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Review Series: Spotlight on Viruses

Potential subversion of autophagosomal pathway by picornaviruses

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The RNA replication complexes of small positive-strand RNA viruses such as poliovirus are known to form on the surfaces of membranous vesicles in the cytoplasm of infected mammalian cells. These membranes resemble cellular autophagosomes in their double-membraned morphology, cytoplasmic lumen, lipid-rich composition and the presence of cellular proteins LAMP 1 and LC3. Furthermore, LC3 protein is covalently modified during poliovirus infection in a manner indistinguishable from that observed during bona fide autophagy. This covalent modification can also be induced by the expression of viral protein 2BC in isolation.

However, differences between poliovirus-induced vesicles and autophagosomes also exist: the viral-induced membranes are smaller, at 200–400 nm in diameter, and can be induced by the combination of two viral proteins, termed 2BC and 3A. Experimental suppression of expression of proteins in the autophagy pathway was found to reduce viral yield, arguing that this pathway facilitates viral infection, rather than clearing it. We have hypothesized that, in addition to providing membranous surfaces for assembly of viral RNA replication complexes, double-membraned vesicles provide a topological mechanism to deliver cytoplasmic contents, including mature virus, to the extracellular milieu without lysing the cell.

Introduction

The process of autophagy, which leads to the destruction of cytosolic constituents, is well-suited to the destruction of foreign invaders. Indeed, autophagy has been shown to play an important role in the innate immune response, helping to clear intracellular bacteria such as *Mycobacterium tuberculosis*[1] and viruses such as herpesvirus and tobacco mosaic virus.[2,3] This realization may have great practical importance in the short term as well as in future antimicrobial strategies. For example, during organ transplant to pediatric patients, major complications can result from the exposure of the young patients to viruses with which the donor was chronically infected, such as Epstein-Barr (EBV) or other herpes viruses. Some evidence has suggested that the lympho-proliferative disorder associated with EBV infection may be less severe if the post-transplant immunosuppressant used is rapamycin, which can also suppress the growth of EBV-transformed cells in tissue culture.[4] Although the mechanism by which rapamycin suppresses viral infection is not yet known, it is tempting to speculate that this might be through the induction of autophagy, and heartening to think that the suppression could extend to other pathogens that chronically infect donor tissue as well.

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Similarities and Differences Between Poliovirus-Induced Vesicles and Autophagosomes

The innate immune response has an extensive arsenal to combat viruses. Successful viruses must evade or subvert this barrage at least well enough to establish transient infections. Spectacular examples of subversion exist, such as mink focus-forming virus, which requires one of the caspases induced by the apoptotic response of infected cells to process a viral capsid protein.[5] As discussed below, our laboratory has argued that picornaviruses subvert the process of autophagosome formation for the generation of elaborate membranous vesicles crucial for viral RNA replication. Here, we discuss the evidence for this hypothesis and some possible interpretations.

Picornaviruses, like all positive-strand RNA viruses, replicate their genomes on the surfaces of intracellular membranes. Cells infected with poliovirus, a well-studied picornavirus, exhibit massive rearrangements of the intracellular membranes into clusters of vesicles 200–400 nm in diameter.[6] Electron microscopy has revealed that these vesicles contain two membrane layers, reminiscent of autophagosomes.[7,8] Furthermore, poliovirus-induced double-membraned vesicles display, in addition to the viral RNA replication machinery, biochemical markers from several intracellular compartments, including the autophagosomal protein LC3.[9,10]

To test whether the induction of double-membraned vesicles is an antiviral response or a process induced by the virus with benefit for the virus, the effects of activation and inhibition of the autophagosomal pathway on viral yield were tested. Stimulation of autophagy by tamoxifen and rapamycin also simulated intracellular yield of poliovirus, and inhibition of autophagy with either 3-methyladenine treatment or the RNAi-mediated decrease in expression of LC3 or Atg12 reduced intracellular virus yield. These data are certainly not compatible with an antiviral role for autophagy or autophagy-related proteins. Instead, these data support a model in which the process of autophagosome formation itself, or the increased availability of autophagy-related proteins such as LC3, facilitate viral replication. A recent publication documents a lack of effect on the yield of rhinovirus 2, a related picornavirus, following treatment of cells with 3-methyladenine, tamoxifen or rapamycin.[11] Therefore, either this

rhinovirus is less dependenton the autophagy machinery, or its slower growth kinetics do not require new production of this machinery.

Molecular Inducers of Double-Membraned Vesicles and LC3 Modification

A particularly interesting detail of the mechanism by which autophagy protein LC3 is recruited to, or aids in the formation of, poliovirus-induced vesicles, is the identity of the viral protein or proteins responsible. Poliovirus encodes three proteins, 2B, 2C and 3A which, when expressed either in isolation or as their stable precursors 2BC or 3AB, localize to intracellular membranes. Co-expression of the unprocessed precursor 2BC and the 87-amino acid protein 3A was found to be sufficient to induce double-membraned vesicles with associated LC3.[9,10]

During poliovirus infection, the lipidation of LC3 correlates with its membrane localization.[12] We were surprised to find that, although both viral proteins 2BC and 3A were shown to be required for co-localization of LC3 and LAMP1 and the formation of double-membraned vesicles, only protein 2BC was required to induce LC3 lipidation.[12] To our knowledge, this is the first example of the uncoupling of LC3 modification from double-membraned vesicle formation. Direct interaction between LC3 and 2BC was not observed in co-immunoprecipitation experiments, although the two proteins are recovered together in the same immunoprecipitate in the absence of detergent, arguing that they were present in the same membranous structure.[12]

Interestingly, when expressed singly and in other combinations, the membrane-associated proteins encoded by poliovirus have been shown to induce other kinds of membrane morphologies. Protein 3A, for example, induces endoplasmic reticulum (ER) swelling and fragmentation, and slows the rate of anterograde transport between the ER and the Golgi.[11] Expression of either the precursor protein 2BC or the 2C portion alone leads to the formation of large, single-membraned vesicles with no visible lumenal contents.[9,14] When 2C and 3A are expressed together, enlarged ER membranes with cytoplasmic invaginations can be observed.[9] These dilated, invaginated ER membranes are similar to structures observed during infections of $Atg5^{-/-}$ ES cells with murine hepatitis virus, another positive-strand RNA virus.[15] It is possible these alternate morphologies are either intermediates or off-pathway products in double-membraned vesicle formation by 2BC and 3A together, and during poliovirus infection.

The known molecular inducers of autophagosomes and double-membraned vesicles and their presumed sites of action are listed in Table 1. Only the proteins encoded by positive-strand RNA viruses, specifically poliovirus and equine arterivirus, are known to act directly at intracellular membranes. Given what is known of autophagic signaling and control, we suspect that these virally encoded proteins recruit LC3, and perhaps other constituents of the autophagosomal pathway, somewhere downstream of the initial signaling events. It is possible, for example, that the polioviral 2BC and 3A proteins intersect the canonical autophagy pathway somewhere between TOR signaling and the protein conjugation pathways that link ATG5 to ATG12 and LC3 to phosphatidylethanolamine. A working model for the formation of double-membraned vesicles by poliovirus proteins 2BC and 3A, and during poliovirus infection, is shown in Figure 2.

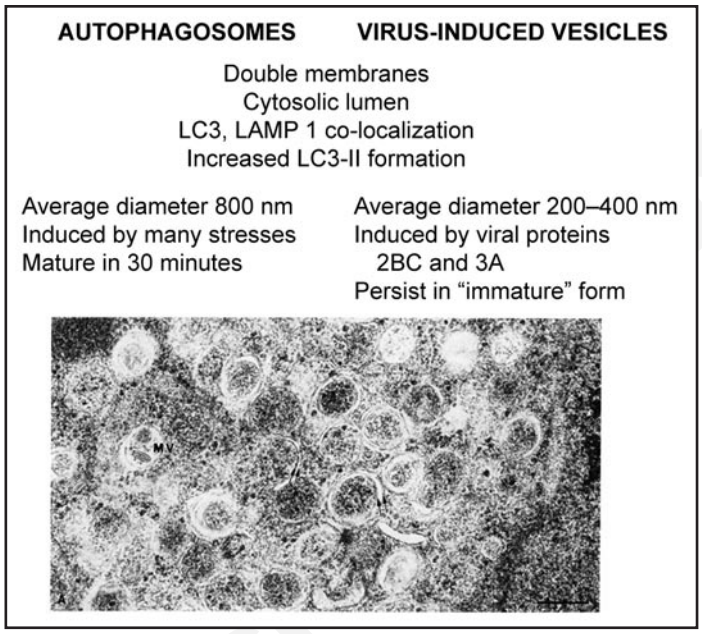

Figure 1. Similarities and differences between poliovirus-induced vesicles and cellular autophagosomes. Electron micrograph was taken by Thomas H. Giddings, Ph.D. (University of Colorado) and is taken from reference[8] with permission.

Why Double Membranes?

The relationship between the life cycle of poliovirus and the normal cellular autophagy pathway is not yet known. As of yet, there has been no evidence of autophagosome-associated protein

Table 1 **Inducers of autophagosomes and double-membraned vesicles**

Molecular Inducer	Site of Induction	Method of Induction	Ref.
G-protein overexpression	Plasma membrane	Effector of autophagy in HT-29 cell background	16
Tamoxifen	Plasma membrane	Signaling inhibits PI-3 signaling and modulates C2 ceramide levels to induce autophagy	18,19
C_2 ceramide	Intracellular	Affects membrane homeostasis and lipid pools to stimulate autophagy	19
Sphingosine Kinase	Intracellular	Affects pools of lipid to stimulate autophagy	20
Rapamycin	Intracellular	Inhibits TOR kinase function to stimulate autophagy	21
Poliovirus 2BC and 3A proteins	Endoplasmic Reticulum	Induces double membrane vesicles and LC3 relocalization	9,10
Equine Arterivirus ns2/ns3 proteins	Endoplasmic Reticulum	Induces double membrane vesicles	22

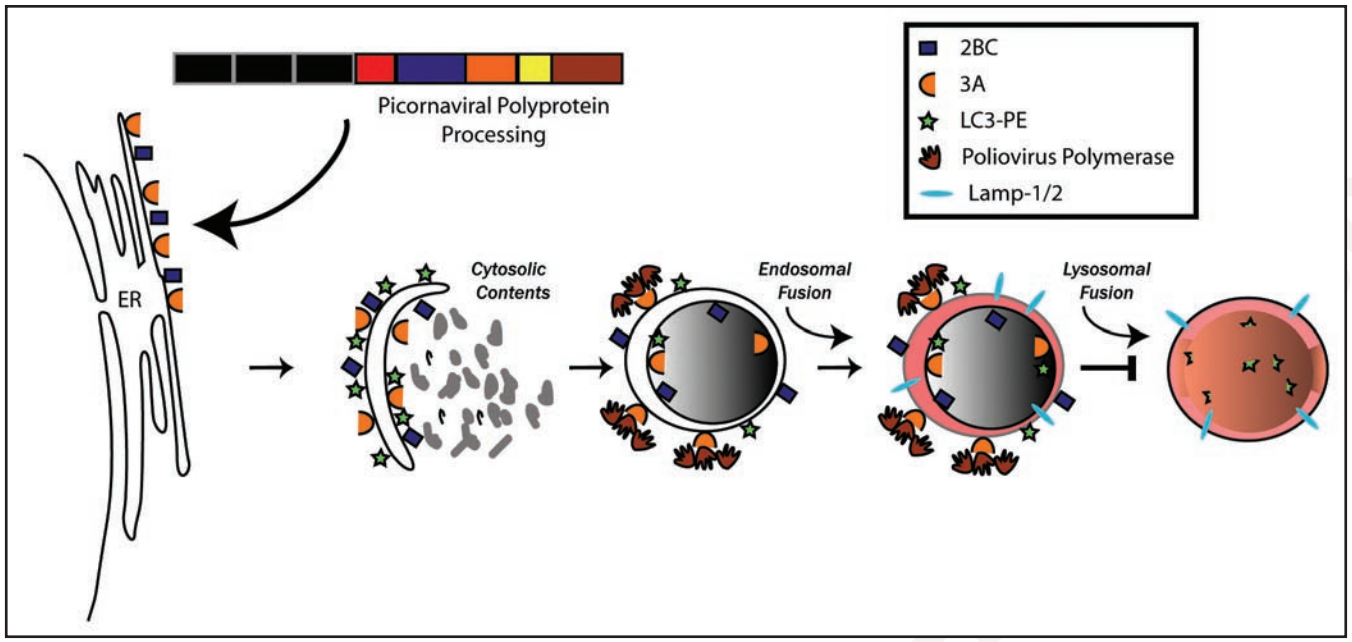

Figure 2. Picornaviral induction of autophagosome-like membranes. Picornaviral genomes are translated into a single polyprotein that undergoes proteolytic digestion to release individual viral proteins. Two viral proteins involved in the polioviral double-membrane induction are 2BC and 3A, which localize to the endoplasmic reticulum (ER) when expressed in isolation. Here, it is postulated that, in the ER, these proteins recruit modified LC3 to form a complete autophagosome-like vesicle. At this stage the RNA-dependent RNA polymerase is recruited to the outer membrane through its interaction with 3AB, a precursor of 3A. The membranous vesicle acquires LAMP1 through a hypothesized fusion with endosomes, but a subsequent block in lysosomal fusion in possible, which would prevent degradation of internal contents.

degradation in poliovirus-infected cells. It is possible that poliovirus subverts the pathway of autophagosome formation, yet blocks the subsequent maturation and degradation of the autophagosomal-like membranes (Fig. 2). Further experimentation is necessary to determine the similarities and differences between bona fide autophagosomes and the structures formed during viral infection.

We have noted that the double-membraned topology of autophagosomes, coupled with the ability to degrade the inner membrane partially or completely, has the topological effect of converting cytoplasm into lumen.[10,16] Does the autophagy pathway, like the mulitvesicular body pathway and exosome formation, provide a mechanism of secretion? We have shown that reduction of LC3 or ATG12 abundance reduced the yield of extracellular poliovirus to a greater extent than intracellular virus,[10] consistent with the idea that autophagosomes, or any kind of double-membraned vesicle, can provide a cellular exit route for any cytosolic protein or complex that can withstand the conditions within such a vesicle. Beyond viral pathogenesis, these findings may lead to new biochemical insights into the membrane remodeling and recruitment events that characterize cellular autophagosome formation and autophagy, and their effects on both the intracellular metabolism and the communication of eukaryotic cells.

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