Flaviviridae Replication Organelles: Oh, What a Tangled Web We Weave

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Abstract

Replication of positive-strand RNA viruses occurs in tight association with reorganized host cell membranes. In a concerted fashion, viral and cellular factors generate distinct organelle-like structures, designated viral replication factories. These virus-induced compartments promote highly efficient genome replication, allow spatiotemporal coordination of the different steps of the viral replication cycle, and protect viral RNA from the hostile cytoplasmic environment. The combined use of ultrastructural and functional studies has greatly increased our understanding of the architecture and biogenesis of viral replication factories. Here, we review common concepts and distinct differences in replication organelle morphology and biogenesis within the Flaviviridae family, exemplified by dengue virus and hepatitis C virus. We discuss recent progress made in our understanding of the complex interplay between viral determinants and subverted cellular membrane homeostasis in biogenesis and maintenance of replication factories of this virus family.

INTRODUCTION

Viral replication factory: spatially defined compartment composed of virus-induced rearranged cellular membranes containing viral and cellular cofactors and metabolites required for replication

Viral replication compartment: all viral replication factories in a single cell

Members of the Flaviviridae family are enveloped viruses containing an ssRNA genome of positive polarity and comprising the genera *Flavivirus*, *Hepacivirus*, *Pegivirus*, and *Pestivirus* (1). Among them are multiple animal and human pathogens that are major causes of morbidity and mortality worldwide. For instance, symptomatic infections by the arthropod vector–transmitted dengue virus (DV), which belongs to the *Flavivirus* genus, currently affect around 100 million individuals per year and are associated with symptoms ranging from mild cold-like disease to life-threatening hemorrhagic fever and shock syndrome (2). To date, there is neither a prophylactic vaccine nor an antiviral treatment available for DV infections. In contrast, therapy for chronic hepatitis C, which is caused by persistent infection with hepatitis C virus (HCV), has improved profoundly, achieving virus elimination in 80% to 90% of treated patients (3). It is estimated that 130 to 170 million individuals are chronically infected with HCV, and unless this virus is eliminated therapeutically, these individuals are at high risk for development of serious liver disease including cirrhosis and hepatocellular carcinoma (4).

Despite considerable differences in transmission, tissue tropism, and pathogenesis, DV and HCV employ similar replication strategies. Being obligate intracellular parasites, all viruses depend on host cell metabolites and machineries to amplify the viral genome and assemble infectious progeny virions. In the case of positive-strand RNA viruses, the intracellular steps of the replication cycle take place in the cytoplasm within or on the surface of virus-induced organelle-like membrane structures that can be regarded as viral replication factories. Analogous to industrial manufacturing units, viral replication factories serve multiple purposes that together facilitate efficient virus propagation. First, they achieve spatial separation of the different steps of the viral replication cycle, namely, RNA translation, replication, and packaging of the viral genome into virions to prevent mutual interference. This functional separation is important, as the viral replicase complex and translating ribosomes move along the viral RNA in opposite directions, thus causing steric clashes. Moreover, interaction of the structural proteins with viral RNA during assembly would interfere with translation and replication. Second, viral replication factories enable a high local concentration of viral replicase complex components and required metabolites such as nucleotides in a physically constrained space, which is needed to ensure highly efficient RNA amplification. One important parameter is the membrane association of the viral replicase proteins, which reduces 3D diffusion to a 2D membrane surface. Third, the shielding of viral RNA from the hostile cytoplasmic environment is another evolutionarily conserved feature of viral replication factories. Exposure of dsRNA replication intermediates to cellular nucleases and sensors of the innate immune surveillance is minimized by protection in membrane-delimited compartments.

Although the viral replication factory concept applies to all animal and plant positive-strand RNA viruses as well as dsRNA viruses (e.g., rotavirus) and some large DNA viruses (e.g., vaccinia virus) (5–7), in this review, we focus on the viral replication compartments of two representative members of the Flaviviridae family: DV and HCV. We highlight the two morphological classes of viral replication factories that are represented by these two viruses, elaborate on the viral machinery engaged in membrane remodeling, and discuss recent findings on how these viruses subvert cellular membrane-active proteins and lipid homeostatic pathways for biogenesis and maintenance of viral replication factories.

ARCHITECTURE OF REPLICATION ORGANELLES

Owing to the small size of viruses, studies in virology have historically been closely linked to developments in electron microscopy methods. Of these, electron tomography in combination with 3D

reconstruction (for technical review see 8, 9) has been instrumental in precisely describing alterations of host membranes in cells infected with DV or HCV (10–13). Despite their relatively close evolutionary relationship, these viruses induce two morphologically distinct subclasses: the invaginated vesicle/spherule type, represented by DV, and the double-membrane vesicle (DMV) type, represented by HCV (**Figure 1**). Interestingly, all positive-strand RNA viruses can be assigned to one of these groups, which probably reflects the involvement of different host cell pathways exploited by these viruses for viral replication factory biogenesis (14). DV and HCV both induce viral replication factories that are derived from the endoplasmic reticulum (ER), but their morphologies are substantially different. By using electron tomography of DV-infected human hepatoma cells, investigators observed single-membrane invaginations into the ER lumen with a diameter of ~90 nm (**Figure 1*a***) in addition to rather unstructured convoluted membranes (CMs) (**Figure 1*b***) (see also **Video 1**) (10). Importantly, the lumen of these invaginations is connected to the cytoplasm via a pore-like opening ~10 nm in diameter. Invaginated vesicles, which appear in conventional electron microscopy as vesicle packets, contain viral replicase proteins and dsRNA (**Figure 1*c***), which is a surrogate marker for replication intermediates and thus for replication sites. Even though CMs do not contain dsRNA, they are enriched for viral proteins (10), suggesting that they might play a role in synthesis or storage of viral proteins. Interestingly, vesicle packets also are observed in DV-infected mosquito cells, whereas CMs are absent, suggesting that CMs play a human-specific but as-yet-undefined role in the viral replication cycle (11). Furthermore, spatial orchestration of DV RNA replication and assembly has been deduced from the juxtaposition of budding events in close proximity to the pore-like vesicle openings (10). Of note, the DV invaginated vesicle morphotype is similar to the viral replication factories of closely related flaviviruses such as West Nile virus (WNV) (15) and tick-borne encephalitis virus (TBEV) (16, 17), suggesting an evolutionarily conserved biogenesis and architecture of flavivirus replication factories.

In the case of HCV, DMVs with an average diameter of ~200 nm accumulate in the cytoplasm of infected hepatoma cells (12, 13) (**Figure 1*e*,*i***). Owing to the sponge-like morphology of DMV accumulations observed at low magnification in cells expressing the HCV polyprotein or containing replicating RNAs, the term membranous web has been coined (18, 19). However, more recent electron tomography–based studies have revealed that DMVs are protrusions (i.e., exvaginations) from the ER and are frequently linked to the ER via their outer membrane (**Figure 1*f***) (see also **Video 2**) (13). Only a minor fraction of DMVs exhibit an opening connecting the DMV lumen with the cytoplasm (**Figure 1*g***). At early stages of infection DMVs predominate, but as infection progresses they develop into more complex membrane rearrangements, including multimembrane vesicles, presumably resulting from a host cell stress response (**Figure 1*h***). Furthermore, purified HCV replication complexes contain viral replicase proteins and HCV RNA that is protected from nucleolytic degradation (20–22). Importantly, DMVs are associated with RNA replicase activity, qualifying them as bona fide HCV replication factories (22). Interestingly, similar DMV-type viral replication organelle morphologies have been described for the distantly related Picornaviridae (23, 24), Coronaviridae (25), and Arteriviridae (26) families, suggesting that similar host cell pathways are utilized by these viruses to induce the formation of viral replication factories.

SPATIOTEMPORAL CONTROL OF THE REPLICATION CYCLE

Compartmentalization of different biosynthetic steps in the Flaviviridae replication cycle facilitates their coordination in time and space (**Figure 2**). After receptor-mediated entry into susceptible target cells (reviewed in 27, 28) and subsequent uncoating in endosomal compartments, the viral RNA genome directly serves as messenger RNA for viral protein synthesis. The 5′

Invaginated vesicle: virus-induced cellular membrane rearrangement with negative membrane curvature and a pore-like opening toward the cytosol

Spherule: invaginated vesicle–type replication factory; a term commonly used for membrane alterations induced by members of the alphavirus-like superfamily

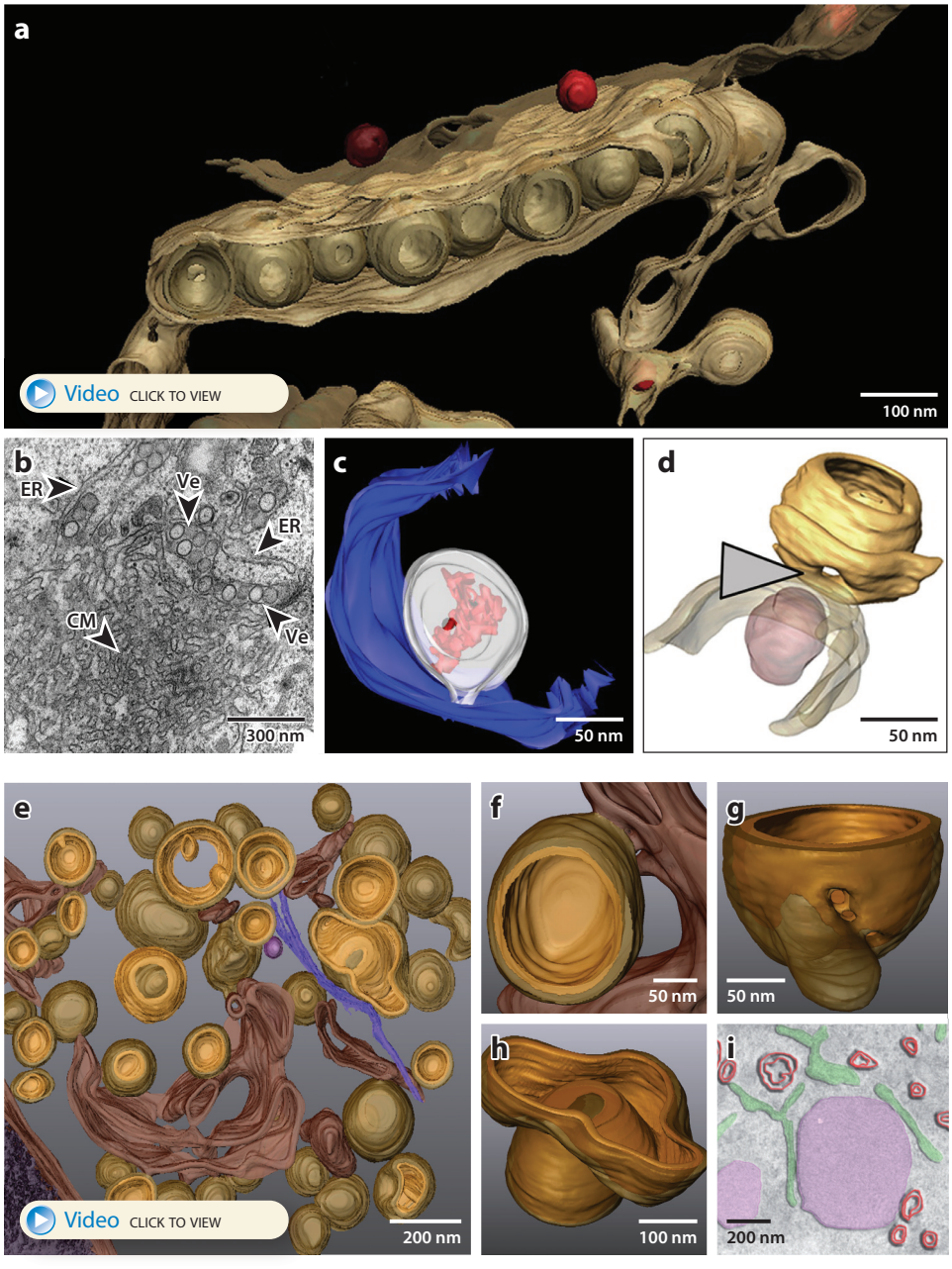
Double-membrane vesicle (DMV): virus-induced cell membrane rearrangement composed of two tightly opposed membranes with predominantly positive membrane curvature

Vesicle packet: accumulation of DV-induced invaginated vesicles in a spatially limited ER subcompartment

Viral replicase: proteinaceous multiunit complex consisting of viral and cellular proteins, providing all enzymatic activities required for viral genome amplification

Membranous web: replication-independent vesicle accumulation in a membranous matrix observed in HCV-containing cells; synonym for HCV replication compartment

cap structure of DV RNA genomes mediates canonical cap-dependent RNA translation (reviewed in 29), whereas an internal ribosome entry site (IRES) contained in the 5′ untranslated region of the HCV RNA genome mediates cap-independent translation at the rough ER (reviewed in 30). This process appears to be highly efficient as deduced from comparative quantification of HCV RNA and protein abundance, indicating that a single positive-strand RNA is translated more than 1,000 times, at least under steady-state conditions (20–22). Upon co- and posttranslational



processing of the viral polyprotein precursor by cellular and viral proteases, membrane-bound viral and co-opted cellular factors induce, in a concerted fashion, membrane rearrangements that lead to the formation of viral replication organelles (described in detail below). There, the positive-strand RNA is copied into a negative-strand intermediate that serves as a template for the production of excessive amounts of new positive-strand RNA molecules. These can be used for polyprotein synthesis or as templates for new negative-strand RNAs or can be incorporated into virus particles. How the switch from RNA translation to replication is regulated remains to be determined. One possibility is the exclusion of ribosomes from the site of viral RNA replication. Alternatively, viral RNA genomes might be circularized to allow regulation of the assembly of translating ribosomes versus active viral replicase. Such a circularization can be mediated by either long-range RNA-RNA interactions (31, 32) or interaction between proteins binding to terminal regions of the viral genome (30, 33). In any case, amplification of the genetic material requires viral RNA recruitment to replicase complexes, which comprise nonstructural (NS) proteins (NS1–5 in the case of flaviviruses and NS3–5B in the case of HCV). Indeed, Flaviviridae minigenomes, so-called subgenomic replicon RNAs, containing 5′ and 3′ untranslated regions and encoding the minimal set of viral replicase proteins, are competent to replicate in cell culture (34, 35).

De novo Flaviviridae RNA synthesis is catalyzed by the RNA-dependent RNA polymerase residing in NS5 of flaviviruses and NS5B of HCV and occurs via a dsRNA intermediate (reviewed in 36, 37) that appears to be shielded in the membrane-delineated compartment (**Figure 2**). Hence, immunodetection of dsRNA has been used as a marker to identify flavivirus replication sites, which were found to reside in the lumen of vesicular invaginations (10, 38); analogous observations have been made for WNV-infected cells by using electron tomography and 3D reconstructions (**Figure 1*c***) (15). However, firm proof of active RNA replication in flavivirus-induced membrane invaginations—for example, by using metabolic RNA labeling of de novo synthesized viral RNA—is still missing. In any case, the pore-like opening (**Figure 1*b***) would allow the import of required metabolites and the export of progeny virus RNA. Yet mobility of TBEV RNA is rather limited, arguing for tight regulation of flavivirus RNA trafficking (17). Although this observation might be due to technical limitations of the RNA detection method, the shielding of viral RNA from cellular innate immune sensors, as demonstrated for TBEV (39), may limit RNA movement. In support of this conclusion, membrane protection of DV dsRNA

Metabolic RNA labeling: incorporation of modified nucleotides into nascent viral RNA, often used for subsequent (immuno)detection of newly synthesized viral RNA

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Figure 1

Morphology of replication organelles. Two distinct classes of Flaviviridae-induced membrane alterations in infected host cells have been revealed by electron tomography and 3D reconstruction. (*a*) Flavivirus infection, as exemplified by dengue virus (DV), induces single-membrane invaginations of the endoplasmic reticulum (ER) (*brown*). Progeny virus particles are highlighted in red. For a 3D view, see **Video 1**. (*b*) Convoluted membranes (CMs), devoid of viral RNA but containing nonstructural proteins, are found in proximity to ER membranes and invaginated vesicles (Ve). (*c*) Viral RNA (*red*) is replicated and stored inside invaginated vesicles (*white*), as illustrated here for West Nile virus. (*d*) Budding of DV progeny virions (*red*) juxtaposed to vesicle openings (*arrowhead*) is frequently observed. (*e*) Membrane rearrangements induced upon hepatitis C virus (HCV) infection, consisting primarily of double-membrane vesicles (DMVs). Outer membranes of DMVs are highlighted in light brown and inner membranes in orange. For a 3D view, see **Video 2**. (*f*) DMVs emerge as protrusions from the ER (*dark brown*) and are thus commonly found to be connected to this compartment. (*g*) A minor fraction of DMVs exhibit a pore-like opening that connects the lumen to the cytoplasmic space. (*h*) More complex membrane structures, including multimembrane vesicles, develop at later stages of infection. (*i*) DMVs (*red*) are frequently found in close proximity to lipid droplets (*pink*) that are surrounded by ER membranes (*green*), as highlighted in this color-coded electron micrograph. Reproduced with permission from References 10 (panels *a*, *b*, and *d*), 15 (panel *c*), 13 (panels *e–h* and **Video 2**), and 79 (panel *i*).

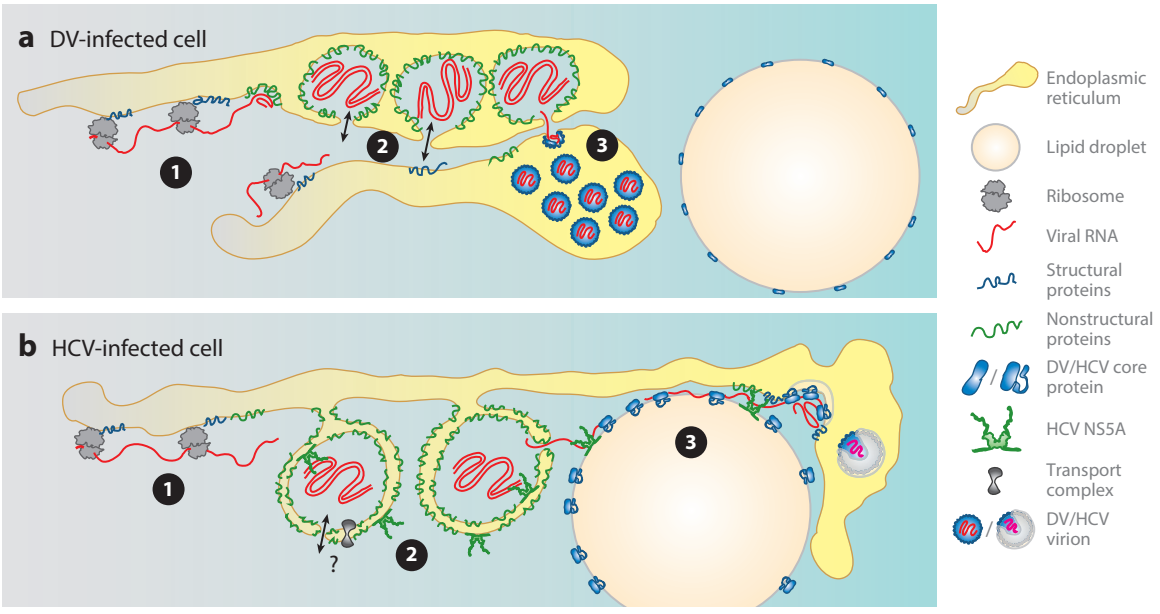

Figure 2

Compartmentalization of the different steps in the Flaviviridae replication cycle. The spatiotemporal orchestration of (*a*) dengue virus (DV) and (*b*) hepatitis C virus (HCV) replication cycles is schematically depicted. (❶) Incoming viral RNA is directly translated at the rough endoplasmic reticulum. (❷) Viral and co-opted host cell factors induce membrane alterations that give rise to replication organelles in which replicating dsRNA is shielded in membrane-delineated compartments. Import of metabolites as well as export of progeny viral RNA is mediated by neck-like openings of DV-induced invaginated vesicles (panel *a*), whereas in the case of HCV-induced DMVs, this transport might occur through DMVs that are not fully closed or may be mediated by a proteinaceous transporter (panel *b*). (❸) Budding of progeny virus particles occurs in juxtaposition with vesicle openings in the case of DV (panel *a*), whereas lipid droplets might represent a cellular hub for coordination of HCV assembly (panel *b*).

is more efficient, concomitant with low levels of innate immunity induction, compared with the related but less pathogenic Japanese encephalitis virus (40).

In contrast to the intuitive topological organization of flavivirus replication factories, the situation for HCV replication organelles is less obvious. HCV-induced DMVs contain dsRNA as well as nascent viral RNA, suggesting an important role in genome amplification (22, 41). Furthermore, the membrane-protected nature of HCV RNA suggests that replication sites are localized on the luminal side of DMVs, which would require extensive transport processes across two membranes. Theoretically, this translocation could be carried out by a proteinaceous transport machinery, as initially proposed for Coronaviridae (25). On one hand, HCV recruits components of the nuclear pore complex that might contribute to a transport mechanism (42, 43). On the other hand, DMVs are presumably highly dynamic structures; active replication in their interior is supported as long as they are connected to the cytosol and might cease upon DMV membrane closure (**Figure 2**) (13). In any case, cellular biosynthetic metabolites such as nucleotides are present in replication organelles. For example, a specific reporter in live cells demonstrated ~5-fold elevated ATP levels within the HCV replication compartment (44).

Viral replication complex: distinct structure comprising the viral replicase, surrounding membranes, and cellular cofactors and metabolites; synonym for viral replication factory

Amplified viral RNA has to be transferred to assembly sites in a presumably regulated manner. In order to spatially separate replication and assembly complexes, (excess) amounts of viral core/capsid protein, which is a structural component of virus particles and binds viral RNA with high affinity, are targeted to distinct cellular compartments, most notably the nucleus or

lipid droplets (LDs), the latter of which have been proposed to play a central role in HCV assembly (45–50). Indeed, DMVs often localize in close proximity to LDs (**Figure 1*i***), which might be mediated by binding of the NS4B N-terminal domain to the LD membrane monolayer (51). In contrast, DV budding is observed in juxtaposition with invaginated vesicle openings without an obvious topological link to LDs (**Figure 1*d***). Hence, the precise role of DV capsid localization to LDs remains to be determined. One possibility is to sequester the capsid protein at sites that are remote from replication sites, because high-affinity capsid-RNA interactions might interfere with RNA replication.

Lipid droplet (LD): dynamic cellular lipid storage organelle composed of a phospholipid monolayer containing proteins and a neutral lipid core containing triglycerides and cholesteryl ester

NS5A serves an important role in HCV particle production by delivering progeny RNA to assembly sites via interactions between its domain III and the core protein (**Figure 2*b***) (52–54). Importantly, in addition to NS5A, several other NS proteins, including NS4B and the NS3 helicase domain, are involved in progeny virus production (reviewed in 55). Moreover, multiple components of the intercellular (very) low density lipoprotein [(V)LDL] lipid transport system are subverted for HCV progeny production, yielding virus particles with unique biophysical properties such as very low density and incorporation of apolipoproteins (reviewed in 56). In contrast, flavivirus particle assembly and release remain less well defined at the molecular level. Interestingly, only actively replicated RNA is specifically packaged (57), presumably as a consequence of the spatial linkage of flavivirus replication and assembly sites (**Figure 1*d***). Similar to the case of HCV, genetic studies suggest that all flavivirus NS proteins have a role in particle production (reviewed in 58), but how the RNA is packaged and what triggers virion budding remain to be defined.

VIRAL DETERMINANTS OF REPLICATION ORGANELLES

In principle, membrane bending in cellular systems is achieved by local modification of membrane lipid composition or asymmetric interactions with proteins (reviewed in 59, 60). The latter includes scaffolding by peripheral membrane proteins, asymmetric insertion of amphipathic α-helices (AHs), oligomerization of (asymmetrically shaped) integral membrane proteins, or interactions with the cytoskeleton and associated motor proteins. Whereas negative membrane curvature is predominant in flavivirus replication organelles, positive membrane curvature toward the cytoplasm prevails for HCV-induced DMVs (**Figure 3**). Despite this distinction, membrane activity of Flaviviridae proteins underlies common principles, and morphological differences among viral replication factories can at least in part be explained by their structural features.

The flavivirus integral membrane proteins NS4A and NS4B have been proposed as main drivers of DV-triggered remodeling events (61–63). Both exhibit complex membrane topologies and contain central helices assumed to lie in plane on the ER-luminal membrane leaflet (62, 64). This arrangement locally increases the membrane surface area, thus favoring negative curvature and inducing invaginated vesicles (**Figure 3*a***). This process likely is sustained by oligomerization of NS4A as well as by its interaction with NS4B (65, 66). However, membrane structures induced by the sole expression of NS4A or NS4B do not resemble those induced in infected cells, indicating that additional viral proteins are involved in flavivirus replication factory morphogenesis. One candidate is NS1, which forms dimers that bind to and remodel membranes in vitro (67) and likely interacts with the NS4A-NS4B complex (**Figure 3*a***) (68, 69). In addition, NS2A, a multispan integral membrane protein (70) with membrane-permeabilizing activity (71), might also be involved in the induction of flavivirus replication organelles, but this remains to be determined.

In the case of HCV, the sole expression of the viral replicase proteins NS3–5B triggers DMV structures that are indistinguishable from those induced in virus-infected cells (**Figure 3*b***). NS4B has been proposed as the main inducer of the membranous web (18). It is a highly hydrophobic

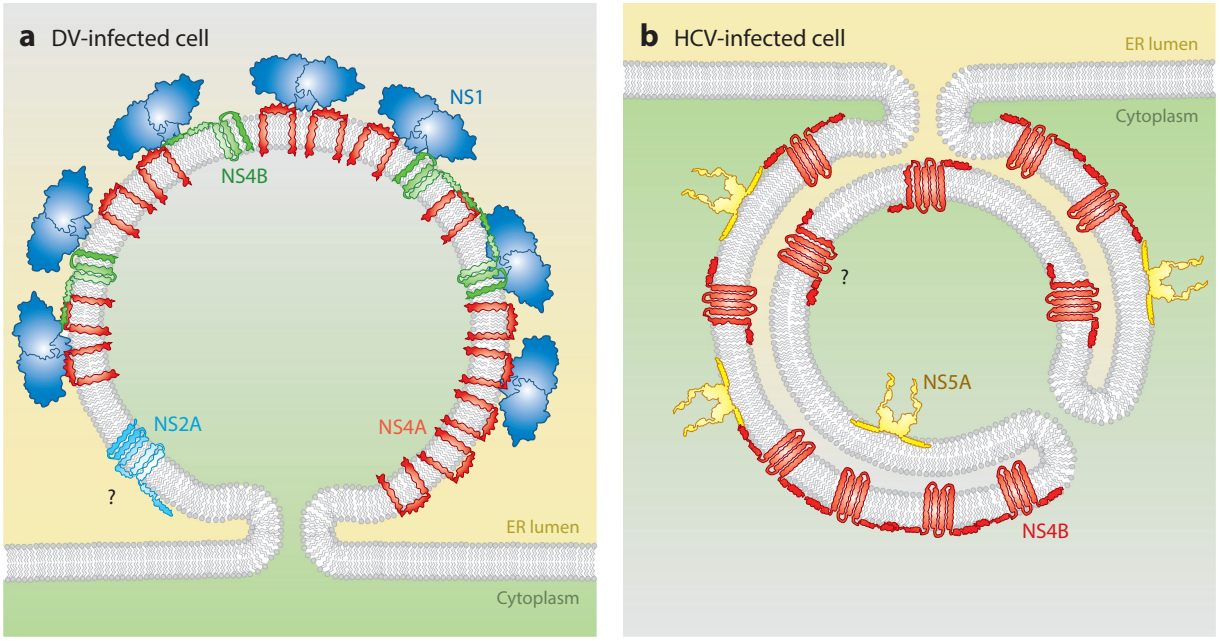

Figure 3

Mechanisms of membrane curvature induction by viral proteins. (*a*) Schematic illustration of the overall negative membrane curvature of flavivirus-induced invaginated vesicles. The integral membrane proteins NS4A and NS4B contain helices that reside in the plane of the endoplasmic reticulum (ER)-luminal membrane leaflet, thereby increasing the surface area. Dengue virus (DV)-induced membrane bending is further supported by homo-oligomerization of NS4A, which additionally interacts with NS4B and NS1. The latter is a homodimeric protein that resides in the ER lumen and possesses membrane-remodeling activity. The possible role of NS2A in membrane alterations remains to be determined, as indicated by the question mark. (*b*) In the case of hepatitis C virus (HCV), the NS4B and NS5A replicase proteins induce double-membrane vesicles (DMVs). Multiple NS4B amphipathic α-helices increase the surface area on the cytosolic side of the ER, inducing positive membrane curvature. This process is further supported by the protein's self-interaction. The contribution of the NS4B N-terminal domain's dual topology to DMV biogenesis remains to be determined, as indicated by the question mark. The contribution of the dimeric (or eventually oligomeric) NS5A could be attributed to its N-terminal amphipathic α-helix or might be mediated by the recruitment of host cell factors.

protein with a complex membrane topology comprising four AHs in its cytoplasmic N- and C-terminal domains flanking four transmembrane-spanning helices (reviewed in 72, 73). Of note, NS4B N- and C-terminal domains interact with membranes and exhibit membrane activity in vitro (74, 75). Thus, it is likely that NS4B AH insertion into the cytoplasmic membrane leaflet facilitates positive membrane curvature. However, NS4B exhibits a dual topology induced by a partial posttranslational flip of the N-terminal domain into the ER lumen (76, 77), which likely regulates NS4B membrane activity. Additionally, NS4B oligomerization, mediated by AH2 and determinants in the C-terminal domain, is required for the induction of functional HCV replication complexes (78, 79), to which positively charged residues within AH1 critically contribute (77). Despite the prominent role of NS4B, the concerted action of HCV replicase proteins is required for DMV biogenesis, with NS5A being another important determinant (13). This protein forms homodimers and, presumably, oligomeric complexes (80, 81) and contains an N-terminal AH (82) that alters properties of model membranes in vitro (83). Accordingly, this AH might directly contribute to HCV-induced membrane remodeling. Alternatively or additionally, NS5A might influence membrane properties by recruiting cellular factors (see below). Furthermore, NS5A interacts with NS4B (84), which likely contributes to DMV biogenesis. In any case, the

importance of NS5A in HCV replication organelle induction is highlighted by the mechanism of action of directly or indirectly acting NS5A-targeting drugs that abrogate membranous web formation, thus representing a new paradigm in antiviral therapy (85, 86).

SUBVERTED CELLULAR FACTORS AND PATHWAYS

In addition to protein-intrinsic membrane-active properties, members of the Flaviviridae family use strategies to selectively hijack the cellular machinery involved in lipid and membrane homeostasis. These activities include the recruitment of cellular membrane-shaping proteins, stimulation of lipogenesis, and repurposing the cellular lipid transport system to specifically modulate viral replication factory membrane lipid composition.

Membrane-Active Proteins

Surprisingly little is known about flavivirus co-opted host cell proteins contributing to the formation of replication organelles. One example is vimentin, a structural component of intermediate filaments of the cytoskeleton that interacts with DV NS4A (87). NS4A-mediated recruitment of vimentin to the viral replication compartment is required for efficient RNA amplification. Interestingly, polymerization of actin filaments plays an important role in membrane-remodeling events at the plasma membrane, such as those involved in endocytosis (reviewed in 88). However, whether vimentin plays a similar role in DV membrane rearrangements or whether it instead stabilizes DV-induced vesicle packets in the cytoplasmic space once formed is not known.

Owing to the lack of information about host cell factors other than vimentin contributing to the biogenesis of flavivirus replication factories, currently we can only make inferences from other positive-strand RNA viruses that induce replication factories of the same morphotype. One prominent example is brome mosaic virus (BMV), which also replicates in ER-luminal invaginated vesicles (89). Biogenesis of these vesicles requires cellular reticulon proteins (90). These ER-resident proteins contain conserved hairpin-like transmembrane segments and are involved in generating and stabilizing high-curvature ER tubules (reviewed in 91). BMV replicase protein 1a recruits reticulon proteins to ER membrane invaginations, which facilitates expansion of the spherule volume by partially neutralizing overall negative membrane curvature or stabilizing positive membrane curvature in neck-like openings toward the cytoplasm (90). Whether reticulon or other cellular membrane-shaping proteins are also involved in the biogenesis of flavivirus replication organelles remains to be investigated.

In contrast to the proviral role of reticulon proteins in BMV replication, this class of membrane-shaping proteins negatively regulates HCV RNA replication (92). Reticulon 3 specifically interacts with AH2 of NS4B, thereby decreasing AH2-mediated NS4B self-interaction. Because this interaction is a prerequisite for HCV-induced membrane remodeling (**Figure 3*b***), silencing of reticulon 3 expression increases HCV replication, whereas overexpression of this protein has a detrimental effect. In this regard, reticulon 3 appears to act as a restriction factor. However, proviral roles of host membrane-shaping proteins have been described for HCV. One example is proline-serine-threonine phosphatase interacting protein 2 (PSTPIP2). This protein belongs to the Bin-Amphiphysin-Rvs (BAR) protein superfamily and supports membrane alterations induced by HCV (93). In cellular homeostasis, BAR domain–containing proteins play important roles in endocytosis and intracellular membrane trafficking (reviewed in 94). Dimerization via the BAR domains yields a concave-shaped structure that scaffolds membranes via positively charged residues that interact with negatively charged phospholipid head groups (reviewed in 95). Indeed, overexpression of PSTPIP2 triggers the induction of cytoplasmic membrane tubules, underlining

its capacity to induce positive membrane curvature (93). PSTPIP2 is recruited to the HCV replication compartment by interaction with NS4B and NS5A, and its crucial role is supported by the abrogation of membranous web formation upon PSTPIP2 depletion.

Due to the close morphological similarity of autophagosomes and viral DMVs, subversion of this cellular catabolic pathway has been proposed to be involved in HCV replication complex biogenesis (96). Indeed, lipidation of LC3, a key event in autophagosome biogenesis, is observed upon HCV infection (97), and the protein is furthermore associated with viral protein–containing membrane fractions (41). However, contradictory functions of autophagy in the replication cycle of HCV and other positive-strand RNA viruses have been proposed (reviewed in 14). Hence, it remains unclear whether the autophagy core machinery, or components thereof, is directly involved in membrane remodeling or whether the pathway is triggered as a secondary cellular (stress) response due to massive alterations of cellular endomembranes.

Lipogenesis

In addition to remodeling existing intracellular membranes, the biogenesis of Flaviviridae replication organelles implies a net increase of intracellular membrane surface and thus requires de novo synthesis of membrane lipids. In fact, high-throughput lipidomic profiling of DV- and HCV-infected cells revealed significant virus-induced alterations of lipid composition, including specific up- or downregulation of main structural and signaling lipid species (98, 99). Cellular lipid homeostasis is tightly controlled at multiple levels, and its dysregulation is involved in multiple human diseases. Transcriptional induction of genes involved in lipid biosynthesis is governed by the sterol regulatory element–binding protein (SREBP) pathway. SREBPs are transcription factors that reside as inactive membrane-bound precursors in the ER, which, upon stimulation, traffic to the Golgi. Proteolytic activation by site 1 protease (S1P) and S2P causes release from the membrane and subsequent trafficking to the nucleus, where mature SREBPs stimulate transcription of genes involved in lipid biosynthesis (reviewed in 100). HCV infection as well as ectopic expression of core or NS4B triggers proteolytic cleavage of SREBPs, leading to elevated levels of lipogenic transcripts such as fatty acid synthase (FAS) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), which is the rate-limiting enzyme of the cholesterol biosynthetic mevalonate pathway (**Figure 4*a***) (101, 102). Notably, the metabolic intermediate geranylgeranylphosphate in protein prenylation is required for HCV replication (103, 104). Furthermore, HCV stimulates SREBP-driven induction of lipogenesis by subverting an unprecedented function of an innate immunity pathway (105). The 3′ untranslated region of the HCV RNA genome interacts with DEAD box polypeptide 3 X-linked (DDX3X), which activates the immune modulator IKK-α. In a noncanonical, NF-κB-independent fashion, IKK-α stimulates the CBP/p300 coactivator that promotes SREBP-mediated transcription. However, as both HCV replication and assembly rely heavily on host cell lipids (56), the relative contribution of SREBP-dependent gene induction to each particular step of the viral replication cycle remains to be determined. In support of a role for the SREBP pathway in HCV particle production, studies with S1P-specific inhibitors suggest that SREBP-driven lipogenesis is required for assembly and release of infectious HCV virions (106).

In contrast, DV does not rely on SREBP-mediated transcriptional activation of lipogenic biosynthetic enzymes but instead uses lipids from intracellular storage sites (**Figure 4*b***) (107). In fact, DV infection induces the autophagy-mediated breakdown of cytosolic LDs to release free fatty acids (108). In turn, free fatty acids might be used as building blocks for new membrane lipids in order to establish viral replication factories or, alternatively, might serve as an energy source in mitochondrial β-oxidation. Additionally, DV subverts FAS to stimulate membrane expansion during replication organelle biogenesis (109). FAS is a large homodimeric

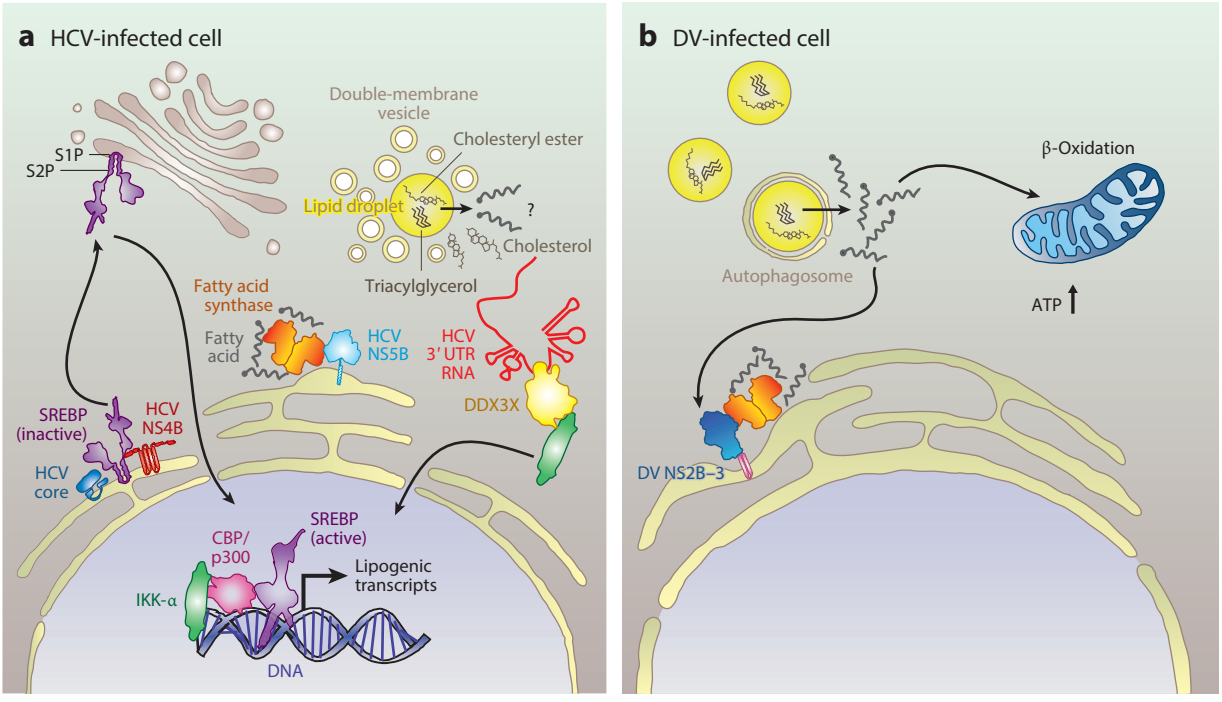

Figure 4

Lipogenesis in hepatitis C virus (HCV)- and dengue virus (DV)-infected cells. (*a*) HCV infection triggers the transcriptional induction of lipid biosynthesis–related genes via the sterol regulatory element–binding protein (SREBP) pathway. The endoplasmic reticulum–localized inactive SREBP precursor traffics to the Golgi upon HCV infection or expression of core or NS4B, where it is proteolytically processed by site 1 protease (S1P) and S2P. The released N-terminal fragment is transported into the nucleus and activates transcription of lipogenic factors. The pathway is further stimulated by an interaction of the 3′ untranslated region (3′ UTR) of the HCV RNA genome with DEAD box polypeptide 3 X-linked (DDX3X). DDX3X activates IKK-α, which in turn stimulates the nuclear CBP/p300 coactivator to promote SREBP-mediated transcription. Whether HCV mobilizes fatty acids from lipid droplets for biogenesis of double-membrane vesicles residing in close proximity remains to be explored (as indicated by the question mark). (*b*) DV infection mobilizes free fatty acids from lipid droplets by subverting autophagy. Apart from a possible role during DV-induced membrane remodeling, lipid release from lipid droplets is required for ATP production by oxidation of fatty acids in mitochondria. The recruitment of fatty acid synthase to sites of DV replication allows the incorporation of newly synthesized lipids during DV replication organelle biogenesis.

protein containing multiple enzymatic activities for de novo lipid synthesis and hence for membrane formation (reviewed in 110). DV NS3 interacts with and thereby recruits FAS to viral replicase complexes during the establishment of replication organelles (109). This recruitment depends on Rab18 (111), which is a small GTPase involved in intracellular membrane trafficking that resides, among other places, on the surface of LDs (reviewed in 112). Interestingly, HCV also co-opts Rab18 via interactions with NS5A to spatially coordinate sites of RNA replication and virion assembly (113). In the case of DV, NS3 stimulates FAS activity, and de novo synthesized lipids cofractionate with the viral RNA (109), suggesting direct engagement of FAS in membrane expansion during the course of replication complex establishment. Importantly, the closely related flavivirus WNV, as well as HCV, also depends on FAS (114, 115), stressing the effect of this evolutionarily conserved function in the biogenesis of Flaviviridae replication organelles.

Modification of Membrane Lipid Composition

Apart from their role as structural components, for example, in the assembly of viral replicase complexes and compartmentalization of viral replication factories, membranes also are more directly involved in biological processes such as signaling events and recruitment of factors. The biological importance of lipids is reflected by the existence of thousands of different lipid species that can serve multiple functions (reviewed in 116). Importantly, lipid asymmetry among the two monolayers as well as lateral segregation into subdomains and among different organelles generates specific lipid signatures of intracellular membranes, which are absolutely required for cell homeostasis. A key example is the complex regulation and signaling functions of phosphoinositide lipids (reviewed in 117), which are required for formation of viral replication factories of the DMV morphotype. In the case of HCV, numerous groups have identified phosphatidylinositol 4-kinase IIIα (PI4KIIIα) and its product phosphatidylinositol 4-phosphate (PI4P) as important factors for the integrity and functionality of the membranous web (**Figure 5**). Whereas the α isoform of this lipid kinase was discovered as a major proviral factor for genotype 2 HCV in multiple high-throughput siRNA screens (118–122), PI4KIIIβ appears in addition to be required for HCV genotype 1 replication (123) and for the replication of picornaviruses (reviewed in 124). Depletion of this protein or pharmacological inhibition of lipid kinase activity profoundly interferes with HCV replication (125, 126). Although these findings qualify PI4K as a target for therapy of chronic hepatitis C, results obtained with knockout mice suggest a high likelihood for cytotoxicity upon interference with this kinase (127). In any case, the HCV replicase factors NS5A and NS5B interact with and thereby recruit PI4KIIIα to replication organelles (122, 128). Moreover, PI4KIIIα lipid kinase activity appears to be stimulated in infected cells, giving rise to massive accumulation of PI4P at intracellular membranes, concomitant with a reduction of PI4P in the plasma membrane (125). Importantly, knockdown of PI4KIIIα expression as well as pharmacological kinase inhibition causes aggregation of DMVs that exhibit significantly reduced diameter (122, 129). These findings imply that PI4KIIIα is dispensable for the induction of DMVs per se but is responsible for modulating DMV lipid content and thus functionality. Intriguingly, nonvesicular transport of other lipids, such as cholesterol and sphingolipids, can be mediated by particular lipid transfer proteins targeting PI4P-enriched membranes (reviewed in 130). One example is oxysterol-binding protein (OSBP)-mediated cholesterol transport, which is harnessed by HCV (**Figure 5**) (129). The N-terminal pleckstrin-homology domain of OSBP mediates PI4P binding, whereas the C-terminal sterol-binding domain mediates cholesterol transport to PI4P-enriched HCV-remodeled membranes (129), consistent with the high cholesterol content of DMVs (**Figure 5**) (22). VAP-A and VAP-B, which bind to NS5A and are required for HCV RNA replication (131, 132), facilitate this process. VAPs interact with OSBP via its FFAT motif, which in uninfected cells stimulates the recruitment of sterol-loaded OSBP to Golgi membranes, where it exchanges cholesterol for PI4P (133). Of note, the FFAT motif of OSBP is required for cholesterol transport to replication sites during HCV infection (129). Blocking OSBP-mediated cholesterol delivery to HCV replication organelles (129), as well as depletion of this lipid from purified DMVs, significantly reduces their size (22), suggesting a structural role for cholesterol in HCV-rearranged membranes. In support of this idea, oxidation of cholesterol by the interferon-inducible cholesterol 25-hydroxylase is detrimental to HCV replication (134). Additionally, HCV subverts another PI4P effector, namely four-phosphate adaptor protein 2 (FAPP2), which facilitates nonvesicular transport of glycosphingolipids (**Figure 5**) (135). Both PI4P and glycosphingolipid-binding domains of FAPP2 are required for HCV replication, but the actual enrichment of this lipid class in HCV-remodeled membranes remains to be revealed. Nevertheless, a functional role for sphingolipids in HCV RNA replication is further

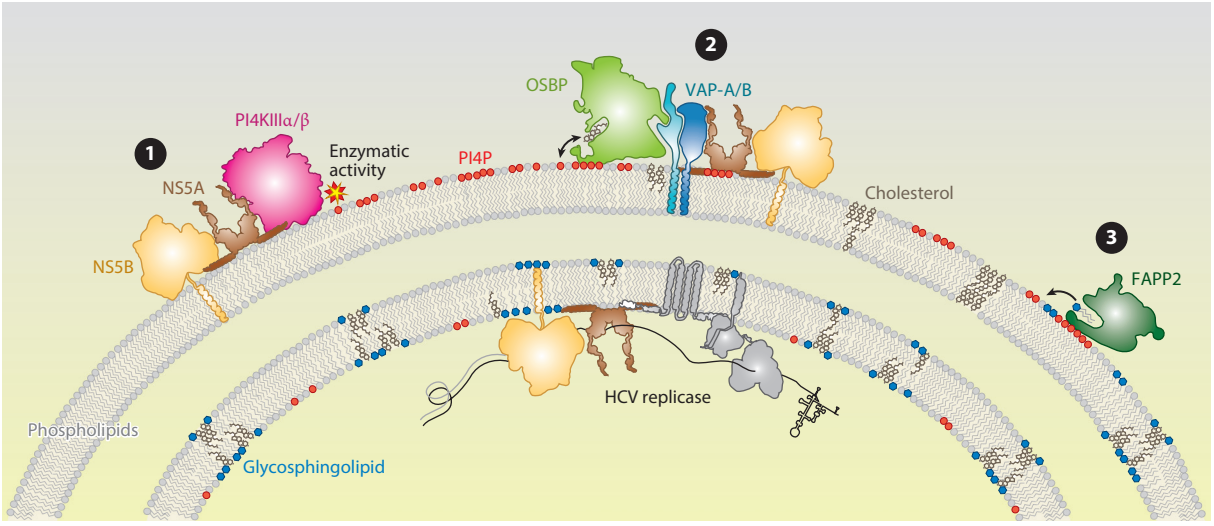

Figure 5

Modification of membrane composition by lipid transfer proteins. Schematic representation of changes in lipid composition occurring at sites of hepatitis C virus (HCV)-induced membrane rearrangements. The HCV proteins NS5A and NS5B recruit phosphatidylinositol 4-kinase IIIα/β (PI4KIIIα/β), likely stimulating phosphatidylinositol 4-phosphate (PI4P) synthesis on site (❶). Subsequently, lipid transfer proteins such as oxysterol-binding protein (OSBP) (❷) or four-phosphate adaptor protein 2 (FAPP2) (❸) are recruited to PI4P-containing membrane microdomains and deliver required lipids to HCV-remodeled membranes. OSBP recruitment also is facilitated by interactions with VAP-A/B and NS5A, and the protein delivers cholesterol in exchange for PI4P (❷), whereas FAPP2 is thought to mediate delivery of glycosphingolipids to HCV-rearranged membranes (❸).

supported by the upregulation of specific sphingolipid species in HCV-infected cells, which in turn stimulate NS5B activity (136, 137). Taken together, these findings likely explain the reported association of the HCV replicase with intracellular detergent-resistant lipid-raft-like assemblies (138).

Whether flaviviruses harness cellular lipid transport proteins in a similar fashion remains to be determined. These viruses do not seem to engage PI4P (114, 122), but a proviral role of cholesterol in the biogenesis of flavivirus replication organelles has been reported (139, 140). Nevertheless, yeast- and plant cell–infecting tombusviruses that induce spherule-like membrane invaginations resembling those of flaviviruses co-opt OSBP-related proteins and VAPs at membrane contact sites to transport cholesterol to remodeled membranes (141). This observation suggests that non-vesicular transport of specific lipids also occurs for viruses that induce invaginated vesicle–type replication organelles. The overall negative membrane curvature of flavivirus-induced vesicular invaginations might additionally be supported by specific lipids such as phosphatidic acid. Due to its conical shape, phosphatidic acid induces local negative membrane curvature, which is required for vesicle fission (142, 143). Indeed, elevated levels of phosphatidic acid are found in DV-infected cells (98), but whether and how this lipid is enriched in DV replication organelles remain to be determined.

CONCLUSION

Although advanced electron microscopy imaging techniques allowed the description of Flaviviridae replication organelles in unprecedented morphological detail, we are only beginning to understand the complex interplay of viral and co-opted cellular factors involved in their biogenesis.

Owing to technical limitations, the dynamics underlying viral replication factory formation are not known, and information, especially about the early stages of their biogenesis, is therefore scarce. These limitations might be overcome by correlative light and electron microscopy, which allows analysis of rare events in a cell with extremely high precision. In fact, this method enabled the 3D visualization of endocytosis with high temporal resolution at the level of single endocytic vesicles (144). Furthermore, technical advances in viral protein and RNA labeling strategies suitable for time-resolved imaging will provide deeper insights into transport processes and the spatiotemporal orchestration of the different steps in the viral replication cycle. Molecular mechanisms underlying viral protein–intrinsic membrane activities are also poorly characterized. However, determination of their 3D structure in a native membrane environment using, for example, solid-state nuclear magnetic resonance spectroscopy (145) combined with functional in vitro studies with model membranes will provide deeper mechanistic insight. Moreover, high-throughput proteomic and lipidomic profiling of viral replication organelles, as performed for transport vesicles (146, 147), will reveal their molecular composition, thereby expanding our view on host factors and pathways implicated in viral replication factory biogenesis and function. Integration of these approaches will certainly reveal the anatomy of these fascinating virus-induced structures. These results may also lead to new avenues for antiviral therapy (85, 86, 148) and reveal new principles of cellular membrane homeostasis.

SUMMARY POINTS

1. Positive-strand RNA viruses, including members of the Flaviviridae family, replicate their genomes in association with remodeled cytoplasmic membranes. These membranous structures are called viral replication factories or replication organelles.
2. The formation of specialized replication compartments allows spatiotemporal control of the viral replication cycle, ensures RNA amplification with high efficiency by mediating a high local concentration of necessary factors, and protects the viral RNA from the hostile cytoplasmic environment.
3. Two morphologically distinct classes of replication organelles, the invaginated vesicle/spherule type, as represented by flaviviruses, and the double-membrane vesicle type, as represented by hepatitis C virus, can be distinguished.
4. Intrinsic properties of Flaviviridae membrane proteins, including membrane-active regions, asymmetric shape, and oligomerization, contribute to the biogenesis of replication organelles.
5. Cellular proteins implicated in direct membrane shaping (e.g., reticulons and BAR domain–containing proteins) or indirect membrane shaping (e.g., proteins that act by altering membrane lipid content) are harnessed by members of the Flaviviridae family.
6. To increase intracellular membrane surface area as required for virus-induced membrane-remodeling events, members of the Flaviviridae family stimulate lipogenesis. Whereas hepatitis C virus subverts SREBP-mediated transcriptional activation of lipid biosynthetic enzymes, dengue virus uses autophagy to mobilize lipids from lipid droplets and co-opts fatty acid synthase.
7. Modification of the endoplasmic reticulum–derived membrane lipid composition of hepatitis C virus replication organelles is mediated by viral subversion of cellular lipid transfer proteins.

FUTURE ISSUES

1. Time-resolved imaging of replication organelles by superresolution microscopy or correlative light and electron microscopy will allow definition of the spatiotemporal regulation of membrane-remodeling events in the viral life cycle.
2. New labeling techniques to specifically track proteins, RNA, and lipids involved in Flaviviridae replication should be employed to determine the dynamics of viral replication factory biogenesis and its regulation in live cells.
3. Detailed 3D structure determination of viral membrane proteins in their native environment is required and should be facilitated by the advent of in vitro expression and reconstitution systems as well as new methods of structure determination requiring small amounts of protein.
4. In vitro studies using model membranes are required to decipher determinants of viral protein–intrinsic membrane activity. This work also will reveal the biochemical and structural features underlying lipid-protein interactions.
5. High-throughput profiling of replication organelles for protein and lipid content is necessary to gain a comprehensive picture of host constituents in viral replication factories and to decipher the cell biology underlying replication factory biogenesis.
6. Phenotypic screens to identify host cell factors required for viral replication factory biogenesis will complement these profiling approaches. In this way, the identification of host cell factors required by multiple viruses for efficient replication might reveal targets for broad-spectrum antiviral drugs.

DISCLOSURE STATEMENT

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