphaviruses [7]. Lee, *et al*. characterized the structure of the Sindbis capsid protein using X-ray crystallography [4]. In this study, a hydrophobic pocket was observed that was occupied by segments of proximal capsid proteins in the crystal structure [4]. It was suggested after aligning the amino acid sequences of capsid and E2 that this hydrophobic pocket could accommodate the carboxy-terminal tail of the E2 protein [4]. This hypothesis has been supported by other studies [1, 5]. It is interesting to note that in a study presented by Sokoloski *et al.* affinities between the capsid protein and genomic viral RNA are different: capsid-RNA interactions in the cytoplasm during early infection are specific while those between capsid and RNA in the mature virus are not [17]. In this study, capsid proteins bearing mutations that disrupt interactions with RNA highlight the importance of capsid-protein interactions in early infection. Disruption of these interactions contributes

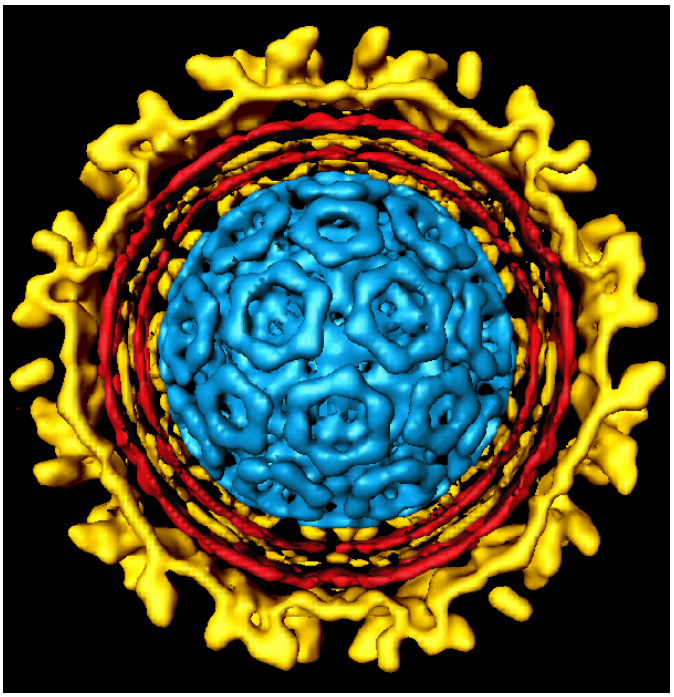

Figure 1. A cryo-electron microscopy (EM) reconstruction of Sindbis Virus. In this reconstruction, the E1/E2 glycoprotein coat is seen in yellow, the lipid bilayer is seen in red, and the nucleocapsid core is seen in blue. Though not seen in this image, the E2 protein crosses the membrane and interacts with the capsid monomers. The capsid exhibits a T = 4 triangulation number like the glycoprotein coat. (Reproduced from Sharp, J., Nelson, S., Brown, D. and Tomer, K. 2006, Virology, 348, 216-223: Copyright 2006, with permission from Elsevier).

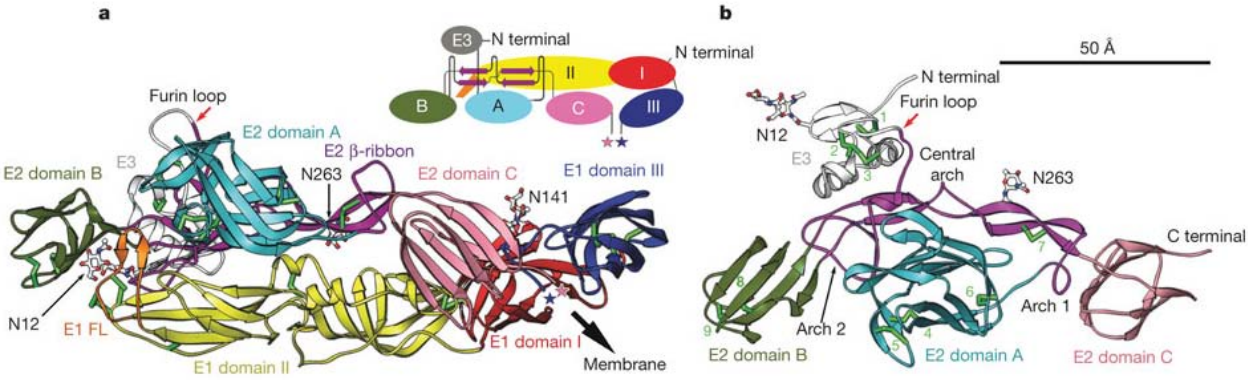

Figure 2. Schematic of E1:E2 interactions before E3 cleavage. (a) Model of the chikungunya virus heterodimer. The domains of the E1 protein are colored as follows: DI, red; DII, yellow; DIII, blue, and the fusion loop is colored orange. The domains of the E2 protein are colored as follows: A, cyan; B, green; C, pink. The E3 protein is colored gray. Glycosylation sites are represented as balls and sticks and the residue bearing the glycosylation is indicated. Green sticks represent disulfide bonds. The large, black arrow represents the orientation of the viral membrane. The C-termini of the glycoproteins are depicted by a pink star (E2) and blue star (E1). The inset shows the relative positioning of the domains of the glycoproteins to each other. (b) A rotation of the glycoprotein heterodimer to show the E3 accessory protein. (Reprinted by permission from Springer Nature: Nature, Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography, Voss, J., Vaney, M., Duquerroy, S., Vonrhein, C., Girard-Blanc, C., Crublet, E., Thompson, A., Bricogne, G. and Rey, F., 2010, Nature, 468, 709).

to an increase in RNA stability during infection, and an increase in intra-cellular immunity of the RNA to cell [17]. Additionally, this study found that the wild-type capsid protein assists in promoting the translation of viral RNA [17].

3. The study of virus entry

Many methodologies have been used in the attempt to study virus entry and many are performed in combination with metabolic inhibitors. To correctly resolve which aspect of the virus replication cycle is being affected in the experiment, it is important that care be taken to directly measure the specific process in question. For instance, if a metabolic pathway (*e.g.*, receptor-mediated endocytosis) is being investigated as a route of entry, the metric of the experiment should be a direct measurement of virus endocytosis, not of virus RNA synthesis, protein synthesis, viral glycoprotein expression on the surface of the cell, nor mature virus production. At best, assay of the biochemical events that are downstream of the actual event in question can be broadly resolved into early or late events in the virus replication cycle. In addition, any experiment that is conducted where observations are made downstream of the event in question should have proper controls in place to eliminate secondary effects of any treatment. For instance, if RNA synthesis is the measure of an experiment with a metabolic inhibitor it should be shown that the inhibitor does not interfere with the function of the viral replication complex itself or any other aspect of RNA synthesis. Unfortunately, the literature contains studies that, while valuable, may not completely describe the specific events being investigated because of these two experimental flaws.

3.1. A brief overview of inhibitors used to study viral entry

Chemical inhibitors have been a staple of studying entry pathways of viruses for decades [18-21]. This section will describe the function of a few inhibitors of the pathways that viruses may use to enter cells and some of the metabolic inhibitors used in the pursuit of this investigation. These inhibitors range from non-specific buffers, like ammonium chloride, that neutralize the low pH of endomembrane compartments [19] to more specific compounds like bafilomycin, Dynasore, PitStop, cytochalasin-D, and monensin. Others exist, however, only a few inhibitors will be discussed here [19, 22-24] as these inhibitors have been used to determine the involvement and mechanisms of endocytosis during the process of alphavirus infection [19-21, 25].

One traditional method to study virus infection by endocytosis has been to neutralize the endosomes using different agents. Bafilomycin (BAF) is a specific inhibitor of the vacuolar ATPase, (V-ATPase) the universal proton pump of eukaryotes [24, 26]. In the cell V-ATPase is localized to the plasma membrane and endomembrane compartments and its main function is to maintain the pH homeostasis of the cell [27]. The V-ATPase is composed of approximately 20 subunits which form 2 domains (reviewed in [28]). The cytosolic V1 domain is responsible for ATP hydrolysis while the membrane bound V_o domain performs proton translocation. Bafilomycin acts on the V-ATPase by antagonizing the c subunit of the enzyme's V_o domain [29, 30]. This antagonism prevents the translocation of protons through the respective membrane thereby allowing disruption of the low pH. Ammonium chloride is a frequently used compound in the field of virology and is classified as a lysosomotropic weak base because of its capability of infiltrating the endomembrane compartments of the cell and neutralizing them [19]. Similar to BAF, the rational for using ammonium chloride is to buffer the pH of the endocytic compartments to neutrality. However, the nonspecific nature of ammonium chloride has been shown to have secondary effects during Sindbis virus infection that prevented the processing of viral non-structural proteins having a direct effect on RNA synthesis [21].

Endocytosis is a complex process that includes pinocytosis, and phagocytosis functionalities (reviewed in [31]). These two sub-pathways of endocytosis have differing functions; pinocytosis facilitates the intake of small extracellular components and water, while phagocytosis allows for the intake of larger materials. Clathrin-dependent and clathrin-independent modes of activity further divide the ways in which pinocytosis can occur [32]. Because of the many different types of endocytosis that can occur and the differing proteins that facilitate them, there are numerous inhibitors that can be used to delineate

which type of endocytosis is used by viruses to gain entry into the cell [18, 22, 23].

The formation of endocytic pits is required for uptake of extracellular materials. The structure of the pits formed by clathrin is through the repeated association of clathrin molecules that deform the cytoplasmic surface of the plasma membrane (PM). These invaginations are subsequently sealed and released into the cytoplasm. The function of clathrin can be inhibited by the treatment of cells with hypertonic sucrose [33] or by depleting the cells of potassium [34]. More specific inhibitors of the function of clathrin-coated pits include Dynasore and Pitstop. These two inhibitors inhibit different aspects of clathrin-dependent endocytosis. Dynasore inhibits the activity of dynamin, which forms rings between the PM and the neck of the clathrin-coated pits thereby inhibiting the GTPase activity of the complex and thus prevents the release of the clathrin-coated vesicles [22]. Pitstop interacts with the N-terminus of clathrin, which prevents the association of endocytic cargo with the clathrin scaffold [23]. Despite secondary effects that the compounds used to study virus entry may have in the infection process, the compounds remain useful and may even provide unexpected insights into the infection, as will be discussed below.

3.2. Biological complications in studying virus entry

As stated previously, the infectivity of the virus sample (as measured by some form of titration) and the cell lines being used can have profound impact on the outcome of an experiment and its interpretation. A study performed by Alfson and colleagues demonstrated that the infectivity of Ebola virus can influence the course of infection in macaques [35]. In this study, virus from two distinct stocks that had differing infectivities (8,400 particles/PFU and 511 particles/PFU) were used and animals were infected with 100, 1, or 0.001 PFU/macaque [35]. All animals died from the infection with the exception of animals infected with 0.001 PFU from the stock of virus with the higher relative infectivity (511 particles/PFU) [35]. Because the 0.001 PFU infection from the stock of virus with an infectivity of 8,400 particles/PFU resulted in death while the same infection done with an infectivity of 511 particles/PFU did not, it was concluded that the particles that are traditionally classified as defective retain some sort of biological significance as there is an increase in animal mortality despite the fact that defective particles are unable to produce cytopathic effects in tissue culture [35]. Differing biological activities between particles of influenza virus that are classically defined as infective or defective have also been observed and may be conditional. Many of these observations have been summarized in a review by Brooke [36].

Despite accepted observations that other enveloped viral species can enter a cell by direct methods at the plasma membrane [37-40], there is still quite a large amount of opposition to any model suggesting that the alphaviruses are capable of similar entry routes that lack pH dependence. A study that underscores the complexity of virus entry was performed by Whitbeck and colleagues using vaccinia virus [41]. This virus contained a beta-galactosidase reporter gene cloned into the viral genome and its expression was assayed during infection in the presence or absence of BAF [41]. In this experiment transcription and translation of the reporter virus genome is required for the expression of the beta-galactosidase gene (β-gal), and the BAF used in the experiment should have no direct effect on enzyme activity. This study, however, demonstrated that differences in beta-galactosidase activity were detected in different cells infected with vaccinia and treated with BAF [41]. This situation shows that BAF is not directly contributing to the decrease in activity of beta-galactosidase implicating other host factors in the expression of β-gal. Using this reporter assay in several cell types, the authors were able to conclude that vaccinia virus is capable of entry by pH-dependent and pH-independent routes. HIV, herpesvirus, coronavirus, and influenza A have also been shown to possess the ability to enter cells using a pH-independent fusion mechanism [37, 38, 40, 42, 43].

In contrast to the alphavirus structural proteins described above that are used for entry by fusion, influenza, coronavirus, herpes virus, and HIV are known to utilize proteins with a vastly different structure. Class II fusion proteins are only known to be used by the arboviruses. While the fusion machinery between the viruses discussed appears

to be different, these particular cases highlight the ability of enveloped viruses to enter cells by means that do not absolutely require endosomal acidification.

4. Evidence for receptor-mediated endocytosis

The model of alphavirus entry by endocytosis states that upon receptor-mediated ingestion of the virus and subsequent delivery into endosomal compartments, the virus takes advantage of the decreasing pH to facilitate conformational changes in the glycoproteins of the virus [44]. The conformational rearrangements then expose a hydrophobic loop of the E1 protein, containing approximately 20 amino acids, proposed to be critical for membrane fusion upon insertion into the endosome membrane [13].

Countless studies have been conducted that seek to demonstrate the ability of alphaviruses to enter cells by receptor-mediated endocytosis [15, 19, 20, 45-47]. In pursuit of this line of study, experiments by Smit and colleagues began to characterize the biophysical properties of membrane fusion between Sindbis virus particles and liposomes that were composed of a mixture of cholesterol and sphingolipids [48]. This study questioned what specific pH exposure was required by the virus to render the particle fusion-competent with the liposomes. It was determined that this value was a pH of 5.0 with some fusion occurring at a pH as high as 6.0. In this experiment it was also determined that Sindbis virus was capable of fusing in this pH range but required artificial membranes containing large amounts of cholesterol despite the lack of proteins or other potential virus receptors in the liposomes [48]. This large cholesterol requirement was explained by the suggestion that the large quantities of cholesterol used could promote membrane curvature that would provide favorable conditions for virus-liposome fusion [49]. However, there are two inconsistencies with the liposome model: 1) the large amount of cholesterol required for fusion and 2) the mechanism of fusion of a small fusion domain with a protein-free lipid bilayer. It is known that membrane lipid composition affects curvature [49]; however insect hosts of alphaviruses are cholesterol auxotrophs and infections in these cells cannot utilize high levels of cholesterol [50]. Additionally, although alphavirus and influenza encode different classes of fusion proteins, one mechanistic quandary remains. The membrane-fusion mechanism of entry for influenza has been suggested to require that the fusion protein penetrates the plasma membrane to at least the interface between the inner and outer leaflets, which is a distance occupied by ~20 Å [51]. The pitch of the α-helical fusion domain being 1.4 Å requires ~14 amino acids to reach the bilayer interface [9]. Thus the attributes afforded by the fusion proteins of the alphaviruses would seem insufficient, in this case, for fusion to occur as only 10 amino acids constitute the fusion loop and would not reach such a depth to maintain productive interactions to induce fusion. Indeed, fusion from without and within still occur in the alphaviruses [52, 53] despite the lack of depth reached by the fusion loop. This observation can be explained by interaction between the viral fusion proteins and various proteins on the plasma membrane (PM) or endosomal membranes which could promote the merging of the viral and host membranes resulting in fusion [41].

The biological properties of virus infection were also investigated using BAF, which inhibits the action of V-ATPase. The pH decrease in endomembrane compartments is accomplished by the action of the V-ATPase during endomembrane maturation; hence perturbing its function was of interest to those studying virus entry by endocytosis. One such study by Pérez and Carrasco questioned the role of the V-ATPase in entry for enveloped viruses like Semliki Forest virus, influenza virus, vaccinia virus, herpes simplex virus-1, and Sendai virus; non-enveloped viruses like polio virus and adenovirus were also represented [54]. By observing events downstream of entry, like RNA and protein synthesis after treatment with BAF, the authors concluded that inactivation of the V-ATPase, and therefore inhibition of fusion after endocytosis, was responsible for the differences seen between treated and untreated experiments [54]. Further, it was concluded that BAF had an effect on all enveloped viruses tested with the exception of Sendai virus. Similar to Sendai virus, all non-enveloped viruses were unaffected in their ability to infect cells after treatment with BAF [54].

However, another explanation of this observation may be that replication of Sendai virus may be less dependent on the activity of the V-ATPase, in view that virus entry was not assayed directly. A more recent study [55] has confirmed V-ATPase requirement as a host factor involved in dengue virus maturation and has been observed with other flaviviruses and influenza virus [56-58]. Further, a study by Duan and colleagues demonstrated that the V-ATPase interacts directly with the prM protein of dengue virus and that this interaction is required for proper secretion of dengue virus from the cell [59]. The observation that dengue virus requires the V-ATPase for secretion was made by disrupting the interaction between prM and the V-ATPase. V-ATPase has been found to be involved in glycoprotein transport in the past [60, 61] and could be involved in dengue maturation. However, the study by Duan evaluated the successful inhibition of entry by BAF treatment with immunofluorescence to assay for the production of glycoproteins on the surface of the cell [59]. As stated, protein translation occurs downstream of entry and conclusions should be drawn with caution.

In addition to the V-ATPase, various other host cell proteins have been implicated in viral infection by receptor-mediated endocytosis and, like the V-ATPase, are vastly associated with the membrane of endosomal compartments. Rab5 and Rab7 have also been implicated in the replication of alphaviruses; however the utilization of these two proteins by membrane-containing viruses may vary [62]. These proteins contribute to viral infection by controlling the trafficking of endomembrane compartments as their association with these compartments is thought to be linked to endomembrane maturation [63-66]. A more recent study by Stiles and colleagues has shown by siRNA screening that TSPAN9 is a host cell protein, whose function is largely unknown, that may be involved with entry by receptor-mediated endocytosis by increasing the ability of alphaviruses to fuse with the membrane of the endosome [67].

5. Evidence for pH-independent entry

An early observation that the alphaviruses are capable of pH-independent entry was made by showing that Sindbis virus-mediated cell fusion takes place as a two-step event requiring the virus to be exposed first to low pH and then returned to neutrality [52, 68]. This situation does not occur in the physiology of cells as the pH of the endomembrane compartments changes unidirectionally from neutrality to around a pH of 4 [45]. Also, many of the studies in the formative years of alphavirus biology assayed biochemical events downstream of entry as the reporter [19, 54]. This was because of inherent difficulties in directly observing and capturing the entry of viruses that have both a known particle-to-PFU ratio and one that optimally approaches unity. The fact that alphaviruses do not produce empty particles because of their maturational properties lends them the advantage over other viruses in that they inherently have a lower particle-to-PFU ratio. However, they could still exhibit higher particle-to-PFU ratios because of defects in the virus particle after release from the cell. The complication of the increased particle-to-PFU ratio has been overcome by the use of the heat-resistant strain of Sindbis virus (SVHR) that was originally isolated by Burge and Pfefferkorn [69, 70] which expresses high structural stability.

Electron microscopy (EM) continues to be an invaluable tool for studying the superstructure of cells and viruses and has been used extensively in the study of infection for many families of virus. However, infectivities of viruses, as discussed, greatly complicate direct observations by electron microscopy, as it is known that exposing the cell to excess ligands can induce endocytosis. Direct observations of virus infections at the entry stage can obscure evidence by other means because of potential artifacts induced by exposing the cell to large numbers of noninfectious virus particles. An EM study by Vancini, *et al.* observed for the first time, in a direct fashion, an alphavirus particle depositing its genome into the host cell (Figure 3) in a manner that is not by receptor-mediated endocytosis [7, 71]. This study, aside from the direct nature of observation, has the advantage over others in that it utilizes SVHR, which if correctly prepared produces nearly 100% infectious virus particles; hence all cellular responses are representative of response to a single, infectious particle. In this study, an apparatus for the transfer

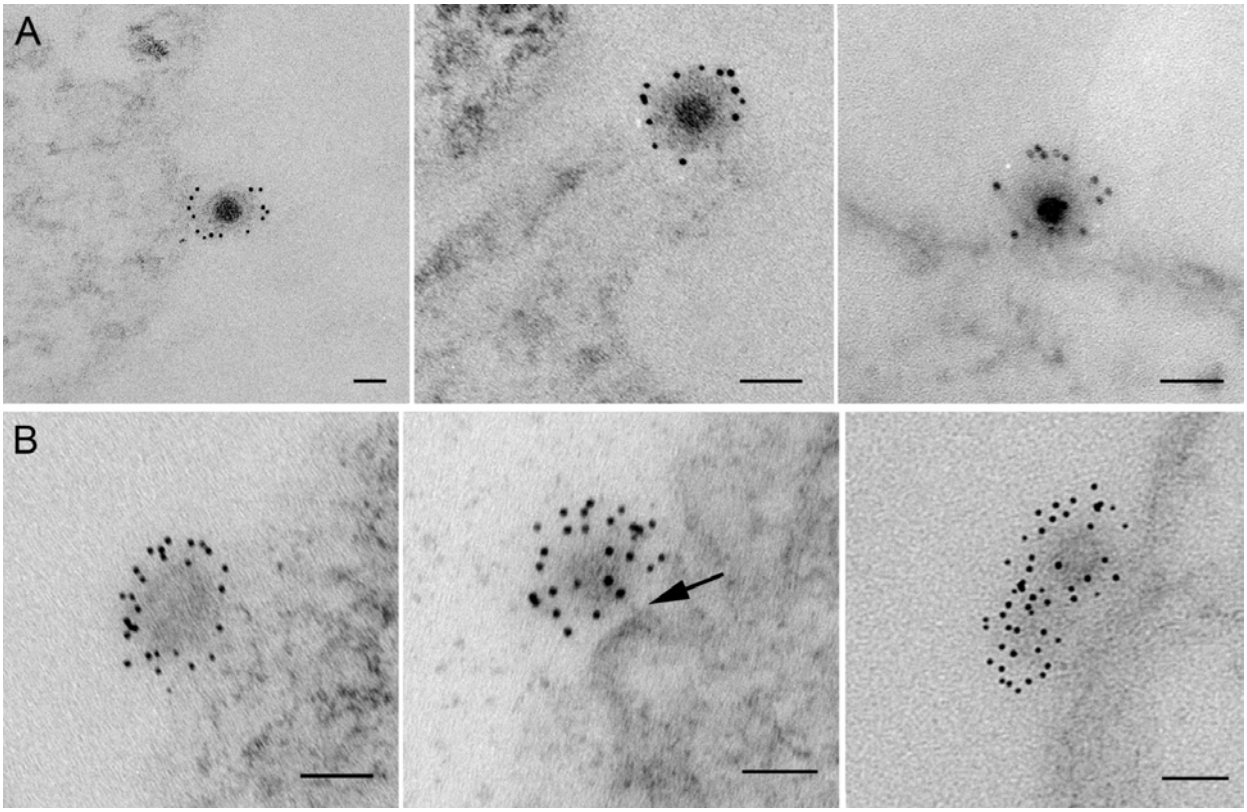

Figure 3. The deposition of the Sindbis virus genome at the plasma membrane. These images depict SVHR at 4 °C (A) and 37 °C (B). All panels have been treated with α-SVHR primary polyclonal antibodies and immune-gold labeled secondary antibodies. The full particles (A) can be seen emptying their genomes (B) while the virus particle remains at the plasma membrane. The arrow indicates a putative apparatus used by the virus to deliver its genome and the scale bars represent a 50 nm measurement. (Reproduced from Vancini, R., Wang, G., Ferreira, D., Hernandez, R. and Brown, D. 2013, J. Virol., 87, 4352-4359 with permission from American Society for Microbiology).

of the genome from virus to cell was also observed [71]. The observation of this apparatus agrees with experiments by Paredes *et al.* that demonstrate that a protrusion forms at the fivefold axis of purified infectious virus after exposure to low pH [7]. Because exposure to low pH did not result in complete disassembly of the glycoprotein lattice, conformational changes induced by exposure to low pH may be indicative of similar changes that occur after interaction with the receptor(s) on the surface of the host cell [7].

An early study in 1991 by Edwards investigated the role of low pH in Sindbis virus infection, by genetic means, in Chinese hamster ovary cells [72]. Despite being defective in endosomal acidification, these cells were still susceptible to Sindbis virus infection. At the time of this study, the genetic cause of the inability of these cells to acidify endosomes at non-permissive temperatures was unknown, but it was speculated that inactivation of the V-ATPase could be a cause. Stemming from this study, the involvement of the V-ATPase in alphavirus infection was recently investigated in the infection of SVHR using a combination of BAF treatment and a GFP reporter gene encoded by the virus under the control of an independent promotor [25]. Despite treatment with BAF, infection was still observed. Importantly, a condition tested in this study examined the effect of BAF treatment on infection after transfecting the virus into the host cells. This particular experiment did not yield a productive infection when BAF was present throughout (pretreatment, during transfection, and after transfection). Because of this, the

V-ATPase is implicated in viral processes that pertain to events in viral replication that are independent of entry. Potential viral processes that are dependent on the function of the V-ATPase are currently under investigation in our laboratory. In these as yet unpublished experiments, we generated a mutant Sindbis virus that was resistant to BAF (BRSV). Importantly, in this study, the mutations that accounted for the mutant phenotype were not in the viral replication proteins. When the RNA replication of wild-type and mutant virus was compared with BAF treatment, BRSV was shown to overcome blockages in replication. Analysis of SVHR viral replication during a time of addition assay showed that BAF inhibits viral replication when cells are treated 5 minutes before infection, a time point which does not allow for endosomal pH neutralization before virus entry can occur (by endocytosis, for example). Further, a time point with BAF treatment 30 minutes after infection resulted in a similar decrease in RNA production, a time in which entry should have been complete [73]. These experiments demonstrate that the effect of BAF is due to an inhibited V-ATPase and not because of neutralization of pH, nor because of off-target effects of the drug on the viral replication proteins.

6. Summary

The field of virus entry has been fraught with many challenges that have led to very intensive questioning. During the course of studying the entry characteristics of the viruses presented in this review, it is apparent that processes in nature are rarely simple and well-defined: many viruses that were thought to enter cells by only one mechanism have been shown to do so by several. Despite the fact that some viruses may exhibit a preferential mode of entry, it is imperative that all potential routes are characterized. Aside from academic pursuits, this knowledge is valuable for applied science, particularly the development of antiviral therapeutics directed toward entry. Simply put, the design of therapeutics for only one route of entry may not prevail if other avenues can establish infection. For these reasons the entry of alphaviruses has been re-examined and a paradigm shift in what was once thought to be a canon in virology has been shown to be not absolute.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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