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Microreview

Virus-induced double-membrane vesicles

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Summary

Many viruses that replicate in the cytoplasm compartmentalize their genome replication and transcription in specific subcellular microenvironments or organelle-like structures, to increase replication efficiency and protect against host cell defences. Recent studies have investigated the complex membrane rearrangements induced by diverse positive-strand RNA viruses, which are of two morphotypes : membrane invagination towards the lumen of the endoplasmic reticulum (ER) or other specifically targeted organelles and double-membrane vesicles (DMVs) formed by extrusion of the ER membrane. DMVs resemble small autophagosomes and the viruses inducing these intriguing organelles are known to promote autophagy, suggesting a potential link between DMVs and the autophagic pathway. In this review, we summarize recent findings concerning the biogenesis, architecture and role of DMVs in the life cycle of viruses from different families and discuss their possible connection to autophagy or other related pathways.

Introduction

Viruses are obligate intracellular parasites, and as such, are dependent on the host cell machinery for the replication of their genome and the generation of progeny virus particles. Many viruses induce subcellular microenvironments or mini-organelles known as 'virus factories' or viroplasm (Fernandez de Castro *et al.*, 2013). These viral factories are thought to create a platform bringing together the replicase proteins, virus genomes and host proteins required for replication, while physically separating replication sites from the cytoplasmic sensors of the innate immune response. A common feature of plus-strand RNA viruses is the ability to usurp and modify host-cell cytoplasmic membranes to form functional site for protein translation, processing and RNA synthesis (den Boon and Ahlquist, 2010; den Boon *et al.*, 2010). Depending of the family and genus to which the virus belongs, these rearranged cellular membranes may be derived from various organelles, including the endoplasmic reticulum (ER), late endosomes/lysosomes or the mitochondrial outer membrane. Plus-strand RNA viruses induce the formation of two types of vesicles: (i) single-membrane vesicles, formed by negatively curved membranes and initiated by invaginations of the pre-existing membrane bilayer, giving rise to spherules or vacuoles toward the lumen of the targeted cell organelle, and (ii) double-membrane vesicles (DMVs), formed by positively curved membranes.

Viruses from the *Togaviridae*, *Bromoviridae* and *Nodaviridae* families induce small invaginations called spherules or larger spherule-lined cytopathic vacuoles, whereas those from the *Coronaviridae*, *Arteriviridae* and *Picornaviridae* families generate a more extensive network of different membrane structures during the temporal and spatial dynamics of viral replication. These membrane rearrangements include not only convoluted membranes and single-membrane vesicles, but also a massive network of DMVs. The various host-cell membrane rearrangements induced by the different families of positive-strand RNA viruses have recently been reviewed in detail (den Boon and Ahlquist, 2010; Chatel-Chaix and Bartenschlager, 2014; Romero-Brey and Bartenschlager, 2014). In this microreview, we focus on virus-induced DMVs and discuss for the possible mechanisms underlying their biogenesis and roles.

Presence and architecture of DMVs in cells infected with viruses from different families

In cells infected with poliovirus, single-membrane tubular vesicles detected at early time points after infection are gradually converted into DMVs, 100–300 nm in diameter, that are presumably filled with cytoplasmic material (Belov *et al.*, 2012). Poliovirus replicase proteins and nascent viral RNA localize at the outer membrane of these single- and double-membrane structures (Belov *et al.*, 2012). It

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has been suggested that these DMVs are formed by the extension of membranous walls and/or collapse of the luminal cavity of the single-membrane tubular structures, which probably originate from *cis*-Golgi membranes. A similar process has been observed for coxsackievirus B3 (CVB3), another member of the *Picornaviridae* family, with closed single-membrane tubules present from the start of infection gradually being converted into DMVs (approximately 160 nm in diameter), and then into complex multimembranous structures, through a wrapping process (Limpens *et al*., 2011). However, the precise location of viral proteins and RNA with respect to these rearranged membranes remains to be determined in this viral model.

By contrast to picornavirus-infected cells, cells infected with coronaviruses such as the severe acute respiratory syndrome coronavirus (SARS-CoV) (Knoops *et al*., 2008) or the mouse hepatitis virus (MHV) (Ulasli *et al*., 2010) display early membrane rearrangements in the form of ER-derived convoluted membranes interconnected with DMVs (about 250 nm in diameter). Similar observations have also been reported for the recently identified Middle East respiratory syndrome coronavirus, for which convoluted membranes are systematically surrounded by DMVs and are observed only in cells at more advanced stages of infection (de Wilde *et al*., 2013). This observation is consistent with the notion that DMV formation precedes the development of convoluted membranes, as previously suggested for SARS-CoV. These coronavirus-induced DMVs may subsequently merge into larger structures called vesicle packets (1–5 μm in size), probably through the fusion of their outer membranes (Knoops *et al*., 2008). Coronavirus replicase proteins are mostly found in convoluted membranes, and are rarely detected in DMVs. By contrast, double-stranded RNA (dsRNA), an intermediate of viral replication that serves as a marker for this process, is predominantly found in DMVs (Knoops *et al*., 2008). Despite the absence of specific morphological structures, such as convoluted membranes and vesicle packets, a similar architecture is observed in cells infected with the equine arterivirus (EAV), a member of the *Arteriviridae* family, with small DMVs (about 90 nm in diameter) interconnected to form a reticulovesicular network in the close vicinity of single-membrane tubular structures (Knoops *et al*., 2012). As for coronaviruses, EAV replicase proteins are associated with DMVs and the surrounding membranes, whereas dsRNA is mostly located in DMVs.

The *Flaviviridae* family includes the dengue and West Nile viruses, which principally induce the formation of single-membrane invaginated vesicles and smooth ER-derived convoluted membranes (Welsch *et al*., 2009; Gillespie *et al*., 2010). By contrast, another member of this family, the hepatitis C virus (HCV), induces the production of large number of DMVs (Ferraris *et al*., 2010; Romero-Brey *et al*., 2012). Thus, despite being unrelated

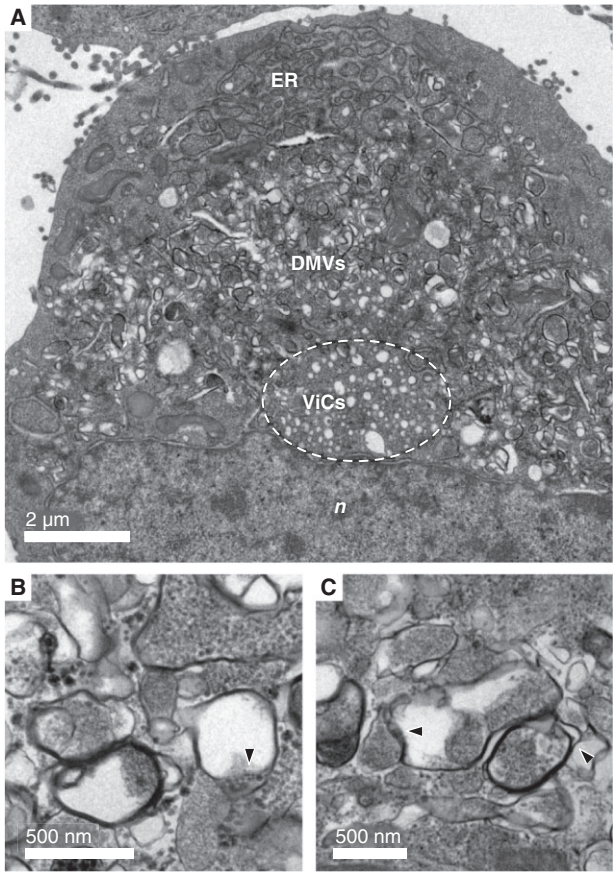

Fig. 1. Membrane rearrangements encountered in HCV-infected cells.
A. At low magnification, a typical HCV-infected cell shows a perinuclear cluster (dotted line) of single-membrane vesicles (ViCs, for vesicles in cluster) surrounded by an area containing numerous DMVs.
B and C. High magnification of DMVs. Careful examination of these structures shows that their thick membrane actually consist of two or more closely apposed membranes as a double membrane can be discerned in some areas (black arrows). n, nucleus.

to picornaviruses, coronaviruses and arteriviruses, HCV may display similarities to these other groups of viruses in terms of the cellular pathways exploited during the viral life cycle, resulting in similar membrane rearrangements. Electron tomography analysis of HCV-infected cells has shown that about 50% of DMVs are linked to the ER via the outer membrane, giving rise to a neck-like structure, but that they subsequently seem to detach from the donor membrane (Romero-Brey *et al*., 2012). Recent investigations have suggested that HCV-induced DMVs may derive from single-membrane vesicles present as discrete clusters in the perinuclear area during the early events of viral infection (Ferraris *et al*., 2013) (Fig. 1). The direct detection of HCV proteins and RNA is difficult in ultrastructural studies, and the precise distribution of the viral components with respect to these rearranged membranes remains unclear in this model.

Role of DMVs in the viral life cycle

For viruses inducing single-membrane invaginated vesicles or spherules, it is generally accepted that the viral replicase complexes reside on the invaginated membrane, and with RNA replication taking place in the vesicle lumen (den Boon and Ahlquist, 2010; den Boon *et al*., 2010). A neck-like connection to the cytoplasm allows the import of all the required metabolites, including nucleotides, and the export of newly synthesized RNA destined for translation or packaging into a nucleocapsid. However, the situation remains unclear for viruses inducing DMVs, as both membranes of these DMVs are often sealed, with no connection to the cytoplasm (Knoops *et al*., 2008; Limpens *et al*., 2011; Romero-Brey *et al*., 2012). For poliovius and CVB3, it has been suggested that viral replication occurs principally on the cytoplasmic side of the single-membrane tubular vesicles, subsequently ceasing after these vesicles are wrapped in membranes to form sealed DMVs (Limpens *et al*., 2011; Belov *et al*., 2012). For SARS-CoV and EAV, the viral RNA is found mostly in the lumen of DMVs, and it remains unclear whether viral replication occurs within the DMVs or on the cytosolic side of these vesicles (Knoops *et al*., 2008; 2012). Replication within the DMVs would require the import of metabolites and export of viral RNA, and the mechanisms by which these processes might occur remain unknown. Even with high-resolution electron tomography, no neck-like membrane structures or translocon-like complexes have been observed in these viral models (Knoops *et al*., 2008; 2012). This has led to suggestions that DMVs are formed to conceal viral RNA, enabling it to escape the dsRNA-triggered host antiviral response (Knoops *et al*., 2008). This hypothesis was supported by the results of a study based on the detection of RNA synthesis by live cell imaging, which showed that the subcellular compartments containing dsRNA (i.e. probable DMVs) were not necessarily the sites of active MHV replication (Hagemeijer *et al*., 2012). Furthermore, a panel of MHV mutants was recently used to demonstrate that viruses producing larger and more numerous DMVs in primary or continuous cell cultures have no fitness advantage (Al-Mulla *et al*., 2014). This suggests that intracellular viral RNA synthesis can be at least partly dissociated from DMV formation.

Biochemical analyses of the purified DMVs induced by HCV have shown these vesicles to contain an enzymatically active viral replicase capable of catalysing *de novo* HCV RNA synthesis (Paul *et al*., 2013). However, it remains unclear whether RNA replication takes place within DMVs or on their outer surface. Interestingly, electron tomography studies of HCV-infected cells have shown that a small subset of DMVs (about 10%) have a pore-like opening to the cytoplasm, whereas most have sealed inner and outer membranes (Romero-Brey *et al*., 2012). This has led to suggestions that HCV replication occurs within the lumen of DMVs while they are linked to the cytosol, with the replication complexes ceasing to be active once the membranes of the DMVs are sealed.

Role of autophagy in DMV biogenesis

DMVs are usually rare in cells, but large double-membrane structures called autophagosomes, which engulf cytoplasmic components targeted for degradation, are induced during autophagy (Rubinsztein *et al*., 2012). Autophagosomes have thus been identified as a possible source of DMVs associated with viral replication. Lipidation of the cytosolic microtubule associated light chain 3 (LC3) protein with phosphatidylethanolamine, generating a membrane-associated species known as LC3-II, is a key event in the induction of autophagy (Xie *et al*., 2008). LC3-II is associated with both the inner and outer membrane of the growing autophagosome, and this association is essential for autophagosome formation. The lipidation of LC3 has been observed after poliovirus and CVB3 infection (Taylor and Kirkegaard, 2007; Kemball *et al*., 2010). In addition, knocking-down expression level of a key player in autophagosome formation, Atg5, has also been shown to decrease viral replication, although this knock-down principally seems to affect the production of progeny viruses, suggesting a key role for autophagy in virus assembly and release (Jackson *et al*., 2005). In the coronavirus model, functional studies based on the knock-down of Atg5 expression have yielded conflicting results. In one study, cells lacking Atg5 displayed levels of viral replication three orders of magnitude lower than those in cells containing Atg5, together with smaller numbers of DMVs (Prentice *et al*., 2004). By contrast, Atg5 had little impact on viral replication in another study (Zhao *et al*., 2007). These discrepancies may be due to differences in these studies in terms of cell lines used. Conflicting observations have also been reported for HCV. It is generally agreed that HCV induces autophagic signalling, but the specific role of autophagy during HCV infection remains unclear. Autophagy has been shown to be essential for translation of the viral genome, but dispensable after the infection has begun in some studies (Dreux *et al*., 2009), whereas others have suggested that it is involved in initiating RNA replication (Sir *et al*., 2008; Guévin *et al*., 2010). In another report, knocking down the expression of autophagy genes was found to have no effect on virus translation and RNA replication, but be essential for HCV particle assembly and release (Tanida *et al*., 2009). It has also been suggested that HCV induces autophagy to down-regulate the innate immune response, thereby

favouring viral replication (Ke and Chen, 2011). Again, the discrepancies between the results of these studies may reflect the use of different subclones of the Huh7 hepatoma cell line.

The role of autophagy in the biogenesis of virus-induced DMVs thus remains unclear. Autophagy is clearly activated in viral infections associated with DMVs, but this does not mean that all virus-induced DMVs are autophagosomes. Other differences between virus-induced DMVs and cellular autophagosomes include the smaller size of these virus-induced organelles and their persistence as double-membrane structures. Cellular autophagosomes mature by fusing with lysosomes. This enables them to acquire degradative machinery, but also causes them to lose their double-membrane structure within as little as 30 min after their formation (Haas, 2007). It is difficult to determine whether the assembly of viral replicase complexes results directly in the formation of DMVs or whether DMVs are actually modified autophagosomes. Nevertheless, Western-blotting on isolated DMVs and immunogold labelling electron microscopy studies have suggested that at least some HCV-induced DMVs may be derived from autophagosomes, as they are strongly positive for LC3-II (Ferraris *et al*., 2010). It has recently been shown that the non-structural nsp6 proteins of various coronaviruses can generate small ER-derived autophagosomes called omegasomes, which are normally formed during the initial stages of autophagy (Cottam *et al*., 2011). These proteins also inhibit the autophagosome expansion observed in regular autophagy processes (Cottam *et al*., 2014). Other studies have suggested that DMVs are instead related to vesicles called EDEMosomes. EDEMosomes are devoted to the elimination of EDEM proteins to tune down the ER-associated degradation (ERAD) pathway (Reggiori *et al*., 2010). In this model, DMVs recruit non-lipidated LC3-I, by a pathway not strictly related to autophagy, but nevertheless requiring components of the autophagy pathway.

It should also be pointed out that the induction of autophagy or related pathways to enhance viral replication is not restricted to DMV-inducing viruses. In addition to its direct contribution to the formation of rearranged membranes by providing support for replication complexes, autophagy can promote viral replication in several other ways. Early studies showed that some viruses induce the autophagic pathway while inhibiting the associated degradation activity, but more recent studies have indicated that, in some cases, viruses can promote the generation of degradative autolysosomes, the final compartments in the process of autophagy (Richards and Jackson, 2013). For example, dengue virus infection leads to autophagy-mediated lipid droplet degradation, a process referred to as lipophagy (Heaton and Randall, 2010). This results in the mobilization of triglycerides, which are used for adenosine triphosphate production via β-oxidation, a process required for efficient viral replication. A role of autophagy in promoting viral assembly and release has also been demonstrated in various viral models including hepatitis B virus (Li *et al*., 2011), human immunodeficiency virus (Kyei *et al*., 2009), but also DMV-inducing viruses, such as poliovirus (Richards and Jackson, 2012) and HCV (Tanida *et al*., 2009).

Conclusion

Ever since the first pioneering ultrastructural observations of virus-induced DMVs, the biogenesis and role of these structures in viral infection have been the subject of debate. Components of the cellular autophagy pathway or related pathways seem to be involved in the life cycle of DMV-inducing viruses, but the link between autophagy and DMV biogenesis remains unclear. In various viral models, the production of viral proteins, either alone or in combination, has led to the identification of proteins sufficient to induce the formation of DMVs (Neuman *et al*., 2014; Romero-Brey and Bartenschlager, 2014). It has also recently been shown, in a cell-free assay, that a single poliovirus protein (3AB) is sufficient to induce the formation of a double-membrane liposome via membrane curvature, leading to the invagination of a single-membrane liposome (Wang *et al*., 2013). It is therefore plausible that, in infected cells, one or several viral proteins replace the host-cell proteins involved in autophagosome biogenesis, or work together with these host-cell proteins, to form the virus-induced DMVs. This may account for the inhibition of autophagy resulting to various effects on viral replication, depending on the cell type used. Once formed, DMVs could act as a scaffold for the assembly of viral replication complexes by providing an organization and environment facilitating viral replication. These DMVs may later be completely sealed, such that the viral RNA they contain is no longer actively engaged in the RNA amplification process. This would enable the virus to escape the dsRNA-triggered antiviral response of the host cell. In parallel, autophagy may enhance the viral life cycle through mechanisms other than the formation of rearranged membranes, including viral assembly and release. The diverse conclusions of recent studies provide an equivocal picture of the sequence of events involved and further studies will be required to determine the roles of viral/cellular factors and the molecular pathways involved in the life cycle of DMV-inducing viruses. This will not only improve our understanding of viral replication, but will probably also shed light on the ways in which autophagy and other related pathways are regulated.

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