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Untangling Membrane Rearrangement in the *Nidovirales*

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All known positive sense single-stranded RNA viruses induce host cell membrane rearrangement for purposes of aiding viral genome replication and transcription. Members of the *Nidovirales* order are no exception, inducing intricate regions of double membrane vesicles and convoluted membranes crucial for the production of viral progeny. Although these structures have been well studied for some members of this order, much remains unclear regarding the biogenesis of these rearranged membranes. Here, we discuss what is known about these structures and their formation, compare some of the driving viral proteins behind this process across the nidovirus order, and examine possible routes of mechanism by which membrane rearrangement may occur.

**Membrane Rearrangement as a General Strategy of Positive Sense RNA Viruses**

Host cell membrane remodeling is a tactic used by many viruses as a means of reaching their end game of using the host cell for viral production. Positive sense single-stranded RNA viruses make use of the host’s internal membrane environment for viral genome replication and transcription (Ahlquist, 2006; Denison, 2008; Miller and Krijnse-Locker, 2008; Netherton and Wileman, 2011). The reasons for doing so include the need to concentrate, localize, and anchor the machinery and precursors required for transcription and aiding in shielding double-stranded RNA intermediates from activating an innate immune response. Membrane rearrangements involved in viral genome replication and transcription for some members of the positive sense RNA viruses have been well characterized and have a wide range of complexity, both of membrane involvement and numbers and types of proteins responsible for the remodeling (Kirkegaard and Jackson, 2005; Mackenzie, 2005; Novoa et al., 2005; Salonen et al., 2005; den Boon and Ahlquist, 2010). The typical method of designating which membranes are involved is by mapping the intracellular localization of the dsRNA replicative intermediates, nascent RNA, and replicase proteins. Once the membranous replicase structures have been identified, further characterization and ultrastructural description can occur, including the more recently applied technique of three-dimensional electron tomography (Subramaniam, 2005; Kopek et al., 2007; Subramaniam et al., 2007).

Although the strategy of membrane remodeling is thought to be conserved in all positive sense RNA viruses, the actual modifications and structures made and the means by which they are formed can drastically vary between viruses. Flaviviruses induce an organized network of interconnected double-walled endoplasmic reticulum (ER)-derived membranes termed “vesicle packets” and “convoluted membranes” (Welsch et al., 2009; Gillespie et al., 2010; Miorin et al., 2013). Picornaviruses have been shown to reorganize ER, Golgi, and lysosomes into both single and double membrane vesicles (DMVs), the latter of which are similar to autophagosomes (Suhy et al., 2000; Limpens et al., 2011; Belov et al., 2012). Alphaviruses induce ~50 nm in diameter single membrane vesicles termed “spherules,” seen to be derived from invaginated ER, plasma membrane, and endosomes/lysosomes depending on the virus (Froshauer et al., 1988; Schwartz et al., 2002; Kopek et al., 2007; Spuul et al., 2010). Nodaviruses reorganize the mitochondrial membrane into small ~50 nm vesicles. These vesicles are single membranes and are positioned between the inner and outer mitochondrial membrane (Miller et al., 2001; Kopek et al., 2010).

**An Introduction to Nidoviruses**

The order *Nidovirales* contains families of positive sense nonsegmented single-stranded RNA viruses featuring an envelope and, notably, a mechanism of discontinuous transcription to produce nested subgenomic mRNAs for which the order is named (Latin nidus means nest) (González et al., 2003; Gorbalenya et al., 2006; Pasternak et al., 2006). This order contains families capable of infecting both vertebrates (*Coronaviridae, Arteriviridae, and Roniviridae*) and invertebrates (*Mesoniviridae*) (Cowley et al., 2000; Lauber et al., 2013). All nidoviruses share a genome with a similar layout with the first two overlapping open reading frames (ORFs1a and 1b) producing two large polyproteins (pp1a and pp1ab) that are co- and post-translationally cleaved free
into the nonstructural proteins (nsps) (Brian and Baric, 2005; Britton and Cavanagh, 2008). Processing of these polyproteins is directed by nsp encoded proteinases, which vary among families and species of these viruses (Ziebuhr, 2006; Snijder et al., 2013).

These nsps are part of the viral replication transcription complex machinery necessary for viral genome replication and transcription, in association with cellular membranes (van Hemert et al., 2008; Hagemeijer et al., 2010). ORFs downstream from ORF1a and 1b encode varying structural and accessory proteins. These structural and accessory proteins are produced from nested 3′ co-terminal subgenomic mRNAs via a discontinuous transcription process (Faaberg, 2008; Hogue and Machamer, 2008). While nidovirus genomes range significantly in sequence and size, from 12.7 kb for the smaller arteriviruses to 31.7 kb for the large coronaviruses, they share some commonalities (Gorbalenya et al., 2001) and amended to reflect known topologies (Baker nsp3, Oostra nsp4, Oostra nsp3+ nsp6) wherever possible. Virus names are abbreviated as follows: Human coronavirus 229E (HCoV-229E), severe acute respiratory syndrome coronavirus (SARS-CoV), infectious bronchitis virus (IBV), porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), cavally virus (CAVV), and gill-associated virus (GAV). The amino-terminal region of the polyprotein is shown for CAVV and GAV because no obvious homolog of nonstructural protein (nsp3) was detected. A jagged line denotes the uncertain position of the amino termini of EToV, WBV, FHMV, and GAV.

Membrane Rearrangement in the Nidoviruses

Ateriviruses and coronaviruses both induce characteristic DMVs in host cells (David-Ferreira and Manaker, 1965; van der Meer et al., 1998; Pedersen et al., 1999; Gosert et al., 2002; Snijder et al., 2006; Knoops et al., 2008, 2012; Ulasli et al., 2010; de Wilde et al., 2013). The DMVs, so named for their distinctive two-layer delineated membranes, are interconnected with regions of convoluted membranes (CMs). Despite the fact that DMVs and CMs often appear in close and/or continuous proximity to the ER and their evidenced partial colocalization with the ER maker protein disulfide isomerase, the precise progenitor pathway from which they are formed remains unclear (Goldsmith et al., 2004; Snijder et al., 2006; Knoops et al., 2008).

For both the coronaviruses and arteriviruses, members of the cellular autophagy machinery have been implicated in DMV formation (Maier and Britton, 2012). The autophagosomes themselves are double-membrane vesicles, which initially suggested the possibility of this pathway’s involvement. Multiple studies have shown activation of autophagy machinery upon coronavirus infection or in the presence of coronavirus proteins (Prentice et al., 2004; de Haan and Reggiori, 2008; Cottam et al., 2011). Additionally, microtubule-associated protein light chain 3 (LC3) has been shown to associate with the DMVs of both arteriviruses and coronaviruses and loss of LC3 had an overall negative effect on DMV formation (Prentice et al., 2004; Reggiori et al., 2010; Monastyrksa et al., 2013). During autophagy, cytoplasmic LC3-I becomes lipidated and studs the autophagosome, serving as a marker for these vesicles. This is in contrast to the LC3 that has been shown to decorate the DMVs, which is the nonlipidated LC3-I form, which has also been implicated in the ER-associated degradation (ERAD) pathway. Additionally, chaperone members of the ERAD machinery were shown to be present in the DMVs, suggesting a role for ERAD in DMV formation (Cali et al., 2008; Reggiori et al., 2010). Absence of Atg5, a critical member of the autophagy machinery, did not cause a defect in DMV formation in coronavirus-infected cells, showing that DMV formation does not require an intact autophagy pathway (Zhao et al., 2007).

Nonstructural Proteins Involved in Membrane Rearrangement: Similarities and Differences

The identity of the specific viral proteins responsible for inducing membrane rearrangement in both arterivirus and coronavirus-infected cells has long been of interest. Both

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**FIG. 1.** Conservation of double membrane vesicle (DMV)-making proteins in the Nidovirales. Domain annotations were based on conserved amino acid sequences (solid colors) or secondary structure patterns (diagonal stripes). Positions of transmembrane and hydrophobic nontransmembrane regions were predicted by TMHMM 2.0 (Sonnhammer et al., 1998; Krogh et al., 2001) and amended to reflect known topologies (Baker nsp3, Oostra nsp4, Oostra nsp3+ nsp6) wherever possible. Virus names are abbreviated as follows: Human coronavirus 229E (HCoV-229E), severe acute respiratory syndrome coronavirus (SARS-CoV), infectious bronchitis virus (IBV), munia coronavirus HKU13 (MuCoV), equine torovirus (EToV), white bream virus (WBV), fathead minnow virus (FHMV), equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV), porcine reproductive and respiratory syndrome virus (PRRSV), simian hemorrhagic fever virus (SHFV), cavally virus (CAVV), and gill-associated virus (GAV). The amino-terminal region of the polyprotein is shown for CAVV and GAV because no obvious homolog of nonstructural protein (nsp3) was detected. A jagged line denotes the uncertain position of the amino termini of EToV, WBV, FHMV, and GAV.
families have three nsps that feature putative transmembrane (TM) domains (Oostra et al., 2007, 2008; Snijder et al., 2013). Arteriviruses have TM domains within nsp2, nsp3, and nsp5, which appear to be homologous to coronavirus TM proteins nsp3, nsp4, and nsp6. These three proteins share conserved features between families and across the Nidovirales, as shown in Figure 1. Due to their presence at the DMVs and CMs and their TM domains, these three nsps were proposed as being important for membrane rearrangement. It was shown that the arterivirus equivalents of coronavirus nsp3 and nsp4 were sufficient for inducing membrane modifications similar to those seen in arterivirus-infected cells (Snijder et al., 2001). Recently, we showed that coronavirus nsp3, nsp4, and nsp6 are all required to induce DMVs resembling those of virus-infected cells (Angelini et al., 2013). Coronavirus nsp3 and nsp4 alone were incapable of forming full DMVs, but they were capable of creating maze-like patterns of paired membrane, agreeing with previously published immunofluorescence studies using expressed nsp4 and a truncated version of nsp3 (Hagemeijer et al., 2011; Angelini et al., 2013). Mutation in coronavirus nsp3 has been shown to lead to virus having a defect in DMV-formation ability, suggesting an important role for nsp5 and polyprotein processing in DMV formation (Stokes et al., 2010). Coronavirus nsp4 and its arterivirus homolog have both been shown to be important for DMV formation through mutagenesis approaches (Posthuma et al., 2008; Gadlage et al., 2010). MHV nsp4 has been shown to be important for DMV formation. As mentioned earlier, nsp6 of coronavirus and nsp5–7 of arterivirus have been shown to induce autophagy when expressed in the absence of virus (Cottam et al., 2011).

Theoretical Mechanism

As the identity of the viral proteins important for membrane rearrangement has become clear and the cellular membranes and pathways involved begin to be distinguished, the mechanism of membrane deformation can be deciphered. DMVs and CMs are comprised of highly curved membranes and presumably this curvature is induced via the TM domains located in coronavirus nsp3, nsp4, and nsp6 (nsp2, nsp3, and nsp5 for arteriviruses) and protein–protein interactions of these nsps with each other, with other viral proteins, and with host cellular proteins. TM domains that feature conical shapes, as a product of this shape, will induce membrane curvature by forcing the membrane to accommodate and deform around the conical region in a wedge-like manner (McMahon and Gallop, 2005; Shibata et al., 2009). Additionally, suites of proteins may form together as a scaffold that has the ability to deform lipid bilayers and steric effects of these membrane-interacting proteins may also aid in deformation (Schley et al., 2013).

Since nsp3, nsp4, and nsp6 (and their Nidovirales homologs) contain TM domains and work together in a scaffold-like fashion, it is possible that both of these approaches work together to induce DMVs and CMs. As we propose in Figure 2, following initial genome polyprotein translation and proteolytic processing, nsp3 (dark blue), nsp4 (teal), and nsp6 (green) remain inserted in the lipid bilayer (Figure 2-upper left inset). The intracellular phenotypes of cells expressing both nsp3 and nsp6 suggest that these proteins may promote membrane curvature, inducing proliferated membranes and vesiculation respectively on their own. Nsp4 alone appears incapable of inducing membrane curvature but, in conjunction with nsp3, is able to produce paired membranes, suggesting that some combination of homotypic and/or heterotypic interactions is driving this pairing (Figure 2-lower right inset). Nsp3–nsp3, nsp4–nsp4, and nsp3–nsp4 interactions have all been previously identified by mass spectrometry-based approaches, yeast two-hybrid assays, and co-immunoprecipitation studies (von Brunn et al., 2007;
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Imbert et al., 2008; Neuman et al., 2008; Hagemeijer et al., 2011. Since this membrane pairing was not observed when using a C-terminal region of nsp3, it is likely that the scaffolding function relies on interaction of nsp4 with some region of nsp3 N-terminal to its TM domain. Addition of nsp6 changes the organization of paired membranes from remarkably consistent maze-like swirls to a mixture of heterogeneous DMVs and CMs that resembles the viral replicative organelles found in infected cells. This suggests heterogeneous DMVs and CMs that resembles the viral nsp6 changes the organization of paired membranes from scaffolding function relies on interaction of nsp4 with some using a C-terminal region of nsp3, it is likely that the 2011). Since this membrane pairing was not observed when certain species of lipids may aid in certain types of membrane modifications and replication complex function. J Virol 86, 302–312.


