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MINIREVIEW

How Positive-Strand RNA Viruses Benefit from Autophagosome Maturation

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The autophagic degradation pathway is a powerful tool in the host cell arsenal against cytosolic pathogens. Contents trapped inside cytosolic vesicles, termed autophagosomes, are delivered to the lysosome for degradation. In spite of the degradative nature of the pathway, some pathogens are able to subvert autophagy for their benefit. In many cases, these pathogens have developed strategies to induce the autophagic signaling pathway while inhibiting the associated degradation activity. One surprising finding from recent literature is that some viruses do not impede degradation but instead promote the generation of degradative autolysosomes, which are the endpoint compartments of autophagy. Dengue virus, poliovirus, and hepatitis C virus, all positive-strand RNA viruses, utilize the maturation of autophagosomes into acidic and ultimately degradative compartments to promote their replication. While the benefits that each virus reaps from autophagosome maturation are unique, the parallels between the viruses indicate a complex relationship between cytosolic viruses and host cell degradation vesicles.

INTRODUCTION TO AUTOPHAGY

While many viruses avoid or suppress host immune responses, several subvert the host immune machinery to promote their own replication (1). The autophagic pathway is one well-characterized effector mechanism of the innate immune response, resulting in a highly regulated lysosomal degradation mechanism by which a cell degrades its own contents. The pathway has been shown to be essential for cellular clearance of several intracellular pathogens, including *Mycobacterium tuberculosis*, *Toxoplasma gondii*, and herpes simplex virus 1 (HSV-1) (2–4). Despite the role of autophagic signaling in innate immunity, several pathogens are capable of subverting autophagy for their own benefit (5, 6). In particular, the replication cycle of positive-strand RNA viruses, which are the causative agents of many diseases, including myocarditis, encephalitis, and hand, foot, and mouth disease (7–9), can be promoted by some aspect of the autophagic pathway. However, there is a difference between a pathogen benefitting from autophagic signaling or the machinery from the autophagic pathway and a pathogen benefitting from the endpoint activity of autophagy, which is the degradation of cytosolic contents. Both are of interest, but this review will focus exclusively on relatively new findings that several pathogens can actually benefit from the degradative activity of autophagy.

Autophagy is a constitutive degradation pathway with important roles in development, differentiation, and stress responses (10). By facilitating the removal of damaged organelles and cytoplasmic protein aggregates, autophagy has proven to be essential for maintaining cellular homeostasis (6). Several signaling pathways induce autophagic signaling, although the mechanism by which these pathways cooperate to promote vesicle formation remains unknown (11). Inhibition of the Akt/mTOR pathway has long been considered essential for induction of autophagic signaling, although recent reports have demonstrated the existence of mTOR-independent autophagy (12, 13). Induction of a ubiquitin-like conjugation system promotes lipidation of the cytosolic microtubule-associated light chain 3 (LC3) protein with phosphotidylethanolamine, generating a membrane-associated species known as LC3-II (14–16). LC3-II is associated with both the inner and the outer membrane of the growing autophagosome, and this association is essential for autophagosome formation (14, 17, 18). LC3-II remains the only protein known to stably associate with completed autophagosomes, and as such it is an invaluable marker for monitoring autophagy. The initial events in autophagosome biogenesis have been well-described elsewhere (18–20).

Autophagosomes are unique vesicles composed of two lipid bilayers which, during their formation, engulf cytosolic contents, including long-lived proteins, intracellular pathogens, and damaged organelles. This cargo is then transported to the lysosome for degradation (10). Double-membraned autophagosomes undergo a stepwise maturation process culminating in their fusion with lysosomes to form degradative autolysosomes (Fig. 1). Autophagosomes mature into amphisomes, a change primarily characterized by the acidification of the lumen of the vesicle and the acquisition of proteins associated with late endosomes and lysosomes (21). Mature amphisomes then fuse with lysosomes to form autolysosomes, in the process losing one lipid bilayer through an unknown mechanism (22, 23). The autolysosome is the compartment in which actual autophagy, the degradation of cytosolic contents, takes place.

The acidification of the amphisome is believed to be the result of fusion with late endosomes bearing vacuolar ATPases (21, 24). Treatment of cells with inhibitors of vesicular acidification, including bafilomycin A1, chloroquine, and ammonium chloride, prevents autolysosome formation (24–26). This indicates that acidification of either the autophagosome, the lysosome, or both is a prerequisite for fusion of the autophagosome with the lysosome. Two proteins, lysosomal-associated membrane protein 2 (LAMP-2) and Rab7, have been reported to be required for autophagosome maturation. Rab7 is a small GTP-binding protein that has functions in late endosomal transport (27, 28). Depletion

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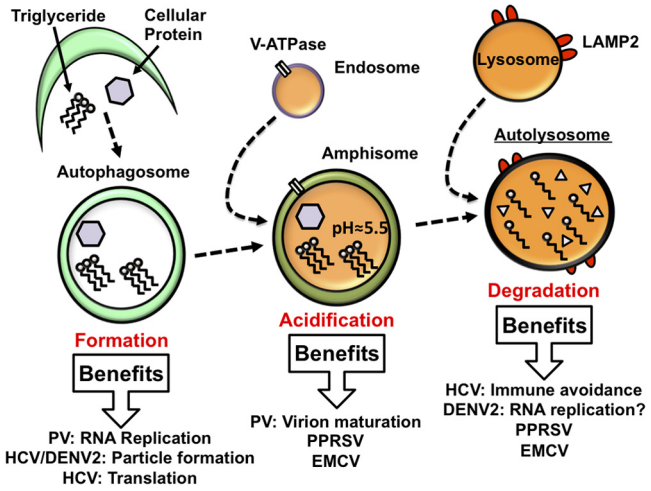

FIG 1 How viruses utilize autophagosome formation and maturation during infection. As the immature autophagosome forms, it captures portions of the cytoplasm. The lumen of the autophagosome acidifies, likely through fusion with late endosomes carrying vacuolar ATPases, to form the amphisome. The amphisome then fuses with the lysosome to form the autolysosome. The replication of viruses that subvert the autophagic pathway is attenuated when autophagosome formation is inhibited. Vesicle acidification is required for efficient PV virion maturation, while inhibition of degradation has no effect on the virus. Degradation of cellular triglycerides by autophagy benefits dengue virus replication. Autolysosome degradation decreases IFN activation following HCV infection. Both PPRSV and EMCV require autophagosome maturation; however, it is not clear if this is due to a requirement for vesicle acidification or autolysosome degradation. V-ATPase, vacuolar (H^+)-ATPase.

of endogenous Rab7 or expression of a dominant negative form results in decreased autophagosome maturation (29, 30). LAMP-2, a lysosomal transmembrane protein, is one of the most abundant lysosomal components (31). Depletion of LAMP-2 prevents autophagosome fusion with lysosomes (32–34). These data indicate that multiple steps of the autophagosome formation and maturation pathway are regulated by the cell and may be subverted by pathogens.

VIRAL SUBVERSION OF THE AUTOPHAGIC PATHWAY

As an obligate intracellular parasite, a virus depends on its ability to evade the host cell's antiviral defenses, as well as its ability to regulate cellular processes that facilitate its own replication, for its success. Subversion of the autophagic pathway, which aids both of these goals, has been the most extensively studied in positive-strand RNA viruses (35, 36). Optimal production of positive-strand RNA viruses depends on the initiation of the autophagic pathway during infection. This is counterintuitive, because the autophagic pathway promotes degradation of cytosolic contents and positive-strand RNA viruses replicate in the cytosol.

The physical hallmark of the autophagic pathway is the formation of cytosolic double-membraned vesicles (19, 37). Positive-strand RNA viruses replicate their genomes in association with cytosolic membranes (38, 39). Therefore, by inducing autophagy, these viruses may be facilitating the creation of scaffolds for their own replication. However, these vesicles are part of a degradative pathway, and if this pathway is unaltered, the vesicles will fuse with lysosomes and their contents will be degraded. Coxsackievirus B3 (CVB3), an enterovirus in the *Picornaviridae* family, appears to have developed a strategy to prevent this. CVB3 relies on autophagosome formation for optimal virus replication (40, 41). However, there is evidence that during both *in vitro* and *in vivo* infections amphisome maturation and autophagic protein degradation are inhibited (40, 41). The mechanism by which CVB3 upregulates autophagosome formation while restricting autophagic degradation is unknown. Treatment of CVB3-infected cells with inhibitors of autophagosome maturation results in increased virus production, indicating that at least a portion of the virus remains sensitive to autophagic degradation (41). Recently it was also shown that rotavirus induces autophagic signals to promote virus replication but that the virus blocks protein degradation (42). As with CVB3, the mechanism by which rotaviruses specifically inhibit autophagosome degradation is not yet known. Further work to identify the specific virus or host cell proteins used by these viruses to prevent degradation will help our understanding of autophagic regulation in general.

A similar antidegradative phenomenon has been observed in bacterial infection models, with the best-characterized being *Legionella pneumophila* infection, which induces replication vesicles that resemble autophagosomes (43). The vacuoles become acidic; however, the bacteria secrete a factor that delays their fusion with lysosomes (44). A recent report indicates that *Legionella* can interfere with the formation of LC3-II, although the significance of this to autophagosome maturation is unclear (45). Inhibition of autophagosome maturation has been observed for several other nonviral pathogens (46).

In the following sections, we discuss recent advances in understanding the interaction of three positive-strand RNA viruses with the late stages of the autophagic pathway. Replication of all three viruses is reduced when autophagy is inhibited. Conversely, stimulation of autophagy increases infectious virus production (47–52). To date, this subset of viruses are the only pathogens shown to induce autophagosome formation to promote their own replication while allowing maturation of the vesicles, fusion of amphisomes with lysosomes, and subsequent cargo degradation. For reference, a brief description of assays used to monitor autophagosome maturation and autophagic degradation is provided in Table 1, and more detail is available in reference 56.

DENGUE VIRUS

Dengue virus, a member of the *Flaviviridae*, is the causative agent of dengue fever, which in a small subset of the population progresses to dengue hemorrhagic fever/dengue shock syndrome (57, 58). Dengue virus (DENV) is comprised of four antigenically related but distinct viruses (DENV-1 to DENV-4) with each virus comprising many distinct genotypes (59). Thus far, only DENV-2 and DENV-3 have been shown to require autophagosome formation for maximum virus replication (52, 57, 60). During infection with either virus, viral proteins involved in translation and replication locate to autophagosomes (52, 60).

While DENV-2 and DENV-3 both subvert autophagosome formation, there are some major differences in the way these viruses interact with the late stages of the autophagic pathway. DENV-3 nonstructural proteins primarily colocalize with autolysosomes during infection, whereas DENV-2 proteins are primarily located on immature autophagosomes (52). During DENV-2 virus infection, autophagy increases the cell's degradative capacity, specifically in regard to cellular lipid droplets (51). The degradation of lipid droplets by autophagy is referred to as lipophagy (61). Increased lipophagy during DENV-2 infection results in

TABLE 1 Assays for autophagosome maturation and autophagic degradation[a]

Assay	Description	Read out
Protease sensitivity of LC3-II	LC3-II is degraded by lysosomal proteases following fusion of the autophagosome with the lysosome (53). Lysosomal protease inhibitors, which inhibit LC3-II degradation by the autolysosome, increase the steady-state level of LC3 II (53, 56).	Protein degradation by the autophagic pathway
p62 Degradation	The p62/SQSTM1 protein directly binds LC3-II on the autophagosome membrane (54). p62 is degraded within the autolysosomes (55). A decrease in the steady state level of p62 following induction of autophagy indicates successful protein degradation through the pathway (55, 56).	Autolysosome formation
LC3-II-lysosome colocalization	The cellular locale of autophagosome-associated GFP-tagged LC3-II can be monitored by fluorescence microscopy (67). During the initial stages of the autophagic pathway, colocalization of LC3-II with lysosomal markers is low. As autophagosomes mature and fuse with lysosomes, colocalization with lysosomal markers increases. Cellular lysosomes can be visualized by staining for protein markers such as LAMP-2, LAMP-1, and cathepsin D (31).	Autolysosome formation
Tandem-tagged GFP-RFP-LC3	Tandem-tagged RFP-GFP-LC3 localizes to the autophagosome membrane following induction of autophagy (58). Only the signal generated by the GFP protein is sensitive to the acidic and/or proteolytic conditions in the lumen of mature autophagosome and lysosomes. Colocalization of GFP and RFP signals is observed on early or immature autophagosomes. As autophagosomes mature, the GFP signal is lost, leading to only RFP fluorescence.	Autophagosome maturation
Transmission electron microscopy	Autophagosomes can by identified by TEM as membrane-bound structures containing cytoplasmic material. Immature autophagosomes (AVi) show a double membrane visible as two membrane bilayers separated by an electron-lucent cleft. These vacuoles contain cytosol and/or organelles that appear morphologically intact (56). Mature or degradative vesicles (AVd) typically show partial degradation of the enclosed cytoplasmic material, as well as increased electron density in the lumen of the vesicle.	Autophagosome maturation

[a] An extensive discussion of known assays for analysis of autophagic signaling, autophagosome formation, and all other aspects of the pathway can be found in reference 53. TEM, transmission electron microscopy.

both a decrease in triglycerides and an increase in β-oxidation. Inhibition of autophagosome formation reduces infectious virus production; however, when cells are supplied with the products of lipophagy, virus production returns to levels observed in cells capable of autophagy. Heaton and Randall speculate that the release of free fatty acids during lipophagy increases ATP generation, which is critical for viral replication (51). A role for lipophagy during DENV-3 infection has not yet been reported.

HEPATITIS C VIRUS

Hepatitis C virus (HCV), another flavivirus, is a major cause of chronic liver disease (62). The HCV RNA-dependent RNA polymerase interacts with the cellular autophagy protein Atg5, and the two proteins colocalize during early time points of infection (63). Additionally, HCV RNA and proteins cofractionate with LC3-II on a discontinuous sucrose gradient (64). Expression of HCV proteins NS5A and NS4B in isolation is sufficient to induce autophagic signaling (65, 66). While there is agreement that HCV induces autophagic signaling, the specific role of autophagy during HCV infection remains controversial. Autophagy was shown to be essential for translation of the viral genome but dispensable once the infection had begun (49). These data contrast with a report that knockdown of autophagy genes had no effect on virus translation and RNA replication but instead was essential for HCV particle formation (47).

HCV-infected cells expressing tandem-tagged green fluorescent protein-red fluorescent protein-LC3 (GFP-RFP-LC3) (Table 1) show predominantly red fluorescence, indicating maturation of autophagosomes into acidic amphisomes (67, 68). The RFP-positive puncta colocalize with LAMP-1, demonstrating fusion of the autophagosome with either late endosomes or lysosomes (68). However, there is at least one report of an incomplete autophagic response to HCV infection. No change in either p 62 levels or long-lived protein degradation was observed following transfection with the HCV replicon (69). This discrepancy may result from a difference between transfection of the viral genome and infection with live virus. However, elevated autolysosome formation has been observed following transfection of the HCV replicon, making this an unlikely explanation (70). An alternative hypothesis is that typical autophagosome cargo, such as p62, is not incorporated into the specialized autophagosomes generated during HCV infection. If this is the case, assays measuring protein degradation levels would not be a reliable measure of autophagosome maturation.

HCV genome replication is attenuated following depletion of LAMP-2 or Rab7, both of which are essential for autolysosome formation (68). Treatment with either bafilomycin A1 or chloroquine, both of which inhibit autophagosome maturation, results in reduced viral RNA and protein expression (68, 71). Investigation of the retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) signaling cascade following HCV infection has revealed a role for autophagic degradation during infection in suppressing the innate immune response to infection (68). Activation of the beta interferon (IFN-β) promoter by ectopically expressed RIG-I was measured in infected cells in both the presence and the absence of autophagic degradation. In control cells, HCV was able to inhibit RIG-I-mediated IFN-β activation. In the absence of autophagic degradation, infected cells showed a significant increase in IFN activation. The varied reports on the effects of autophagy in

HCV production lead us to conclude that the process may play multiple roles in promoting viral replication.

POLIOVIRUS

Poliovirus (PV), the causative agent of poliomyelitis, is a member of the *Picornaviridae* family. It is one of the most well-characterized members of this family in terms of its molecular and cellular biology, biochemistry, structure, life cycle, and pathogenesis and therefore represents an important model virus (72). By 5 h postinfection, infected cells exhibit extensive accumulation of autophagic vacuoles throughout the cytoplasm (50, 73). Viral RNA replication proteins localize to the autophagosome membrane during infection (50, 74). LC3 and LAMP-1 also colocalize during infection, indicating autophagosome fusion with late endosomes and/or lysosomes (50). Staining infected cells with monodansylcadaverine (MDC), a lysosomotropic agent that is concentrated in acidic compartments by an ion-trapping mechanism, reveals that the lumen of autophagosomes acidifies relative to the cytosol (50, 75). Infection promotes autophagic protein degradation; however, unlike for dengue virus and HCV, degradation is not necessary for virus replication, since lysosomal protease inhibitors have no impact on the intracellular virus titer (76).

Our group recently showed that poliovirus utilizes both autophagosome formation and maturation of the autophagic vacuole to promote two separate and distinct steps in the virus life cycle. Inhibitors of autophagosome formation limit viral RNA replication (76). However, if autophagosome formation proceeds normally but vesicle acidification is inhibited, virus production remains attenuated. In the absence of acidic vesicles, viral entry, translation, RNA replication, and genome encapsidation all occur normally. Acidic vesicles are, however, required for the last step in the production of an infectious virion, marked by the internal cleavage of capsid protein VP0, which results in the maturation of a noninfectious provirion to an infectious virion (76). This cleavage step is attenuated in the absence of vesicle acidification, resulting in a decrease in the number of infectious virions produced. How an acidic vesicle can promote the maturation of a presumably cytosolic, nonenveloped virus is a current question of research focus.

POSSIBLE CONNECTIONS AMONG VIRUSES THAT BENEFIT FROM AUTOPHAGOSOME MATURATION

We have presented here three examples of how viruses benefit from autophagosome maturation. However, there are indications that some of these benefits may be conserved among the viruses. Of the viruses discussed, only HCV has been definitively shown to use autophagic degradation to downregulate immune signaling during infection (68). However, preliminary results indicate that the immune response to dengue virus infection may also be attenuated by autophagic degradation (68). Recently, Japanese encephalitis virus (JEV) has been shown to subvert autophagy as a viral immune evasion strategy. The mechanism may be similar to that used by HCV, since type I IFN activation is increased when JEV infects cells deficient in autophagy (77). Infection with JEV increases autolysosome formation *in vivo*, and this formation is essential for maximum virus production (77). Is it not yet known if autophagic degradation is responsible for restriction of the immune response during JEV infection. Interestingly, autophagy is essential for JEV production even in an IFN-defective background, indicating that the virus may have multiple uses for the autophagic pathway during infection.

A recent publication investigated the role of autophagy in lipid metabolism during HCV infection. As with dengue virus, infection with HCV results in the appearance of autophagosomes filled with lipid cargo (78). Inhibition of autophagosome maturation through bafilomycin A1 treatment results in an accumulation of cholesterol in both HCV replicon cells and cells infected with HCV strain JFH1 (78). The purpose of increased autophagic breakdown of cholesterol during HCV infection remains elusive. One hypothesis is that the autophagic flux of cholesterol is needed for lipid droplet biogenesis during HCV infection. This would be very intriguing, as the lipid droplet area is decreased by autophagic degradation during dengue virus infection (78, 79). The differences in the proposed roles of autophagic degradation of lipids during infection may be a product of the different requirements each virus has for lipid droplets during infection. Unlike for dengue virus, lipid droplets are required for HCV virion assembly (79); therefore, increased surface area of these lipid droplets may aid HCV during replication. Conversely, dengue virus may promote destruction of these lipid pools to provide energy for virus replication occurring at an alternate site in the cell.

The data gathered thus far regarding the role of autophagy during poliovirus and dengue virus infections are almost exclusively from *in vitro* systems. Therefore, the effects of autophagic degradation on the host immune response to infection have not been assessed. If it is found that immune signaling during infection is attenuated through autophagic degradation, then autophagic degradation could play multiple proviral roles during infection. Recently, impairment of autophagosome formation has been shown to hamper formation of infectious dengue virus particles while having minimal effects on viral RNA replication (80). This suggests that both poliovirus and dengue virus may be utilizing the environment within a mature autophagosome to promote the final steps in production of viral progeny. It is not yet know if inhibitors of autophagosome maturation have an effect on dengue virus particle formation.

CONCLUSION

It is now appreciated that some viruses, such as poliovirus, dengue virus, and HCV, rely on the degradative activity of the autophagic pathway for efficient replication. While this review focused on the three viruses for which the role of autophagic maturation during infection has been elucidated, the story is far from complete. For example, both encephalomyocarditis virus (EMCV) and porcine reproductive and respiratory syndrome virus (PRRSV) subvert the autophagic pathway for optimal virus production while promoting autophagic protein degradation (81, 82). Replication of both PRRSV and EMCV has been shown to be sensitive to treatments known to restrict autophagosome maturation (83, 84). There is currently no model for the role that maturation of the autophagosome is playing during infection with either of these viruses. It will be interesting to learn if either virus shares a mechanism with one of the viruses presented in this review or if novel roles for autophagosome maturation are discovered. There is also recent evidence that treatment with the cathepsin inhibitor pepstatin A results in a decrease in influenza A virus production, which the authors of the study have linked to pepstatin A altering the regulation of autophagy (85). Together, these data indicate that multiple viruses may utilize autophagic degradation.

The question remains: how do these viruses thrive in a highly degradative environment? One possibility is that they have evolved to be resistant to degradation within the autolysosome. Alternatively, they may have developed a mechanism to avoid being trapped within the degradative vesicles. Finally, viral replication may occur outside the degradative autophagosome and thus be unaffected by the degradative environment within the vesicle.

It is often in the best interest of a virus to maintain the host cell's integrity until progeny virus production is complete. For example, many viruses have evolved mechanisms to prevent cellular apoptosis, a pathway that is linked to autophagy (86, 87). The way in which a virus interacts with the autophagic pathway may have important implications for cell viability throughout the infection. A number of studies have indicated that autophagy is induced by endoplasmic reticulum (ER) stress (88, 89). In both yeast and mammalian cells, autophagy has been shown to have prosurvival effects when the ER is overloaded with misfolded proteins (90–92). Inhibition of the autophagic pathway increases cell death following ER stress. This effect is due to the degradation of protein aggregates and misfolded proteins (90). Both HCV and EMCV have been shown to increase ER stress (68, 69, 93–95). By allowing autophagic degradation to proceed unperturbed, these viruses may be minimizing the risk of cell death prior to the completion of the replicative cycle.

Finally, a great deal has been learned about the functions of the host cell by studying the ways in which viruses regulate cellular pathways. A clearer picture of the mechanism by which viruses inhibit or promote autophagic degradation will not only improve our understanding of virus replication but also shed light on the ways in which the late stages of autophagy are regulated.

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