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(+) RNA virus replication compartments: a safe home for (most) viral replication

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This review describes recent advances in our understanding of the mechanisms by which (+) RNA viruses establish their replication niche.

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Introduction

A hallmark of all (+) RNA viruses is their ability to sequester host intracellular membranes to generate replication compartments (RCs). These RCs contain viral RNA and proteins as well as several recruited host proteins and lipids that create a favorable environment for RNA replication. RCs may serve as platforms to concentrate viral RNA, proteins, and nucleotides, creating an appropriate replicase topology. Additionally, RCs form a barrier between viral RNA replication and the cytosol, which contains innate immune sensors and RNA degradation machinery. In this paper, we review in mechanistic detail the formation of viral RCs and highlight recent findings that have advanced our understanding of (+) RNA virus replication.

Replication compartment morphology

RCs are derived from different sources, depending on the virus, including the endoplasmic reticulum (ER), Golgi, peroxisomes, endosomes, mitochondria and plasma membrane [1]. For some viruses, the source of membranes is unimportant. Flock house virus (FHV) replication is unperturbed by targeting RCs to a different subcellular location [2]. However, other (+) RNA viruses such as hepatitis C virus (HCV) have distinct but spatially linked sites of replication and virion assembly that likely require specific localization of RC formation [3,4•]. Multiple lines of evidence support a role for RCs as the site of viral replication, as opposed to a cellular response to viral infection. Immuno electron microscopy (EM) detects BrUTP incorporation into FHV viral RNA localized inside RCs [5], while long (>40 nucleotide) dsRNA, indicative of the RNA replication intermediate, is detected in RCs for several (+) RNA viruses [6–11,12•].

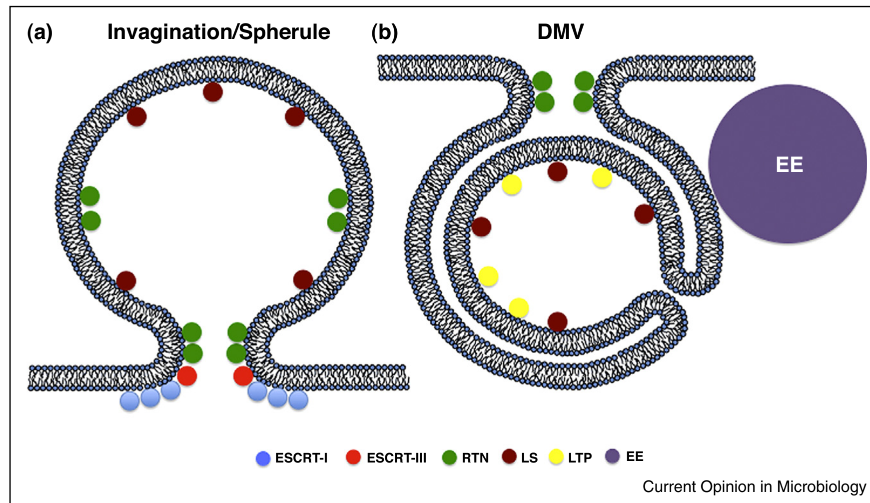
Although viral RCs have been described using standard EM for a long time, we have only begun to appreciate their fine structural details in the last decade. Advances in EM tomography (EMT) have enabled a high-resolution description of viral RCs for multiple viruses [3,5,7,11,12•,13–20]. Despite the evolutionary distance between these (+) strand RNA viruses, they appear to induce two general classes of membrane modifications: invaginations/spherules or double membrane vesicles (DMVs), suggesting conserved mechanisms behind their formation [21].

Invagination RCs were first visualized by EMT for FHV, which induces them at the outer mitochondrial membrane [5]. Invaginations entail the induction of negative membrane curvature (bending away from the cytosol) on an intracellular membrane to yield a vesicle budded into the luminal space (Figure 1a). These invaginations have portions of positive membrane curvature (bending towards the cytosol) at their neck that keeps the vesicle attached to the organelle from which it originated [22].

Capsids have not been observed in RCs, leading to a model wherein viral RNA is replicated inside RCs and then (+) RNAs are transported outside to sites of virion assembly. EMT of dengue virus (DENV)-infected cells revealed invaginations into the ER lumen that are connected to the cytoplasm via a neck-like opening [7,19]. On the cytosolic side of the neck are nucleocapsids, suggesting that viral (+) RNA is transported through the neck for capsid assembly immediately adjacent to the RC [7]. ER invaginations that retain a pore to the cytosol have also been shown for other flaviviruses, including tick borne encephalitis virus [18], West Nile virus (WNV) [13] and Langat virus [17].

Other viruses that induce invaginations include brome mosaic virus (BMV), tombusvirus and the togaviruses (Table 1). Togavirus infection leads to formation of cytopathic vacuoles, which are modified endosomes, lysosomes and internalized plasma membrane fragments of around 600–2000 nm that accommodate invaginations to create replication compartments [14,23,24].

Figure 1



Host cell pathways involved in replication compartment formation of (+) RNA viruses. **(a)** Schematic illustration of an invagination replication compartment with overall negative membrane curvature. **(b)** Schematic illustration of a DMV replication compartment with overall positive membrane curvature. RTN: reticulon; LS: lipid synthesis; LTP: lipid transfer protein; ESCRT: endosomal sorting complexes required for transport; EE: early endosome.

The other class of RCs is DMVs, which are more complicated structurally and frequently accompanied by other membrane rearrangements (Figure 1b). Prototype viruses of the DMV RC class include the coronaviruses, picornaviruses, and hepatitis C virus (HCV). HCV DMVs are ER-derived vesicles that appear sealed from the cytoplasm [3]. Since there is not an obvious neck, as with the invagination RCs, it is unclear how HCV RNA could traffic from the RC to the site of virion assembly, the lipid droplet. It has been proposed that components of the nuclear pore complex are recruited to HCV RCs to regulate traffic into and out of the RC [25,26•]. HCV also induces single-membrane and multi-membrane vesicles that are not associated with replicating viral RNA, but

Table 1

(+) RNA viruses and their replication compartments

Family	Bromoviridae	Flaviviridae	Flaviviridae	Nodaviridae	Togaviridae	Togaviridae	Tombusviridae
Genus	Bromovirus	Flavivirus	Flavivirus	Alphanodavirus	Alphavirus	Rubivirus	Tombusvirus
Species	BMV	DENV	WNV	FHV	SFV	RUBV	TBSV
Replication compartment	Invaginations	Invaginations	Invaginations	Invaginations	Invaginations	Invaginations	Invaginations
Membrane source	ER	ER	ER	Mitochondria	PM/Endosomes	Endosomes/ Lysosomes	Perixosomes
Viral proteins	1a [32]	NS4A, NS4B [65–67]	NS4A, NS4B [68,69]	Protein A [70]	P123 [71]	P150, P90 [72]	p33 [73]
Host Pathways	RTN, ESCRT, LS	LS	LS	LS	LS	LS	ESCRT, LS

Family	Arteriviridae	Coronaviridae	Dicistroviridae	Flaviviridae	Picornaviridae	Picornaviridae
Genus	Arterivirus	Coronavirus	Cripavirus	Hepacivirus	Enterovirus	Cardiovirus
Species	EAV	SARS-CoV	Drosophila C	HCV	PV, CBV3, EV71	EMCV
Replication compartment	DMV	DMV	DMV	DMV	DMV	DMV
Membrane source	ER	ER	Golgi	ER	Golgi/ER	Golgi/ER
Viral proteins	nsp2, nsp3 [74,75]	nsp3, nsp4, nsp6 [76]	—	NS4B, NS5A [77,78]	2B, 2C, 3A [79,80]	2B, 2C, 3A [81]
Host pathways	ERAD	ERAD	LS	LS, LTP	LS, LTP, EE, RTN	LS, LTP

BMV: brome mosaic virus; DENV: Dengue virus; WNV: West Nile virus; FHV: Flock-house virus; SFV: Semliki Forest Virus; RUBV: Rubella virus; TBSV: Tomato bushy stunt virus; ER: endoplasmic reticulum; PM: plasma membrane; nsp: non-structural protein; RTN: reticulon; LS: lipid synthesis; LTP: lipid transfer protein; ESCRT: endosomal sorting complexes required for transport; EAV: equine arterivirus; SARS-CoV: severe acute respiratory syndrome coronavirus; HCV: hepatitis C virus; PV: poliovirus; CVB3: COXSACKIEVIRUS B3; EMCV: encephalomyocarditis virus; DMV: double membrane vesicle; ERAD: endoplasmic reticulum associated degradation; EE: early endosome.

may represent endosomes and/or autophagosomes, which are sometimes observed in the proximity of RCs [10,27–29].

For enteroviruses, the replication compartments are a mix of single and double membrane vesicles that may originate from multiple cellular membrane sources. EMT analysis revealed that early in infection (~2 hpi) RCs consist of mainly single-membrane tubules that transform into larger DMVs and subsequently into multilamellar structures as infection progresses [15]. Formation of DMVs is observed during coronavirus and arterivirus replication as well [16,30]. Coronavirus DMVs range in size from 150 to 300 nm and appear as a network of membranes continuous with the rough-ER [11,31]. The DMV inner membrane appears as a closed compartment and it is not clear how import of substrates or export of RNA is achieved.

Mechanisms of replication compartment formation

Induction of membrane curvature via viral and cellular proteins

Multiple processes that curve cellular membranes have been described, such as insertion of proteins or irregularly shaped lipids [1]. Many viral proteins have been implicated in the formation of RCs (Table 1). These may alter membrane shape directly, by associating with membranes and inducing curvature; or indirectly, by recruiting cellular factors to alter membrane morphology. A direct role for viral proteins altering membrane curvature has been challenging to prove because RC formation has not been reconstituted *in vitro* for most viruses. Many of the viral proteins implicated in RC formation have properties that may alter membrane morphology, such as multiple transmembrane domains and/or amphipathic helices, in addition to protein oligomerization.

Two examples of recruiting cellular machinery to modify membrane curvature have been defined thus far: reticulons and the endosomal sorting complexes required for transport (ESCRT) proteins. BMV 1A protein, which lines the interior of the spherule and is postulated to induce negative membrane curvature, recruits reticulons to invaginations [32]. Reticulons induce positive membrane curvature, which may promote two functions: (i) counter-balance the 1a negative membrane curvature to enable spherule expansion or (ii) promote positive membrane curvature at the neck of the spherule (Figure 1a). Reticulons may also be involved in DMV RC formation since multiple enteroviruses encode 2C proteins that interact with reticulon 3 within its RC to promote replication [33].

It has been noted that there are structural, and perhaps mechanistic commonalities between invaginations and retroviral budding [34]. Given these similarities, the role of the ESCRT machinery, which is required for retroviral budding, has been investigated in two plant viruses that form invaginations: tomato bushy shunt virus (TBSV) and BMV. TBSV p33 recruits cellular ESCRT-I proteins to induce formation of its RC [35,36], while ESCRT-III proteins are required for proper RC morphology [36,37]. This suggests a role for ESCRT-I in initiating invagination, with ESCRT-III required to produce the correct RC morphology, possibly closing the neck of the invagination via positive membrane curvature with incomplete membrane scission. Thus, there are similarities between TBSV RC formation and the steps of retroviral budding before membrane scission. BMV also co-opts ESCRT-III factors for proper BMV spherule formation [38•]. Unlike TBSV and HIV, BMV does not require components of ESCRT-I. Presumably, its 1a protein can initiate RC invagination without the need for ESCRT-I.

De novo lipid synthesis

A second strategy for modulating membrane curvature is to modify its lipid composition, either via lipid transfer proteins (LTPs) or *de novo* lipid synthesis. This can involve the insertion of lipids that (i) increase membrane fluidity (cholesterol, sphingomyelin, or unsaturated phospholipids), (ii) induce positive membrane curvature (ceramide), or (iii) induce negative membrane curvature (lysophosphatidylcholine). Modifying membrane lipid composition is likely crucial for RC formation, virion envelopment (and possibly assembly), and virion infectivity. A key node for viral manipulation of lipid synthesis is fatty acid synthase (FASN), which generates palmitate (C16:0). Palmitate is either post-translationally linked to proteins or further modified by lipid synthetic enzymes to produce the bulk of membrane lipids. Replication of multiple (+) RNA viruses requires FASN [39–44]. HCV increases the expression of FASN, although FASN does not appear to localize to RCs [43]. FASN is redistributed to RCs during DENV [39,45] and WNV infection [42]. DENV NS3 binds FASN, recruits it to RCs stimulates its activity and *de novo* lipid synthesis [39]. This is required for DENV replication and lipid alterations at the RC, including increases in sphingomyelin and ceramide upon DENV infection of mosquito cells [41].

Lipids downstream of FASN, including phosphatidylcholine (PC) and phosphatidylethanolamine (PE), play essential roles in the replication of multiple viruses. Picornaviruses stimulate the activity of the fatty acid modifying enzyme, long chain acyl CoA synthetase 3 (Acsl3), which results in increased PC accumulation at RCs [46]. Although PC and PE are increased during HCV infection [47], only PC accumulates in HCV RCs [48•]. In the case of BMV RC formation, the BMV 1a protein interacts with and recruits choline requiring 2 (Cho2p), a cellular enzyme involved in PC synthesis, to sites of viral replication, which is essential for BMV replication [48•]. Alternatively, TBSV RC formation relies on PE. TBSV p33 promotes cellular redistribution of PE and *in vitro*

reconstitution of TBSV RCs from liposomes revealed an exclusive role for PE in maintaining TBSV replication. [49•]. Modulation of lipid synthesis is thus a general strategy for formation of both classes of RCs.

Phospholipid kinases and lipid transfer

The picornaviruses and HCV, both of which form DMVs, convergently evolved strategies that coopt phosphatidylinositol (PI)-4 kinases to stimulate PI(4)P production at RCs. PI(4)P recruits LTPs to RCs leading to alterations in the lipid composition of the RC. HCV NS5A binds and activates the ER resident kinase, PI4K-IIIαat RCs, which is essential for HCV replication and appropriate RC morphology [50–54]. Two LTPs that bind PI(4)P are implicated in HCV replication: four-phosphate adaptor protein 2 (FAPP2) and oxysterol-binding protein (OSBP). FAPP2 is recruited to viral RCs where it might supply glycosphingolipids [55]. OSBP interacts with NS5A and its silencing leads to inhibition of both HCV replication and particle secretion [56–58]. OSBP is recruited to HCV replication compartments in a PI(4)P-dependent manner and mediates transport of cholesterol to RCs [56]. PI4K-IIIα has two other functions in HCV replication. It modulates NS5A phosphorylation and the downstream product of PI(4)P, PI(4,5)P_2, also accumulates at RCs and is bound by NS5A, which may be important for appropriate replicase topology [59,60].

Picornaviruses require distinct PI4 kinases, which differ based on their subcellular location. Enteroviruses and human rhinoviruses manipulate the Golgi-localized PI4K-IIIβ [61,62], while encephalomyocarditis virus (EMCV) requires PI4K-IIIα for RC formation [63•]. Similarly to HCV infection, a primary role for these kinases is to recruit OSBP to promote cholesterol accumulation [62,63•].

Picornaviruses modulate two other lipid transport pathways to promote RC formation. Long chain fatty acids that are imported in the infected cell are diverted from storage as triglycerides in lipid droplets, and instead are transported to the viral RCs where they are substrates for PC synthesis [46]. A second source of cholesterol for RCs, in addition to OSBP, is retrograde trafficking from the plasma membrane to RCs via early endosomes during enterovirus infection [64]. This may also occur in HCV infection, since HCV RCs are rich in cholesterol and early endosomes localize in proximity of RCs [9,27–29].

Transient replication before replication compartment formation?

A spatiotemporal analysis of HCV replication using single molecule RNA detection of viral (+) and (−) RNA uncovered a surprise. Low levels of HCV RNA replication occur soon after infection, which are then shutoff before the beginning of detectable RC formation [4•]. The interpretation was that a few 'backup copies' of HCV RNA are made to lessen the reliance on the integrity of the initially infecting genomic RNA. RC formation is then required for robust RNA replication. If this interpretation is correct, that leaves interesting unanswered questions as to the subcellular location of these RNAs and how they are protected from innate immune sensors and/or RNA degradation machinery.

Conclusions

Much progress has been made in defining the structural composition of (+) RNA virus RCs and mechanisms associated with their formation. In particular, our understanding of the formation of invaginations in model plant (+) RNA virus infection is becoming quite advanced. The development of a cell free system for TBSV invaginations will allow biochemical confirmation of much of these proposed mechanisms. However, numerous questions remain, particularly in the formation of DMVs. How is DMV structure achieved? Additionally, the role of PI4 kinases in DMV infection is not entirely clear. Enteroviruses can easily mutate to replicate in their absence, and they appear to have other roles in HCV infection beyond LTP recruitment. Finally, can the success of targeting proteins involved in RC formation as antiviral strategies in cell culture be extended to patient therapeutic strategies?

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