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Coupling of Replication and Assembly in Flaviviruses

Swapna Apte-Sengupta, Devika Sirohi, and Richard J. Kuhn

Markey Center for Structural Biology, Department of Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, Indiana, USA.

Abstract

Flaviviruses affect hundreds of millions of people each year causing tremendous morbidity and mortality worldwide. This genus includes significant human pathogens such as dengue, West Nile, yellow fever, tick-borne encephalitis and Japanese encephalitis virus among many others. The disease caused by these viruses can range from febrile illness to hemorrhagic fever and encephalitis. A deeper understanding of the virus life cycle is required to foster development of antivirals and vaccines, which are an urgent need for many flaviviruses, especially dengue. The focus of this review is to summarize our current knowledge of flaviviral replication and assembly, the proteins and lipids involved therein, and how these processes are coordinated for efficient virus production.

Introduction

Flaviviruses (Family- *Flaviviridae*) are one of the major causes of arthropod-borne illness in humans. Flavivirus pathogenesis ranges from mild illness such as fever, rash and joint pain, to more severe symptoms such as hemorrhagic fever and fatal encephalitis. The Flavivirus genus consists of more than 70 enveloped, positive-strand RNA viruses including yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). Altogether flaviviruses affect hundreds of millions of individuals every year. Despite the availability of vaccines against YFV, JEV and TBEV, diseases resulting from these viruses are still prevalent worldwide. Protective vaccines or therapies are not yet available against the more pathogenic flaviviruses and prevention from insect bites remains the major defense against some of these viruses. Therefore, a better understanding of the flavivirus life cycle is essential in order to develop effective strategies for antiviral intervention and for development of novel vaccines.

The virus enters host cells by receptor-mediated endocytosis and the ~11 Kb positive-sense RNA genome gains entry into the cytoplasm by viral glycoprotein-mediated membrane

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Corresponding Author: Richard J. Kuhn kuhn@purdue.edu Phone: 765-494-4407, Fax: 765-496-1189 Mailing Address: 240, S. Martin Jischke Drive West Lafayette IN 47907.

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fusion. Flavivirus replication begins when the genome is recognized as messenger RNA and translated by host cell machinery to yield a single polyprotein. The polyprotein is co- and post-translationally cleaved by viral and cellular proteases into 10 gene products (Figure 1). The structural proteins capsid (C), precursor membrane (prM/M) and envelope (E) are incorporated into the virion, whereas the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 serve to coordinate the intracellular aspects of virus replication, assembly and modulation of host defense mechanisms. NS1 is essential for virus replication and inhibition of complement-mediated immune response [1]. NS3 contains serine protease, Nucleoside 5' triphosphatase (NTPase), RNA helicase, and 5' RNA triphosphatase (RTPase) activities, while NS2B serves as a cofactor for the protease activity of NS3. NS5 contains methyltransferase and RNA-dependent RNA polymerase (RdRp) domains required for genome replication and capping of nascent RNA [2]. Three non-enzymatic, integral membrane proteins NS2A, NS4A and NS4B are poorly understood. NS2A is required for virus replication and assembly [3-5]. NS4A induces membrane rearrangement [6,7] and autophagy to enhance viral replication [8], whereas NS4B modulates host immune response by suppressing the α/β interferon signaling and the helicase activity of NS3 [9,10].

Polyprotein processing into component proteins takes place on ER membranes (Figure 1). The replication complex (RC) is formed on modified ER membranes where negative-sense RNA is copied from the genomic RNA template. The process of RNA synthesis is asymmetric favoring positive-sense RNA [2]. The RNA genome then interacts with C protein, which buds through the glycoprotein-containing ER membranes as immature virus particles into the lumen. The immature virus particles then go through maturation steps in the ER and Golgi complex and are released as mature particles from the cell. The biogenesis of the RC and its assembly involve modifications of cellular metabolic and structural components. This review presents an overview of the current knowledge of interactions between the viral and cellular components to promote cellular changes required for replication and assembly and how these two processes are coupled. Replication and assembly are important aspects of the virus life cycle that are targeted for future antiviral development.

Flavivirus Translation and Replication

Virus-induced membrane rearrangements

It is now established that flavivirus replication and viral RNA synthesis occurs on an extended network of modified ER membranes. At least three distinct membranous structures are found in flavivirus-infected cells: membranous sacs or vesicle packets (Vp), membrane vesicles (Ve) and convoluted membranes (CM) (Figure 2) [11-16]. Vps are small clusters of Ve formed by modification of ER membranes and used as sites of replication by the virus [13,14]. Ve are open to the cytoplasm via a small neck. The CMs are suggested to form the sites of translation and polyprotein processing and/or storage sites for viral proteins [14,15]. These structures are formed by membrane remodeling that can be induced by changes in lipid composition, influence of integral membrane proteins, activities of cytoskeletal protein and microtubule motors, scaffolding by peripheral and integral membrane proteins [17]. Certain lipids such as lysophosphatidic acid and phosphatidic acid favor negative membrane

curvature [18]. Similarly, enzymes such as flippase can also introduce membrane asymmetry [19].

It is still unclear what triggers the membrane modifications in flavivirus infection, viral proteins along with modulation of host lipid metabolism and cytoskeletal components are involved. Expression of KUNV and DENV NS4A containing the uncleaved carboxyl-terminal transmembrane domain, 2K, induced ER membrane rearrangements, while a role of NS4B in membrane rearrangement has also been suggested [6,7,20]. The amino terminal amphipathic helix of NS4A induces homo-oligomerization and is also predicted to form a scaffold in the membrane to induce membrane bending [21]. Additionally, NS4A of DENV was shown to rearrange vimentin intermediate filaments at perinuclear sites. This rearrangement of vimentin along with vimentin phosphorylation was implicated in enrichment of RCs [22]. KUNV NS2A has been implicated in membrane rearrangements, as a mutation at residue 59 (I59A) blocked membrane rearrangements and virus assembly [5]. Recently, NS1 was also shown to have membrane remodeling ability [23]. When purified NS1 was mixed with liposomes rich in cholesterol and phosphatidyl serine, they were fragmented into small nanoparticles suggesting that NS1 possesses an ability to remodel lipid membranes [23].

Comparable membranous structures were reported in both insect and mammalian cells infected with flaviviruses. However, CMs were not observed in DENV-infected insect cells [15]. A higher number of closed ended tubular structures were seen in the TBEV-infected tick cells suggesting that there exist differences between acute and persistent infections with tubular structures being predominant in the latter case [16]. Further studies are required to understand the molecular mechanisms these proteins employ to bring about the structural changes in the ER membrane, the host components involved in the process and the nature of interactions between the viral and host components.

RC arrangement on the membrane

Evidence suggests that viral RNA, NS3 and NS5 are localized within Ve and open to the cytoplasm [13,14,24-27]. The luminal side of the membrane contains NS1, while NS2A, NS2B, NS4A and NS4B are integral membrane proteins. It is still unclear how these proteins are arranged with respect to each other, but specific interactions among nonstructural proteins have been proposed based on genetic, localization and biophysical studies [5,10,28]. Recent genetic studies suggest that alteration of the hydrophobic “greasy finger” loops of NS1 inhibited DENV replication, highlighting the importance of the hydrophobic loop and its potential role in membrane interactions [23]. Additionally, reports demonstrating interactions between WNV NS1 and NS4B as well as between YFV NS1 and NS4A suggest that NS1 interacts with the RC via NS4A and NS4B [29,30]. Based on these observations, the model depicts the NS1 dimer docking on the membrane and interacting with NS4B via the NS1 β -roll (di-peptide RQ10) sequence. It is possible that NS1 stabilizes the replication complex on the membrane by holding the NS4A and NS4B proteins together and creating a scaffold for the replication machinery. NS2A was also predicted to be a transmembrane protein that colocalized with dsRNA and other proteins of the RC [4,25]. In addition, NS3 interactions with NS2A, NS4A, and NS4B [5,10,31] on membrane were

predicted based on genetic studies on YFV, DENV and WNV. Figure 2A represents how RC is assembled on Ve membranes based on the above observations. This model depicts the viral components and their orientation with respect to the membrane. It does not represent their exact topology, oligomeric state or their interactions with host factors. An overview of the replication sites, Vp and Ve membranes, is shown in figure 2B, where RCs are shown on the Ve membrane along with nascent RNA being transcribed.

Flavivirus Assembly

We understand exceedingly little about the assembly process of flaviviruses. A packaging sequence on the genome of flaviviruses has not been identified with C-RNA interactions believed to be non-specific and electrostatic [32,33], yet the genomic RNA is specifically packaged into virions. It has been suggested that this specificity is achieved by coupling genomic replication and encapsidation. There is evidence to indicate that only actively synthesized RNAs are packaged, hinting that replication and virion assembly are closely intertwined processes [34]. Spatial coordination of the two processes has been observed by electron tomography in both DENV-infected mammalian and insect cells. As pointed out earlier, replication is believed to occur within the lumen of the Ve, which have openings/pores that possibly allow entry of building blocks from the cytoplasm and the subsequent release of genomic RNA for packaging (Figure 2B). Budding of virions from ER sites directly opposite to the Ve pores has been visualized and a connection between the Ve pore and the neck of the budding virions has also been seen [14,15]. It should be pointed out that not all virus-induced Ve structures identified in positive-strand RNA viruses have open pores [35-37]. Therefore, it would be interesting to probe the mechanism of formation and molecular architecture of these pores. To this end, it has been suggested that the small hydrophobic non-structural proteins (2A, 2B and 4B) of JEV have viroporin-like activity or capability for membrane permeabilization [38] but this needs to be explored further. It is also unclear how viral RNA is transported from the lumen of Ve to the sites of assembly. Genetic evidence suggests the involvement of several viral non-structural proteins and host proteins in the assembly pathway (Table 1), but their exact role needs to be elucidated. Based on the current evidence, it appears that NS2A, alone or in complex with NS3, may play a role in genome transport.

The cellular location of nucleocapsid formation (C-RNA complex) has also been debated in the field. Lipid droplets (LDs) have been implicated as sites for nucleocapsid assembly since C was found to localize to lipid droplets and mutations in the $\alpha 2$ helix (L50S-L54S) that disrupt C association with LDs abrogated virion production [39]. This suggestion conflicts with tomographic data as well as the observation that DENV assembles in cell lines that are not rich in LDs. The significance, mechanism and dynamics of C-LD interaction are not clear. The role of this association in sequestration of C to prevent premature binding to RNA has been speculated since mistargeted mutant C (L50S-L54S) displayed a dominant negative effect on DENV replication when expressed in *trans* [39]. C-RNA interaction studies have been challenging since nucleocapsids have not been visualized in flavivirus-infected cells though nucleocapsid-like particles of irregular geometry have been assembled *in vitro* from purified C [33,40,41]. Cryo-electron microscopy based single particle reconstruction of flaviviruses has also demonstrated a lack of an organized C-RNA nucleocapsid core [42-44].

Failure to visualize assembly intermediates suggests that the assembly processes are rapid and/or simultaneous with replication and budding.

Lack of tangible interaction between C and the prM/E glycoproteins is another confounding aspect of the flavivirus assembly. Budding of viral particles appears to be nucleocapsid independent, since subviral particles (SVPs) can be produced by over-expressing only the prM/E glycoproteins [45]. Mechanisms that ensure efficient nucleocapsid incorporation into budding viral membranes are not well understood. Requirement of a sequential cleavage of C by NS2B-3 on the cytosolic side (releases mature capsid) before cleavage by host signalase on the ER side (C-prM junction) had been postulated to be a mechanism for temporal regulation of virus assembly [46-48]. One possibility is that the first cleavage allows C-RNA interaction through NS2B-3 (and its interacting partners), and the delayed second cleavage allows nucleocapsid incorporation into budding membranes containing viral glycoproteins.

Following budding, through a complex interplay of events requiring conformational changes in E and furin cleavage of prM, non-infectious, spiky, immature particles get converted to smooth infectious virions as they pass through the secretory pathway. The maturation process of flaviviruses is outside the scope of this review but readers are referred to excellent reviews on this subject [49,50].

Roles of Cellular Lipids in Replication and Assembly

Based on studies so far, it appears that the high demand for lipids due to membrane remodeling and budding virions in flavivirus-infected cells is met in several ways. Reabsorption of LDs, the natural reservoirs and processing centers of lipids in the cell by the ER membrane was observed in DENV-infected cells [51]. Redistribution of fatty acid synthase (FASN), in a Rab18-dependent manner, by DENV NS3 at the sites of replication appears to be a mechanism utilized by the virus to maintain the supply of fatty acids [52,53]. FASN is a multienzyme protein that catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADH into long chain saturated fatty acids. WNV replication also requires fatty acid synthesis [54]. Additionally, DENV has been shown to induce autophagy that leads to break down of LDs and triglycerides into fatty acids leading to higher β -oxidation and ATP release [55]. An increase in lysophosphatidylcholine that induces membrane curvature, was also noticed in DENV-infected mosquito cells along with increased levels of palmitic and stearic acids, indicating lipogenesis in the infected cells [56]. In YFV, DMVJC14 acts as a chaperone and targets the proteins of the RC to detergent-resistant membranes or lipid rafts [31].

Cholesterol and membrane rafts play an important role in flavivirus attachment and entry [57-62], but their role in virus replication has only come to light recently. Modulation of cholesterol biosynthesis is important for DENV replication, as inhibition of mevalonate diphospho decarboxylase (MVD) by siRNAs reduced Den-Rluc replication in A549 cells. Interestingly, other genes in the cholesterol pathways did not affect viral replication [63]. Surprisingly, supplementing cholesterol at the virus adsorption stage externally blocked both entry and replication of JEV and DENV2 in N18 cells [64]. When the cholesterol

biosynthesis inhibitor, lovastatin, was used ER membrane biogenesis remained unaffected. However, production of infectious DENV particles was abrogated suggesting that cholesterol has additional functions in replication [51,65]. Furthermore, increased levels of cholesterol were induced in the early stages of DENV infection and an increase in LDL particle uptake and HMG-COA reductase activity was seen [58]. Several drugs that affect cellular lipid biosynthesis affect flavivirus replication (Table 2). Interestingly, no involvement of cellular pathways such as the unfolded protein response (UPR) and sterol regulatory element binding protein (SREBP-2) pathways that primarily mediate cholesterol and fatty acid biosynthesis was seen in DENV infection [51].

Levels of a cellular sphingolipid precursor, ceramide, were found to be elevated in DENV-infected mosquito and mammalian cells but its role in the infection process is unclear [56]. Increased levels of ceramide in the cell were shown to inhibit JEV replication [66]. Thus, it is not clear whether increased levels of ceramides are induced by DENV for its advantage or if it's a cellular stress response and has a role in the induction of apoptosis [56]. Additionally, the importance of neutral sphingomyelinases (nSMases) has been observed in WNV biogenesis. Inhibitors of nSMases reduced the formation of Vps and SVP [67]. Follow up studies are required to understand the role of sphingolipids and ceramides in flavivirus infection.

Conclusions

A common feature of flaviviruses is to remodel ER membranes to generate distinct organelle-like structures. These structures may be utilized by the virus for specific steps of its life cycle to ensure efficient replication, protection of viral RNA from cellular defense mechanisms and well-coordinated assembly and budding processes. Although there is evidence indicating the role of viral and host factors, the exact molecular mechanisms behind these membrane modifications are still poorly understood. Biochemical evidence is required to study the composition and the distinct roles of the individual membranous structures in replication and assembly. Several steps in the process such as how RNA is translocated from the site of replication to budding, and what host factors are involved in the process are not well known. Cellular lipids are highly involved in the replication and assembly process but how the virus modulates the levels and locations of various lipids is obscure. More studies to understand the key components of the lipid metabolic pathways and their roles in modulation of cell cycle and survival in the context of flavivirus infection are required.

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Highlights

- Flavivirus replication occurs on modified ER membranes called vesicle packets (Vp) and membrane vesicles (Ve).
- Flavivirus non-structural proteins are implicated in membrane rearrangements.
- Viral replication and assembly appear to be tightly coupled.
- Non-structural proteins are important for virus assembly and budding.
- Cellular lipids play an essential role in membrane rearrangements, virus assembly and budding.

Figure 1. Flavivirus polyprotein processing

Translation of the viral genome results in a polyprotein, which is cleaved by cellular and viral proteases. The proposed topologies of the viral proteins with respect to the ER lumen and cytoplasm and the proteases responsible for their cleavages are indicated. The membrane bilayer is shown in pink, transmembrane domains for individual proteins are shown as cylinders spanning the membrane and connecting loops are shown as lines. Black, blue and red arrow / arrowheads represent signal peptidase, NS2B-3 protease and furin protease cleavage sites respectively. Open arrow represents a protease cleavage site that required additional characterization.

Figure 2. Flavivirus genome replication and assembly

A) Spatial model for RC on Ve membranes. Surface representation of DENV NS1 dimer (PDB: 4O6B) [23] located in the ER bound to the Ve membrane through its “GF” loops. Dipeptide RQ10 residues of NS1 potentially interact with the NS4B dimer. NS1 also interacts with NS4A. NS2A is represented as a 5 membrane-pass protein interacting with NS3. NS4A and NS4B are also transmembrane proteins interacting with NS3 on the cytoplasmic side. Surface representations of DENV NS3 (PDB: 2VBC) [68], NS5 Methyltransferase domain (PDB: 1L9K) [69] and RdRp domain (PDB: 2J7W) [70] are shown towards the cytoplasmic side of the membrane. The transmembrane domains of membrane proteins are shown as cylinders and the connecting loops are shown as black lines. This model does not depict the exact topology or oligomeric state of the RC proteins and their interactions with host factors.

B) Coupling of Flavivirus replication and assembly. Genome replication takes place on virus-induced Ve membranes that have opening towards the cytoplasm. The replication complex is composed of NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 along with genomic RNA. The newly synthesized RNA (shown in red) exits the pore and is captured by C (shown as green ovals) attached to LDs or localized on the cytoplasmic side of the ER in close vicinity of the Ve pore to form nucleocapsid. The nucleocapsid complex acquires the viral glycoproteins expressed on the ER membrane by budding into the ER lumen. The spiky immature particles (shown in blue) undergo maturation as they traffic through the TGN and become smooth mature particles (shown in gray) by conformational changes in E protein and furin cleavage of prM protein. C: Capsid, CM: Convuluted Membranes, GF: Greasy Finger, LD: Lipid Droplets, ER: Endoplasmic Reticulum, PM: Plasma membrane,

RC: Replication complex, tGN: Trans-Golgi Network, Vp: Vesicle packets, Ve: membrane vesicles.

Table 1

Proteins implicated in flavivirus assembly.

Protein	Flavivirus	Suggested Role in assembly	Ref.
Viral			
NS1	WNV/DENV (RepliVAX)	Elimination of NS1' decreases packaging efficiency *	[71]
NS2A	YFV, KUNV	Nucleocapsid formation/incorporation	[3,5,72]
	DENV	Formation of intracellular virions	[4]
NS3	YFV	Interaction with NS2 for virion morphogenesis	[5]
	KUNV	Required in <i>cis</i> for assembly and/or release	[73-75]
	YFV	Not required for assembly in <i>cis</i>	[76]
	YFV	Nucleocapsid formation/incorporation	[77]
NS2B-3	YFV, MVEV **	Sequential capsid cleavage for efficient nucleocapsid incorporation into budding viral membranes	[46]
NS5	KUNV	Coupling between genome replication and assembly – Active replication is required for packaging	[34]
Host			
Alix	YFV	Interaction with NS3 for virion morphogenesis	[78]
DDX56	WNV	Interaction with C for virion morphogenesis	[79,80]
Nucleolin	DENV	Interaction with C for virion morphogenesis	[81]

* NS1' is an alternative gene product in WNV resulting from a ribosomal frameshift

** MVEV: Murray Valley encephalitis virus

Table 2

Inhibitors used to target cellular lipids and their effects on flavivirus lifecycle.

Inhibitor	Target	Virus	Effect on Virus	Ref.
U18666A	Cholesterol transport	DENV	Inhibits entry and replication	[82]
C75	FASN	DENV WNV	Reduces replication	[54,56,82]
Cerulenin	FASN	WNV	Reduces replication	[54]
GGTI	Geranylgeranylation	WNV	Reduced replication	[26]
AY9944	Conversion from 7-dehydrocholesterol to cholesterol	WNV	Reduced replication	[26]
Methyl-beta cyclodextrin (MBCD), Nystatin	Cholesterol incorporation into the membrane (cholesterol depletion)	DENV	Reduced replication	[83]
SFV785	Kinase Inhibitor, suppressor of flaviviridae 785	DENV	Reduced virus assembly	[84]
GW4869, Spiroepoxide	nSMase	WNV	Subviral particle release	[67]
Lovastatin	HMG-CoA reductase, blocks conversion from HMG CoA to mevalonate	DENV	Reduces virus assembly	[65]
Spautin-1	Autophagy	DENV	Reduced virus maturation	[85]