Chapter 11

DIGESTING ONESELF AND DIGESTING MICROBES
Autophagy as a Host Response to Viral Infection

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1. INTRODUCTION

The cellular pathway of autophagy is as ancient as the origins of eukaryotic life. Derived from the Greek and meaning to eat ("phagy") oneself ("auto"), the term autophagy refers to a lysosomal pathway of self-digestion, involving dynamic membrane rearrangement to sequester cargo for delivery to the lysosome, where the sequestered material is degraded and recycled. For decades, it has been known that autophagy is the primary intracellular catabolic mechanism for the degradation and recycling of long-lived cellular proteins and organelles. For decades, it has also been known that the recycling function of autophagy is an important adaptive response to nutrient deprivation and other forms of environmental stress. However, only recently have we discovered that autophagy may also be an important mechanism for the degradation of intracellular pathogens and that autophagy may also be important in cellular protection against the stress of microbial infection. Not surprisingly, we have also recently learned that some successful intracellular pathogens have devised strategies either to block host autophagy or to subvert the host autophagic process to foster their own replication. In this chapter, we will review recent progress in understanding

the interrelationships between viruses, autophagy, and innate immunity (Figures 1 & 2).
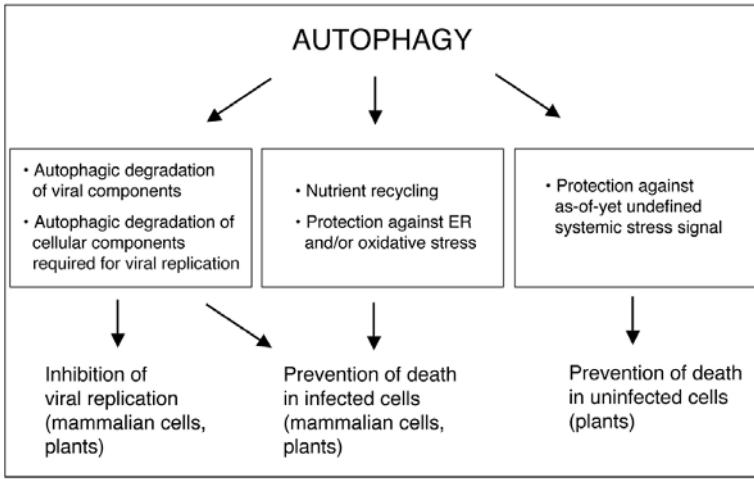

Figure 1. Conceptual overview of protective roles of autophagy in mammalian and plant viral infections. Areas in boxed regions represent potential mechanisms by which autophagy exerts each type of protective effect. See text for details.
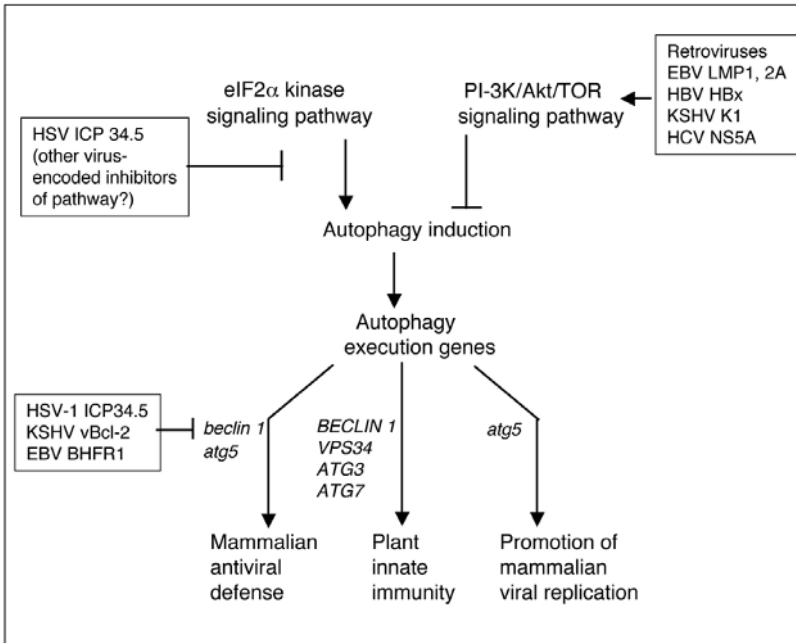

Figure 2. Conceptual overview of the interrelationships between autophagy signaling pathways, autophagy genes, autophagy functions in viral infections, and viral inhibitors of autophagy. See text for details.

2. INTRODUCTION TO THE MOLECULAR AND CELL BIOLOGY OF AUTOPHAGY

Before discussing the interrelationships between viruses, autophagy, and innate immunity, we will provide a brief overview of the molecular and cell biology of autophagy. While this subject has been covered extensively in a recent book and numerous recent review articles[2-5] we will highlight the aspects of this subject that may have particular relevance for viral infections. The process of autophagy was first described more than forty years ago, however, for many decades our understanding of autophagy was based largely on morphological observations from electron microscopy (reviewed in [6]). The field has expanded considerably within the last 15 years after the cloning and molecular characterization of the yeast *AuTophagGy (ATG)* - related genes (reviewed in [7]). The analysis of sequenced genomes of higher eukaryotes has identified *ATG* homologues in mammals, *C. elegans*, *Drosophila*, *Dictyostelium*, and plants, and many of these genes have been shown to be essential for autophagy function in higher eukaryotes (reviewed in [2]) (Table 1). In addition to the identification of the autophagy genes, significant progress has been made in the past decade in understanding some of the signaling events that regulate autophagy (reviewed in [8,9]). Interestingly, some of the signaling molecules that regulate autophagy, as well as some of the autophagy genes, play a role in the host antiviral innate immune response (Table 1, Figure 2).

Table 1. Orthologs of yeast autophagy-related genes in higher eukaryotes, including genes that play a role in the host response to viral infections.[a]

Gene designation							Ref	Protein characteristics
ATG	At/Nb[a]	Ce	Dd	Dm	Hs	Mm		
Regulation of induction								
1	---	unc-51	DdATG1	---	---	---	20, 59, 125	Protein kinase
Vesicle nucleation								
6	**BECLIN 1**	bec-1	DdATG6	---	beclin 1	**beclin 1**	20, 23, 24, **37-40,** 59, 126	Component of PtdIns 3-kinase complex
Vesicle expansion and completion								
3	ATG3	---	---	DrAUT1	hatg3	---	**40**, 42, 127, 128	E2-like enzyme conjugates PE to Atg8
4	---	---	---	ATG4/AUT2	---	atg4B	48, 129, 130	Cysteine protease cleaves at C terminus of Atg8
5	---	---	DdATG5	---	hatg5	**atg5**	**38**, **51**, 58, 131-133	Conjugated to Atg12 through internal lysine
7	ATG7	M7.5	DdATG7	---	HsGSA7 hatg7	atg7	20, **40**, 58, 61, 134, 135	E1-like enzyme activates ubiquitin-like protein Atg8 and Atg12
8	---	lgg-1	DdATG8	---	MAP1LC3[b]	atg8 APG8L	11, 20, 46, 59, 128	Ubiquitin-like protein conjugated to PE
10	---	---	---	---	---	atg10	136, 137	E2-like enzyme conjugates Atg12 to Atg5

2.1 Formation and structure of autophagosomes

The initial step of autophagy is the formation and elongation of the isolation membrane. The isolation membrane invaginates and sequesters cytoplasmic constituents including mitochondria, endoplasmic reticulum (ER) and ribosomes, and the edges of the membrane fuse with each other to form a double-membrane structure called an autophagosome. The outer membrane of the autophagosome fuses to the lysosome/vacuole with subsequent delivery of the inner vesicle or autophagic body into the lumen of the degradative compartment. The source of the autophagosomal membrane is still unclear, but presently, it is thought that the pre-autophagosomal structure (PAS) acts as the site of vesicle formation during autophagy[10-12]. The PAS is thought to form *de novo*, but the source of the vesicle membrane is not known. It seems likely that the "typical" autophagosomes observed during viral infection that contain a mix of virions and self-cytoplasmic constituents, originate from the PAS. However, it is not yet known whether the PAS also serves as the site of vesicle formation for the formation of "atypical" autophagic-like double-membrane vesicles that function as replication sites for certain RNA viruses (e.g. poliovirus, mouse hepatitis virus, equine arterivirus) (reviewed in [13]). More likely, these double-membrane vesicles arise directly from the endoplasmic reticulum (ER)[13].

Autophagosomes are lipid-rich, protein-poor vesicles that vary in size and membrane thickness depending on the organism and cell type. The composition and abundance of proteins sequestered within autophagosomes reflects the relative composition and abundance of proteins in the surrounding cytoplasm[14]. This observation has led to the concept that autophagosomes indiscriminately sequester cytoplasmic content. However, in yeast, there are well-established pathways of specific autophagy, including the biosynthetic cytoplasm-to-vacuole targeting pathway and pexophagy (reviewed in [15]), and in mammalian cells, mitochondria-specific autophagy has been reported[16]. Although molecular determinants of cargo recognition have been identified in yeast pathways of specific autophagy, virtually nothing is known about the specificity of cargo recognition in higher eukaryotes. In circumstances where there is degradation of viruses observed inside "typical" autophagosomes that also contain cellular constituents, the sequestration step may lack specificity. However, in circumstances where viruses utilize components of the autophagic machinery for the formation of "autophagic-like" double-membrane structures that exclusively contain viral constituents, the sequestration step is likely to have exquisite specificity. The identification of the viral and cellular determinants of this specificity will be an important advance in

understanding the cell biology of these types of RNA virus infections and may eventually lead to the identification of novel antiviral therapeutic targets.

2.2   Regulation of autophagy

Autophagy is tightly regulated by nutritional, hormonal, and other environmental cues. It occurs as a cellular response to extracellular stimuli (e.g. nutrient starvation, hypoxia, overcrowding, high temperature, hormonal or chemotherapeutic treatment), and intracellular stimuli (e.g. accumulation of damaged, superfluous or unwanted organelles, accumulation of misfolded proteins, invasion of microorganisms). Although it is not yet known whether different stimuli act through parallel, convergent, or divergent pathways to trigger autophagy, significant progress has been made within the past decade in identifying different signaling molecules that function in the positive (e.g. eIF2α kinases, Class III PI-3 kinases, PTEN, death-associated protein kinases) or negative (e.g. Tor, insulin-like growth factor signals, Class I PI-3 kinase, Rho/Ras family of GTPases) regulation of autophagy (reviewed in [8,9]).

The identification of a role for these signaling molecules in autophagy regulation has implications for understanding antiviral immunity, and more speculatively, generates hypotheses about novel principles of virus-host interactions. The recently defined evolutionarily conserved role of the eIF2α kinase signaling pathway in autophagy induction suggests that autophagy regulation may contribute to the antiviral function of the interferon-inducible eIF2α kinase, PKR. PKR and other eIF2α kinases induce a general translational arrest by phosphorylating the serine 51 residue of eIF2α (reviewed in [17]). Genetic studies in yeast and mammalian cells have also shown that the eIF2α kinase signaling pathway is required for starvation and herpes simplex virus-induced autophagy[18]. While further analyses are required to dissect the relative contributions of autophagy induction vs. translational arrest in mediating the antiviral effects of PKR, these findings link a new cellular function (i.e. autophagy) with interferon signaling.

Although the eIF2α kinase signaling pathway is the only as-of-yet defined autophagy regulatory signaling pathway that has known antiviral functions, it is interesting to note that most autophagy regulatory signals play a role in other important cellular processes, including cell growth control, cell death, and aging. Some of these effects may be the consequence of divergent downstream targets of these regulatory signals and some of these effects may be directly mediated through autophagy. As will be discussed below, given the evidence for a role of autophagy in innate immunity, it is likely that viruses have evolved different strategies to antagonize host

autophagy, which, at least in the case of herpes simplex virus (see Sections 4.1 and 5) include the targeting of upstream autophagy regulatory signals[18]. Therefore, it is tempting to speculate that some of the effects of viruses on cell growth control and cell death may be either direct or indirect consequences of the evolutionary pressure that viruses face to modulate host autophagy.

As one example, the insulin-like/Class I PI-3K/Akt signaling pathway inhibits autophagy[19,20], promotes oncogenesis (reviewed in [21]), and decreases lifespan (most likely through autophagy-inhibitory effects)[20]. Certain retroviruses have recruited the catalytic subunit of PI3-K and its downstream target Akt and these viral gene products function as oncoproteins (reviewed in [22]). The emerging link between these signaling molecules and autophagy inhibition raises the interesting hypothesis that the initial acquisition of these molecules by viruses was perhaps related to the selective advantage of autophagy inhibition in viral growth. In view of recent evidence supporting a role of autophagy in tumor suppression[23-26], the presence of these genes in retroviral genomes could contribute to oncogenesis at least, in part, through inhibition of autophagy signaling, as well as through modulation of other downstream pathways.

2.3 Autophagy genes

The *ATG* genes encode proteins important for responding to upstream signaling pathways as well as proteins needed for the generation, maturation, and recycling of autophagosomes (reviewed in [4,5,15,27]). The Atg proteins can be grouped into four functional groups, including a protein kinase cascade important for responding to upstream signals, a lipid kinase signaling complex important for vesicle nucleation, ubiquitin-like conjugation pathways important for vesicle expansion, and a recycling pathway important for the disassembly of Atg protein complexes from matured autophagosomes. The role of some of the Atg proteins, including ones that act in the lipid kinase signaling complex and in the ubiquitin-like conjugation pathways, has been studied in plant and mammalian viral infections (see Table 1).

2.3.1 Protein kinase signaling

Autophagy is a dynamic process that is tightly regulated by protein kinases and phosphatases. One of the first *ATG* genes identified in yeast, *ATG1*, encodes a serine/threonine kinase[28]. The Atg1 kinase maintains a weak interaction with a hyperphosphorylated Atg protein, Atg13, in nutrient-rich conditions. Upon starvation conditions or stress, Atg13 is

dephosphorylated resulting in a tighter association with Atg1[29]. Atg13 binding is essential for autophagy since *atg13* mutants unable to bind to Atg1 are completely defective in autophagy[29]. Atg17, which is also thought to play a role in Atg1 activation, also interacts with Atg1[29]. Downstream targets of Atg1 have not been identified, although Atg1 interacts with other proteins independently of its kinase activity[30]. The upstream kinase, Tor (target of rapamycin), indirectly or directly results in Atg13 hyperphosphorylation, which is one presumptive mechanism by which Tor kinase inhibits autophagy. Of note, the Atg1 component of the yeast autophagy induction complex plays a conserved role in autophagy in higher eukaryotes. However, as-of-yet, the role of Atg1 in antiviral immunity has not been evaluated.

2.3.2   Lipid kinase signaling

VPS34, which encodes a phosphatidylinositol-3 kinase (PI3-K), phosphorylates the 3' hydroxyl group inosotiol ring of phosphoinositides[31]. Although there is only one PI3-K in yeast, there are three classes of PI3-K in higher eukaryotes; class III PI3-K has been shown to be analogous to yeast VPS34. The importance of Class III PI3-K signaling in autophagy has been demonstrated pharmacologically and genetically. The nucleotide derivative, 3-methyladenine, inhibits Class III PI3-K activity and is widely used to inhibit autophagosome formation in mammalian cells[32,33]. A null mutation in *VPS34* causes defects in autophagosome formation in yeast[34,35] and microinjection of an inhibitory antiVps34 antibody blocks autophagy in cultured mammalian cells[36].

Vps34 functions through the association with other Atg proteins in a large complex that includes Vps15, Atg6/Vps30 and Atg14[35]. This complex is thought to be important in vesicle nucleation by mediating the localization of other Atg proteins at the PAS[10,35]. Vps34 and Atg6/Vps30 are conserved in higher eukaryotes. Importantly, the mammalian (*beclin 1*) and plant (*BECLIN 1*) homologues of yeast *ATG6/VPS30* have been the most extensively studied *ATG* genes in viral infections. As will be discussed in more detail below, mammalian *beclin 1* restricts viral replication, protects against virus-induced cell death, and is a target of inhibition by different virally-encoded gene products[37-39]. Furthermore, both plant *BECLIN 1* and its binding partner, Class III PI3-K/VPS34 prevent the spread of programmed cell death during the plant antiviral hypersensitive response[40]. Thus, the lipid kinase complex plays an evolutionarily conserved role in antiviral innate immunity.

2.3.3 Ubiquitin-like conjugation reactions

Autophagic vesicle expansion and completion involves conjugation machinery analogous to the ubiquitin conjugation needed for proteasome-mediated protein degradation. Autophagy utilizes an E1-like enzyme (Atg7), two E2-like enzymes (Atg10 and Atg3) that facilitate the conjugation, and activation and localization of different ubiquitin-like modifiers (Atg5 and Atg8). The conjugation modification of Atg proteins is necessary for the formation of an autophagosome of appropriate size and shape[41]. However, the precise molecular functions of the conjugation reactions are not known and remain a critical unanswered question in autophagy research.

The first conjugation system involves the lipidation of Atg8, a ubiquitin-like protein whose close mammalian homologues have three-dimensional structures very similar to ubiquitin[42-45]. Both Atg8 and the mammalian homomlogue LC3 are cleaved post-translationally by the cysteine endopeptidase Atg4[46,47]. The cleavage of Atg8/Lc3 is essential for conjugation and further maturation of the autophagosomes[48]. In yeast and mammalian systems, the cleaved Atg8/LC3 is immediately activated by Atg7, an E1-like enzyme; transferred to Atg3, an E2-like enzyme; and finally conjugated to the lipid molecule phosphatidylethanolamine (PE)[42,47,49,50].

The second ubiquitin-like reaction is the conjugation of Atg12 to Atg5. Atg12 is an ubiquitin-like protein that is activated by Atg7 (E1-like enzyme), transferred to Atg10 (E2-like enzyme), and subsequently conjugated to Atg5 through an isopeptide bond[41,49]. The conjugation of Atg5 to Atg12 is necessary for autophagosome formation but not necessary for localization to the PAS[10].

Almost all of the components of the autophagy machinery that participate in the protein conjugation systems have orthologs in at least some higher eukaryotes. However, only mammalian Atg5 has been studied in the context of its role in viral infections. The contrasting phenotypes of *atg5* null cells infected with two different RNA viruses illustrate two distinct mechanisms by which viruses interact with the autophagic machinery. The murine coronavirus, mouse hepatitis virus, which replicates in association with double-membrane vesicles, has severely impaired growth in *atg5* null embryonic stem (ES) cells[51], suggesting that *atg5* is required for the formation of coronavirus replication complexes. In contrast, the prototype alphavirus, Sindbis virus, replicates to higher titers in *atg5* null murine embryonic fibroblasts (MEFs) than in wildtype controls[38], suggesting that the autophagic machinery functions to restrict Sindbis virus replication.

2.3.4   Atg protein recycling

In yeast, Atg proteins that act at the stage of vesicle formation are not associated with the completed autophagosome, with the exception of Atg8. This suggests that Atg proteins are retrieved at some point prior to, or upon, vesicle completion, and then reutilized in the generation of new autophagosomes. The process of recycling requires the action of Atg2 and Atg18, which allow the recycling of Atg9, the only transmembrane protein that is part of the autophagic machinery[52,53]. Atg9 and Atg18 have orthologues in higher eukaryotes, but their function in antiviral responses has not been studied.

The unique association of Atg8/LC3 with the mature autophagosome has led to an important technical advance in autophagy research. Atg8/LC3 is presently the most widely used and reliable marker for labeling autophagosomes[10, 11,20,50,54] and with the recent availability of transgenic mice that express GFP-tagged LC3[54], it is now possible to study autophagy induction *in vivo* during viral infections.

3.   INTRODUCTION TO THE BIOLOGICAL FUNCTIONS OF AUTOPHAGY

In addition to its emerging role in innate immunity, autophagy plays a role in diverse other biological processes, including survival during starvation, differentiation and development, tissue homoeostasis, aging, cell growth control, and certain forms of programmed cell death. These biological functions of autophagy have been reviewed in detail elsewhere[2,55,56]. In this section, we will however, briefly discuss selected biological functions of autophagy that have relevance either to understanding the mechanisms by which autophagy protects cells against virus infections (Figure 1) or to understanding the potential consequences for the host of viral evasion of autophagy (Figure 3).
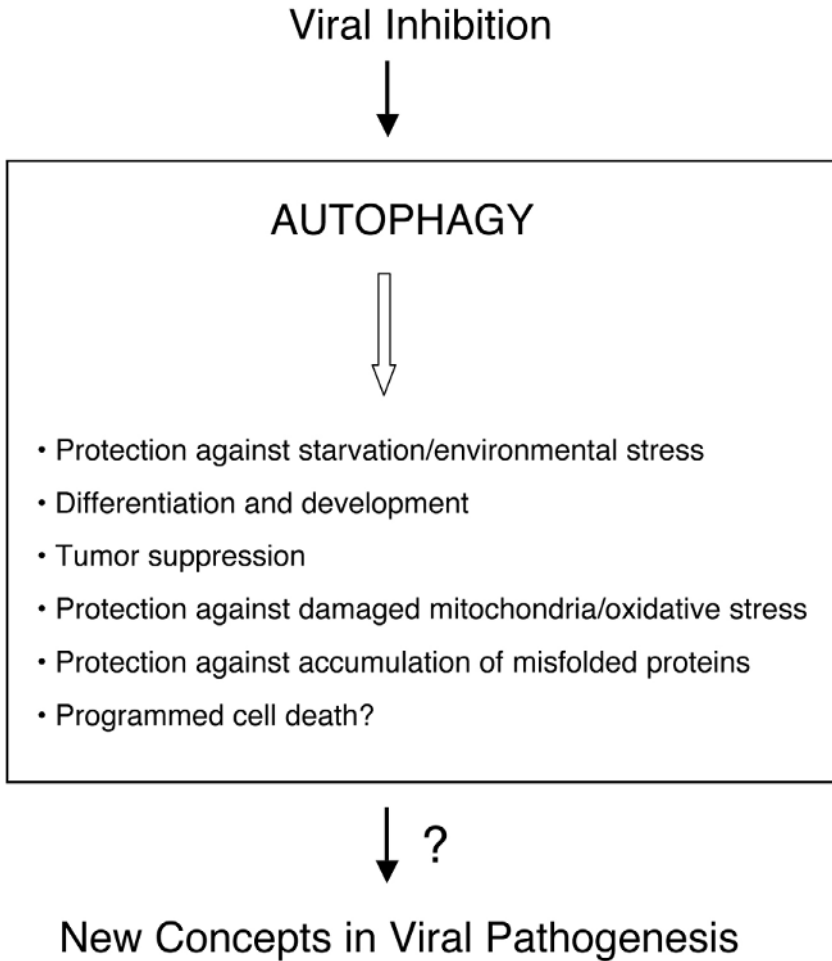
Viral Inhibition

↓

AUTOPHAGY

⇩

• Protection against starvation/environmental stress

• Differentiation and development

• Tumor suppression

• Protection against damaged mitochondria/oxidative stress

• Protection against accumulation of misfolded proteins

• Programmed cell death?

↓ ?

New Concepts in Viral Pathogenesis

Figure 3. Conceptual overview of the biological functions of autophagy (other than antiviral defense). Viral inhibition of autophagy may block these functions, representing novel potential mechanisms of viral pathogenesis. See text for details.

3.1   Role of autophagy in protection against nutrient starvation

Perhaps the primordial function of autophagy is its ability to recycle nutrients and help sustain life during periods of starvation. Several decades ago, starvation was noted to be a potent inducer of autophagy in rodent liver (reviewed in [57]), leading to the hypothesis that autophagy is an adaptive response to starvation. Following the identification of the conserved autophagy genes, genetic studies in different species have confirmed that autophagy genes are required for the maintenance of eukaryotic life in the face of limited environmental nutrient supply. This principle was first demonstrated in yeast, i.e. all *ATG* gene mutant yeasts grow normally in nutrient rich conditions, but unlike wild-type yeasts, die rapidly during carbon or nitrogen starvation[28]. Similarly, *Dictyostelium discoideum* that lack *ATG* genes also grow normally in the presence of their food, nonpathogenic bacteria, but die rapidly when subjected to starvation[58,59]. During nitrogen or starvation, *atg7* and *atg9* mutant plants display two phenotypes that are thought to result from a defective ability to mobilize nutrients through autophagic delivery, including enhanced chlorosis (yellowing of leaves due to a loss of chlorophyll) and accelerated leaf senescence[60,61]. In addition, mammalian cells deleted of the autophagy genes, *atg5* or *beclin 1*, also undergo accelerated cell death in response to starvation as compared to their wild-type counterparts[62].

The pro-survival function of autophagy during starvation is thought to be related directly related to its ability to recycle nutrients to generate a sufficient pool of amino acids required for the synthesis of essential proteins. While the eIF2α kinase signaling pathway shuts off general translation during starvation, at least in yeast, Gcn2 signaling simultaneously stimulates the transcription of essential starvation response genes, including autophagy genes[63,64]. Thus, this signaling pathway provides a coordinated method to effectively generate new amino acids by autophagy and redirect the host cell synthetic machinery to use its limited amino acid supply specifically for the synthesis of essential starvation response proteins.

Although a downstream transcription factor like yeast Gcn4 (which is downstream of yeast eIF2α and transcriptionally transactivates autophagy genes), has not yet been identified for mammalian PKR signaling initiated during virus infection, it seems likely that there are functionally homologous molecules that direct virus-infected cells to mount an adaptive and selective transcriptional and translational response during virus infection. Even in the absence of this postulated arm of PKR signaling, the mere recycling of nutrients in virus-infected cells would be predicted to have a beneficial function for the host. Although few studies have compared cellular amino

acid pools during nutrient starvation and virus infection, acute viral replication involves the parasitism of not only the host cell's translational machinery, but also the host cell's translational building blocks. Therefore, it seems likely that acute viral replication induces what can be thought of as a state of "pseuodostarvation". According to this model, the prediction is that the nutrient recycling function of autophagy plays a similar protective function during viral infection as it plays during nutrient deprivation.

3.2   Role of autophagy in differentiation and development

Differentiation and development both require cells to undergo significant phenotypic changes and must entail a mechanism for the breakdown and recycling of obsolete cellular components. Genetic studies have revealed an essential role for components of the autophagic machinery in differentiation and developmental processes in several different organisms, including sporulation in yeast, multicellular development in *Dictyostelium*, dauer development in *C. elegans*, and embryonic development in mice (reviewed in [2]). In addition, the mammalian autophagy gene, *beclin 1*, appears to play a role in epithelial cell differentiation, since the mammary glands in *beclin 1* heterozygous-deficient mice display striking morphological abnormalities[23]. Since viral gene products can inhibit the autophagy function of Beclin 1 (see below) and potentially other autophagy proteins[65], it is possible that autophagy blockade represents a mechanism by which viruses can affect cellular differentiation. For example, the Bcl-2-like BHRF1 protein encoded by EBV binds to Beclin 1[39] blocks its autophagy function[39], and also perturbs epithelial cell differentiation[66]. Further studies are needed to determine the role of Beclin 1 binding in the perturbation of epithelial cell differentiation by BHRF1, as well as to investigate the effects of other viral inhibitors of autophagy on cellular differentiation and multicellular development.

3.3   Role of autophagy in cell growth control

Many different viruses, including retroviruses, gammaherpesviruses, papillomaviruses, and hepatitis viruses are oncogenic. Studies of the mechanisms of viral oncogenesis have largely focused on the ability of viruses to alter mitogenic signaling, cell cycle regulation, and/or apoptosis. However, new evidence is emerging that autophagy plays a role in tumor suppression and that autophagy is antagonized by gene products encoded by certain oncogenic viruses. Accordingly, it will be important to evaluate the role of viral inhibition of autophagy in viral oncogenesis.

Normal cell growth requires a well-coordinated balance between the cell's biosynthetic machinery (e.g. protein synthesis and organelle biogenesis) and its degradative processes (e.g. protein degradation and organelle turnover). In the 1970's, it was first proposed that protein catabolism through autophagy is a major determinant of cell growth[67,68]. According to this model, both cell mass and the rate of cell growth is a balance between the amount of protein synthesized and the amount of autophagic protein degradation. Although this model has received little attention in recent years, interest in the role of autophagy in cell growth control has reemerged in light of new biochemical and genetic links between autophagy and the negative regulation of tumorigenesis.

As stated above in Section 2.2, several different oncogenic signaling molecules, including members of the insulin signaling pathway (e.g. Class I PI-3K, Akt) and members of the Rho and Ras family of GTPases negatively regulate autophagy in mammalian cells and the PTEN tumor suppressor positively regulates autophagy (reviewed in [69]). Furthermore, the autophagy inhibitor, Tor, is an important positive regulator of cell growth in diverse organisms, and the Tor inhibitor, rapamycin, has promising anti-tumor effects in human clinical trials (reviewed in [70]). Oncogenic viruses have developed multiple different strategies to activate autophagy-inhibitory signaling pathways. These strategies include encoding viral oncoproteins that represent activated forms of the corresponding cellular proto-oncogene (reviewed in [22]) or upregulating Rho/Ras or Class I PI-3K/Akt/TOR signaling through alternative mechanisms[71-77].

Components of the autophagic machinery may also play a direct role in tumor suppression. The *beclin 1* gene is monallelically deleted in a high percentage of cases of human breast, ovarian, and prostate cancer (reviewed in [78]) and has tumor suppressor function in cultured mammary carcinoma cells[79,80]. Heterozygous disruption of *beclin 1* in mice increases the frequency of spontaneous tumorigenesis (including papillary lung carcinomas, B cell lymphomas, and hepatocellular carcinomas) and accelerates the development of hepatitis B virus-induced pre-malignant lesions[23,24]. In addition, *atg5* null ES cells are more tumorigenic in mice than their wild-type counterparts and result in teratomas that are less well-differentiated[81]. Together, these findings lead to the concept that autophagy genes may represent a novel class of tumor suppressor genes and that genetic disruption of autophagy may represent a novel mechanism of tumorigenesis. As will be discussed in more detail below, two different classes of viral gene products have been identified thus far that bind to Beclin 1 and inhibit its autophagy function, including the alphaherpesvirus-encoded neurovirulence protein, HSV-1 ICP34.5, and the gammaherpesvirus-encoded Bcl-2-like proteins, KSHV vBcl-2 and EBV BHRF1[38,39]. The gammaherpesviruses are

oncogenic viruses that are etiologically linked to a variety of different malignancies, including lymphoma, nasopharyngeal carcinoma, and Kaposi's sarcoma. At present, the precise role of gammaherpesvirus Bcl-2-like proteins in viral oncogenesis is uncertain. Nonetheless, given the well-defined role of cellular Bcl-2 in oncogenesis and the emerging evidence that Beclin 1 is a tumor suppressor protein, it will be important to evaluate whether viral Bcl-2 antagonism of Beclin 1 function plays a role in gammaherpesvirus oncogenesis. Of note, preliminary data indicates that KSHV may also encode other gene products that interact with other components of the autophagic machinery[65]. Thus, oncogenic gammaherpesviruses may have multiple mechanisms to disarm host autophagy. It will be of interest to determine whether other oncogenic DNA viruses, especially human papillomavirus, also directly inhibit the host autophagic machinery.

3.4   Role of autophagy in lifespan extension

In many tissues in the adult organism (especially post-mitotic cells), protein and organelle turnover by autophagy plays an essential cellular homeostatic or housekeeping function, removing damaged or unwanted organelles and proteins. For many decades, it has been presumed that this homeostatic function of autophagy represents an anti-aging mechanism, perhaps by reducing reactive oxidative species and other toxic intracellular substances that contribute to genotoxic stress (reviewed in [82]). The conserved effects of protein caloric restriction (a dietary inducer of autophagy) on lifespan extension has provided further fuel for this concept (reviewed in [83]). Recent genetic studies, especially those performed in *C. elegans*, provide more direct evidence for a role of both autophagy regulatory signals and components of the autophagic machinery in anti-aging pathways. Loss-of-function mutations in autophagy-inhibitory insulin-like signaling pathway extend lifespan (reviewed in [84]), and inactivation of the *C. elegans* ortholog of yeast autophagy gene, *ATG6/VPS30*, blocks this lifespan extension[20].

While the precise mechanisms by which autophagy extends lifespan are unknown, one theory is that autophagy selectively removes damaged mitochondria, resulting in decreased levels of intracellular reactive oxygen species and cellular protection against oxidative damage. Viral infections, as well as the inflammatory response to viral infections, can damage mitochondria and/or increase the intracellular generation of reactive oxygen species, and these effects may contribute to viral pathogenesis. For example, the mitochondrial damage that occurs in HIV infection (even in the absence of antiretroviral treatment) is thought to be a major contributory factor to the metabolic abnormalities and cardiomyopathy that occur in patients with

AIDS (reviewed in [85]). As another example, in a transgenic mouse model of hepatitic C virus (HCV) infection, oxidative stress in the absence of inflammation has been implicated in HCV-associated hepatocarcinogenesis[86]. Similarly, studies in transgenic mice and cultured cells indicate that pre-S1/S2 mutant hepatitis B virus surface antigens, which accumulate in late stages of HBV infection, cause oxidative stress and DNA damage[87]. Therefore, it is possible that the mechanisms by which autophagy functions as an anti-aging pathway may be relevant to potential roles that autophagy may play in protecting cells against adverse sequelae of oxidative stress during virus infection.

3.5   Role of autophagy in preventing diseases associated with protein aggregates

Diseases associated with an accumulation of misfolded and aggregated proteins, including neurodegenerative disorders and α_1-anti-trypsin liver disease, are associated with an increase in the accumulation of autophagic vacuoles (reviewed in [56]). In these diseases, it has both been argued that autophagy plays a protective role (i.e. by removing protein aggregates and damaged mitochondria) and a pathologic role (i.e. by promoting liver dysfunction in α_1-anti-trypsin deficiency through excessive mitochondrial autophagy[88] or by promoting autophagic cell death). Although both of these roles may be operative in different diseases or even in different facets of a single disease, recent studies provide compelling evidence that autophagy plays a protective role against the toxic effects associated with protein aggregation. For example, mutant α-synuclein (associated with early onset Parkinson's disease), and aggregate-prone proteins with polyglutamine expansions (associated with Huntington's disease) are targeted for autophagic degradation[88,89]. Rapamycin, which stimulates autophagy, not only enhances the clearance of aggregate-prone proteins but also reduces the appearance of the aggregates and the cell death associated with expression of mutant Huntington's proteins[89]. Furthermore, induction of autophagy with rapamycin protects against neurodegeneration in both a fly and mouse model of Huntington's disease[90].

Recent advances have also been made in understanding the mechanisms by which autophagy is induced in response to misfolded protein aggregates. In cell models, transgenic mice, and samples from human brains of patients with Huntington's disease, mTOR is sequestered into polyglutamine aggregates. This sequestration impairs its kinase activity, leading to induction of autophagy[90]. Although it has not yet been evaluated, it is likely that the accumulation of misfolded protein aggregates also induces autophagy through activation of the ER stress response, which is mediated

by the eIF2α kinase, PKR-like ER resident kinase (PERK) [91,92], since other stress stimuli (e.g. starvation and virus infection) that activate other eIF2α kinases (e.g. Gcn2 and PKR) induce autophagy through this same signaling pathway[18].

These observations are potentially relevant to understanding the role of autophagy in protection against virus-induced diseases in which protein misfolding and ER stress are thought to play pathogenetic roles. Similar to genetic neurodegenerative disorders, there is increasing evidence that murine retrovirus-associated spongiform-like neuronal degeneration is also associated with protein misfolding and ER stress. For example, viral envelope proteins from avirulent strains are processed normally and fail to induce ER stress, whereas envelope proteins from neurovirulent strains are misfolded and activate ER stress response pathways[93-95]. In addition, it has been proposed that the mechanism by which pre-S mutant HBV surface antigens promote oxidative stress and DNA damage is through the accumulation of misfolded mutant proteins and activation of ER stress[87,96]. Thus, based on recent studies with non-viral associated neurodegenerative disorders, the prediction is that autophagy induction might be beneficial in attenuating diseases associated with misfolded viral proteins, such as retrovirus-associated spongiform encephalopathy and hepatitis B virus-induced liver damage. As a corollary, the possibility that these viruses might possess mechanisms to evade host autophagy could be an exacerbating factor in the pathogenesis of these infections.

An interesting question is whether viral protein aggregates trigger autophagy by mechanisms that are similar to those involved in autophagy induction initiated by cellular protein aggregates. Different viral glycoproteins are known to activate the ER stress-related eIF2α kinase, PERK[97,98], although a role for PERK in autophagy induction has not yet been formally demonstrated. It is completely unknown, however, whether viral protein aggregates, like polyglutamine aggregates in Huntington's disease, sequester and thereby inactivate the autophagy-inhibitory kinase, mTOR. If so, this would represent a highly novel mechanism by which viruses trigger intracellular innate immune responses.

4.   AUTOPHAGY AND INNATE IMMUNITY TO VIRUSES

Autophagy is emerging as a newly described mechanism of antiviral innate immunity that is targeted by viral virulence gene products. Although there are not yet many published articles in this area, there are several observations that support this concept. First, during herpes simplex virus

infection, the interferon-inducible antiviral PKR signaling pathway regulates the autophagic degradation of cellular and viral components[18,99]. Second, mammalian autophagy execution genes, including *beclin 1* and *atg5*, regulate Sindbis virus replication and Sindbis virus-induced cell death[37,38]. Third, plant autophagy execution genes, including *BECLIN 1*, Class III *PI3-K/VPS34*, *ATG3*, and *ATG7*, restrict tobacco mosaic virus replication and limit the spread of cell death during the innate immune response[40]. In this section, each of these observations will be described in more detail.

4.1   PKR-dependent autophagy degrades cellular and viral components

The interferon-inducible dsRNA-dependent protein kinase R (PKR) plays an important role in innate immunity against viral infections. PKR activation leads to phosphorylation of the α subunit of eukaryotic initiation factors 2 (eIF2 α) and a subsequent shutdown of host and viral protein synthesis and viral replication (reviewed in [100]). To avoid this translational shutdown, many viruses have evolved different strategies to antagonize PKR function. These include interference with the dsRNA-mediated activation of PKR or PKR dimerization; blockade of the kinase catalytic site or PKR-substrate interactions; alterations in the levels of PKR; direct regulation of eIF2α phosphorylation; and effects on components downstream of eIF2α (reviewed in [100,101]). The importance of viral antagonism of PKR function in viral pathogenesis has been most clearly demonstrated using a herpes simplex virus type 1 (HSV-1) model system[102,103]. The HSV-1 neurovirulence protein, ICP34.5, binds to protein phosphatase 1α and causes it to dephosphorylate eIF2α, thereby negating the activity of PKR[104,105]. A neuroattenuated HSV-1 mutant lacking ICP34.5 exhibits wild-type replication and virulence in mice genetically lacking *pkr*[103], proving that the ICP34.5 gene product mediates neurovirulence by antagonizing PKR-dependent functions.

In addition to regulating host translation during viral infection, the PKR signaling pathway also regulates the autophagic degradation of host proteins[18]. As mentioned above, molecules in the yeast eIF2α kinase signaling pathway (e.g. the eIF2α kinase, Gcn2, the eIF2α Ser51 residue, and the transcriptional transactivator, Gcn4) are required for nitrogen starvation-induced autophagy. Interestingly, the autophagy defect of *gcn2* null yeast can be rescued by mammalian *pkr* transformation[18]. Direct evidence that viruses can induce PKR-dependent autophagy has been provided by studies done with herpes simplex virus type 1 (HSV-1) infection in genetically engineered MEFs[18]. A herpes simplex virus (HSV-1) mutant virus lacking the ICP34.5 inhibitor of PKR signaling (termed HSV-1Δ 34.5), but not wild-

type HSV-1, is able to induce the autophagic breakdown of long-lived cellular proteins in wild-type MEFs. However, HSV-1Δ34.5 infection is not able to induce the autophagic breakdown of long-lived cellular proteins in MEFs lacking *pkr* or with a nonphosphorylatable mutation in Ser-51 of eIF2α. These findings indicate the PKR-dependent signaling events regulate the autophagic breakdown of host proteins during viral infection and that this function of PKR is antagonized by the HSV-1 ICP34.5 neurovirulence gene product. As discussed in section 3.1, the breakdown of cellular proteins may help protect host cells against the effects of "pseudostarvation" induced by viral infection.

More recent studies indicate that PKR-dependent signaling also regulates the breakdown of viral components during HSV-1 infection[99]. Ultrastructural analyses of wild-type and *pkr*-deficient MEFs and sympathetic neurons infected with wild-type and HSV-1Δ34.5 demonstrate that HSV-1 is degraded in autophagosomes by a *pkr*-dependent process. In wild-type cells infected with wild-type HSV-1, the majority of intracytoplasmic virions are either randomly dispersed in the cytoplasm or are contained within "viral vacuoles," a structure thought to represent an important intermediate in the egress of HSV-1 from the nucleus out of the cell[106]. In contrast, in wild-type cells infected with HSV-1Δ34.5, most cytoplasmic virions are localized within autophagosomes that contain a mix of different cytoplasmic constituents (Figure 4A). *Pkr*-deficient MEFs or *pkr*-deficient neurons infected with HSV-1Δ34.5 have very few autophagosomes, and appear similar to wild-type HSV-1-infected wild-type cells, with randomly dispersed intracytoplasmic virions and numerous viral vacuoles. Together, these observations demonstrate that HSV-1 is degraded by autophagy, that HSV-1 ICP34.5 antagonizes the cellular autophagic degradation of HSV-1, and that this process requires PKR.

Recent biochemical analyses confirm that PKR signaling and HSV-1 ICP34.5 regulate viral protein degradation[99]. HSV-1 protein degradation is significantly accelerated in wild-type MEFs infected with HSV-1Δ34.5 as compared to wild-type MEFs infected with wild-type HSV-1, indicating that ICP34.5 delays viral protein degradation. However, in autophagy-deficient *pkr*^{-/-}MEFs or eIF2α S51A mutant MEFs, the rate of HSV-1 protein degradation is similar in HSV-1- and HSV-1Δ34.5-infected cells, indicating that HSV-1 protein degradation is positively regulated by the PKR signaling pathway.
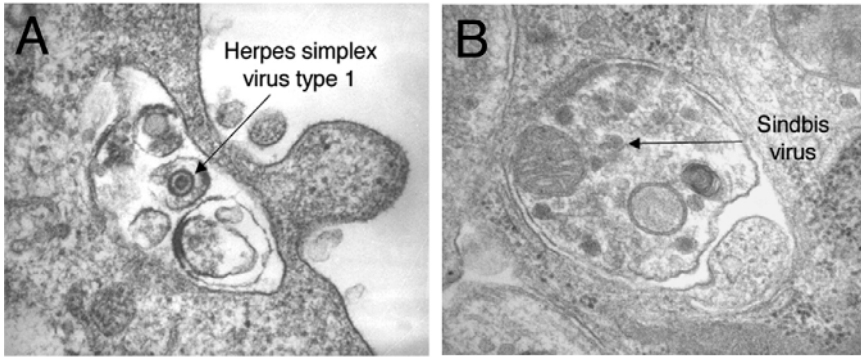
Figure 4. Electron micrographs demonstrating autophagic degradation of herpes simplex virus type 1 (A) and Sindbis virus (B) in neurons. The HSV-1 strain is a mutant lacking the virus-encoded PKR inhibitor, ICP34.5. Similar structures are not observed in neurons infected with wild-type HSV-1 (containing ICP34.5), or in HSV-1-infected or Sindbis virus-infected *pkr* neurons. See text for details.
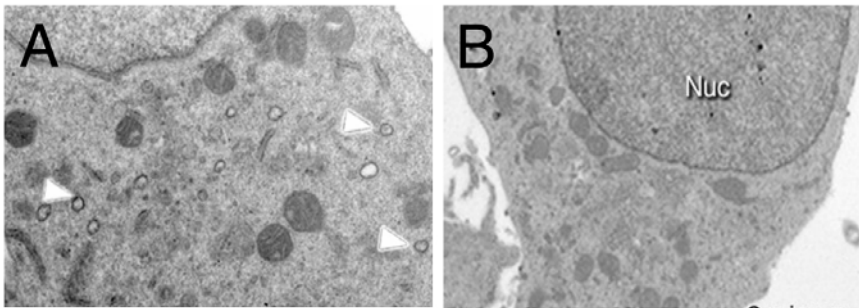

Figure 5. Electron micrographs demonstrating murine hepatitis virus replication complexes associated with double-membrane vesicles (white arrowheads) in wild-type ES cells (A) but not in *atg* ES cells (B). Adapted with permission from[51]. See text for details.

Thus, the eIF2α kinase-dependent autophagy signaling pathway not only regulates the degradation of long-lived cellular proteins but also regulates the degradation of viral proteins. Accordingly, it seems logical to speculate that PKR-dependent autophagic degradation of viruses inhibits viral replication and is an antiviral defense mechanism. However, the relative contributions of the effects of PKR on viral protein synthesis and the effects of PKR on viral protein degradation in the regulation of the HSV-1 replication have not yet been assessed. For this purpose, it will be necessary to selectively inhibit the autophagic protein degradation machinery and/or have HSV-1 mutant viruses that selectively block specific downstream

functions regulated by PKR. It will also be important to determine whether PKR-dependent autophagy degrades and inhibits the replication of viruses other than HSV-1. Preliminary observations indicate that PKR-dependent autophagy does lead to the degradation of another neurotropic virus, the enveloped, positive-strand RNA virus in the alphavirus genus, Sindbis virus[107] (Figure 4B).

4.2 Mammalian autophagy genes play a role in host antiviral defense

The role of PKR in antiviral innate immunity is well established, but PKR regulates many different cellular processes, and it is not yet known exactly what role autophagy induction plays in the antiviral effects of PKR. However, the concept that autophagy is important in innate immunity is more directly supported by studies involving components of the mammalian and plant autophagic machinery.

The first identified mammalian autophagy gene product, Beclin 1, was isolated in a yeast two-hybrid screen, in the context of studies of the mechanism by which the antiapoptotic protein, Bcl-2, protects mice against lethal Sindbis virus encephalitis[37]. Similar to the neuroprotective effects of Bcl-2[108], enforced neuronal expression of wild-type Beclin 1 in a recombinant chimeric Sindbis virus vector reduces Sindbis virus replication, reduces Sindbis virus-induced apoptosis, and protects mice against lethal Sindbis virus encephalitis[37]. Mutations in the Bcl-2-binding domain of Beclin 1 and mutations in other regions of Beclin 1 that block its autophagy function also block its protective effects during Sindbis virus infection[37,62]. Thus, it appears that both the interaction with Bcl-2 and the autophagy function may be required for the antiviral effects of Beclin 1. However, further studies are required to define the precise mechanism of how Beclin 1 inhibits viral replication and virus-induced apoptosis and to identify the precise role of Bcl-2-Beclin 1 interactions in these processes.

Preliminary studies with *beclin 1* null ES cells and *atg5* null MEFs indicate a role for these two endogenous autophagy genes in innate immunity against Sindbis virus infection. Sindbis virus replicates to higher titers and results in accelerated death in *beclin 1* null ES cells as compared to wild-type control ES cells and in *atg5* null MEFs as compared to wild-type control MEFs[38]. In the case of Sindbis virus infection, it is not known whether the acceleration of virus-induced death in *beclin 1* null or *atg5* null cells is a result of increased viral replication or of independent effects of *atg* gene deficiency on cell death. However, studies comparing HSV-1 infection in wild-type and *beclin 1*[-/-]ES cells suggest that *beclin 1* can protect against

virus-induced cell death in the absence of inhibitory effects on viral replication[38].

Together, the studies of Sindbis virus infection in neurons overexpressing *beclin 1* or in cultured cells lacking *beclin 1* or *atg5* demonstrate a role for mammalian autophagy genes in both restricting viral replication and in protection against virus-induced cell death. It will be important to examine whether other autophagy genes have a similar antiviral function and to examine whether autophagy genes also protect against other types of virus infections. The mechanisms by which autophagy genes exert protective effects in Sindbis virus infection are not yet known. Presumably, the autophagic breakdown of viral components leads to decrease viral yields. However, it is also possible that autophagy leads to the breakdown of cellular components required for viral replication. As noted above, the protective effects against cell death may be secondary to inhibitory effects on viral replication. Alternatively, the protective effects may relate to the nutrient recycling functions or "damage control" functions of autophagy, or in the case of *beclin 1*, to interactions between autophagy proteins and anti-apoptotic pathways. It is also possible that autophagy may protect against cell death by degrading specific viral proteins (e.g. the Sindbis virus E1 and E2 envelope glycoprotein's[109]) that are involved in triggering the apoptotic pathway.

The protective effects of *beclin 1* and *atg5* on virus-induced cell death are consistent with the "pro-survival" effects of autophagy during nutrient starvation and other forms of environmental stress. It is not yet clear how to reconcile these pro-survival effects with the view that autophagy represents an alternative form of non-apoptotic programmed cell death (reviewed in [2,25,110]). While the primary basis for this view has been morphologic correlations between the presence of autophagic vacuoles and dying cells (reviewed in [2,111]), recent genetic experiments establish a more direct role for autophagy genes in certain types of programmed cell death. Mammalian *Atg7* and *beclin 1* RNAi blocks cell death in fibroblast and macrophage cell lines treated with the caspase inhibitor, zVAD[112], and *atg 6*, *7*, and *12* RNAi blocks salivary gland destruction during *Drosophila* development[113]. Thus, the relationship between autophagy, cell survival, and cell death is quite complex and likely varies according to the cell type and the specific physiological or pathophysiological setting. It remains to be determined whether autophagy genes primarily play a protective role in preventing cell death during virus infection, or whether they also participate directly in cell death that is induced by certain viruses.

4.3   Plant autophagy genes play a role in the plant innate immune response

The plant homologue of *beclin 1* also functions in antiviral host defense in plants. Similar to mammalian *beclin 1*, plant *BECLIN 1* restricts viral replication; tobacco mosaic virus replication is increased in *BECLIN 1*-silenced tobacco plants as compared to vector-treated control plants[40]. However, in contrast to mammalian *beclin 1, which* prevents the death of Sindbis virus- and HSV-1-infected cells, plant *BECLIN 1* plays an interesting role in preventing the death of uninfected cells[40].

In plants, the innate immune response during virus infection is characterized by a hypersensitive response which is a programmed cell death response that occurs around the infected areas (reviewed in [114-116]). This hypersensitive response limits virus spread and confers pathogen resistance. It is triggered by a pathogen-encoded avirulence protein, which is recognized by a specific plant cognate resistance protein, termed an R protein. In plants lacking R proteins, there is uncontrolled virus spread and pathogen sensitivity.

A tobacco mosaic virus infection model has recently been used to study the role of autophagy genes in plant innate immunity. During tobacco mosaic virus infection, the hypersensitive response is triggered by tobacco mosaic virus protein, TMV p50, which is the helicase domain of the viral replicase[117]. TMV p50 is recognized by an R protein (called the N protein) of *N. benthamiana*, which is composed of a Toll/interleukin 1 receptor domain, a nucleotide binding domain, and a leucine-rich repeat domain[118]. Therefore, in tobacco plants containing the N protein ($N^{+/+}$), there is local cell death in cells that are either infected with TMV or that express the TMV p50 protein but no systemic illness is observed.

BECLIN 1 silencing in $N^{+/+}$ tobacco plants reveals a striking role for this autophagy gene in limiting the spread of cell death during the hypersensitive response[40]. During TMV infection of *BECLIN 1*-silenced $N^{+/+}$ plants, cell death begins as discreet and defined foci but continues to spread beyond the site of TMV infection until there is death of the entire inoculated leaf and other uninoculated leaves. A similar spreading cell death phenotype is seen with local expression of the TMV p50 protein, suggesting that the cell death occurs in response to a specific signal triggered by the pathogen encoded avirulence protein, and is not due to increased TMV replication or altered virus movement. In addition, in plants that lack the *N* gene, *BECLIN 1* silencing does not lead to cell death after TMV infection. Moreover, *BECLIN 1* silencing also results in spreading cell death during the hypersensitive response triggered by bacterially-encoded pathogen avirulence proteins. Together, these observations demonstrate that the

spreading cell death phenotype in *BECLIN 1*-silenced plants is mediated by *R* gene-mediated innate immune responses and that *BECLIN 1* is an important negative regulator of cell death during the plant innate immune response.

A similar role for other autophagy genes in limiting the spread of programmed cell death during TMV infection has also been observed. As discussed in Section 2.3.3, PI3-K/Vps34 is a protein that physically interacts with Atg6/Beclin 1 in yeast and mammals and is essential for proper autophagosome formation. Interestingly, silencing of the plant class III *PI-3K/VPS34* in $N^{+/+}$ plants results in a spreading cell death phenotype during TMV infection that is similar to that observed with *BECLIN 1* silencing. As discussed in Section 2.3.3, yeast and mammalian *ATG3* and *ATG7* are essential for conjugation reactions needed for autophagosome formation, and silencing of the plant homologues of these genes also results in a spreading cell death phenotype after TMV infection. Thus, multiple different autophagy genes, including those that act in the vesicle nucleation stage (e.g. *BECLIN 1*, Class III *PI3-K/VPS34*) and those that act in the vesicle expansion stage (e.g. *ATG3* and *ATG7*) are necessary to prevent the spread of cell death during the plant innate immune response.

While plant autophagy genes protect uninfected cells against death whereas mammalian autophagy genes protect infected cells against death during virus infection, the plant data nonetheless further support a "pro-survival", rather than "pro-death" function of autophagy genes during viral infections. At present, it is not yet clear how autophagy genes protect uninfected cells against death during the plant hypersensitive response. One possibility is that the absence of autophagy genes in uninfected cells somehow modifies the *R* gene-mediated signal transduction pathway in a way that instructs uninfected cells to die. An alternative, perhaps more likely, possibility is that the absence of autophagy genes in uninfected cells renders them more susceptible to pro-death signals emitted from infected cells. Regardless of the mechanism, this newly defined role for autophagy genes in preventing the spread of cell death during plant innate immunity has significant implications for understanding the role of autophagy in systemic protection against viral infections. An important question is whether autophagy genes play a similar role during animal virus infections.

5.   EVASION OF AUTOPHAGY BY VIRUSES

The evolutionarily conserved function of both mammalian and plant autophagy genes in restricting viral replication and/or protection against cell death suggests an essential role for autophagy in innate immunity. This

concept is further supported by recent observations indicating that the herpes simplex virus neurovirulence protein, ICP34.5, possesses multiple mechanisms to disarm host autophagy. It can both antagonize the PKR signaling pathway required for autophagy induction and inhibit the function of one component of the autophagic machinery, Beclin 1.

As discussed in Section 4.1, ICP34.5 blocks PKR-dependent, eIF2α Ser-51-dependent autophagic degradation of cellular and viral components in HSV-1-infected MEFs and neurons[99]. One predicted mechanism by which ICP34.5 blocks PKR-dependent autophagy is through its known ability to promote the dephosphorylation of eIF2α via interactions with PP1α[105]. However, new evidence also suggests a second potential mechanism. Roizman et al. isolated the mammalian autophagy protein, Beclin 1, in a yeast two-hybrid screen using ICP34.5 as a bait[119]. Subsequent studies have shown that ICP34.5 directly interacts with Beclin 1 in mammalian cells and inhibits the ability of Beclin 1 to rescue autophagy in autophagy-defective *atg6* null yeast and in autophagy-defective human MCF7 breast carcinoma cells[38]. Since ICP34.5 binds to Beclin 1 via a domain that is distinct from its PP1α-binding domain, it should be possible to construct HSV-1 viruses containing mutations in ICP34.5 that help differentiate between the role of PP1α-binding (and eIF2α phosphorylation) and the role of Beclin 1-binding in HSV-1 ICP34.5-mediated neurovirulence.

Besides HSV-1 ICP34.5, there are numerous other viral proteins or RNAs that suppress PKR signaling through a variety of different mechanism (reviewed in [100,101]). For example, vaccinia virus E3, influenza virus NS1, HSV-1 Us11, reovirus σ3, and rotavirus NSP3 encode double-stranded (ds) RNA-binding proteins that prevent PKR activation. Adenovirus VAI RNAs and HIV Tar RNAs bind to dsRNA substrates and inhibit PKR. Hepatitis C virus NS5A protein inhibits the dimerization of PKR, and influenza virus recruits a cellular protein, P58[IPK], that directly interacts with PKR and inhibits its dimerization. The vaccinia virus K3L, hepatitis C virus E2, and HIV Tat proteins act as pseudosubstrates of PKR. As-of-yet, the role of these other viral RNAs and proteins in autophagy inhibition has not been investigated. However, given the evolutionarily conserved requirement for an intact eIF2α kinase signaling pathway in autophagy induction, the prediction is that these other viral inhibitors of PKR, like HSV1 ICP34.5, also function as antagonists of host autophagy. Further studies are needed to test this prediction and to study the role of this predicted antagonism of host autophagy in the pathogenesis of diseases caused by these other important viral pathogens that encode putative autophagy inhibitors.

Not only may other viruses antagonize the autophagy function of PKR, but other viral gene products may also antagonize the autophagy function of specific mammalian *atg* genes. Beclin 1 was originally isolated in a yeast

two-hybrid screen with the cellular anti-anti-apoptotic protein, Bcl-2[37]. Subsequently, Beclin 1 has also been shown to interact with viral Bcl-2-like proteins encoded by different gammaherpesviruses, including EBV-encoded BHRF1, KSHV-encoded v-Bcl-2, and murine γHV68-encoded M11[39]. Like ICP34.5, these viral proteins can also inhibit the autophagy function of Beclin 1 in yeast and mammalian assays. In addition, preliminary evidence indicates that other KSHV-encoded proteins may interact with other specific Atg proteins[65].

An as-of-yet explored area is whether viruses also inhibit autophagy by activating autophagy inhibitory signaling pathways. As noted in Section 2.2 and 3.2, the Class I PI-3K/Akt signaling pathway negatively regulates autophagy in both mammalian cells and *C. elegans*[19,20,120] and several different viruses activate this pathway. Certain oncogenic retroviruses encode the catalytic subunit of PI3-K and Akt (reviewed in [22]). In addition, the EBV latent membrane proteins, LMP1 and 2A, the hepatitis B virus protein, HBx, the Kaposi's sarcoma virus protein, K1, and the hepatitis C virus protein, NS5A all activate the PI3-K/Akt signaling pathway[72-77]. Presumably, such activation plays a role in autophagy inhibition, although this has not yet been formally tested.

While further studies are required to more precisely define the interactions between viral gene products and autophagy regulatory signals and autophagy proteins, there is, however, accumulating evidence that viruses do target multiple different steps of the host autophagy pathway. This observation strongly suggests an evolutionary advantage for viruses to inhibit host autophagy, and by extrapolation, a beneficial role for host autophagy in defense against viral infections.

6.   SUBVERSION OF AUTOPHAGY BY VIRUSES

Some viruses appear to have even further outsmarted host autophagy. Rather than merely devising strategies to block host autophagy, certain positive-strand RNA viruses have figured out ways to "co-opt" elements of the autophagy pathway to promote their own replication. This subject has been recently reviewed in detail elsewhere[13] and will therefore only be briefly summarized in this section.

As early as 1965, electron microscopic studies of poliovirus-infected cells demonstrated the presence of large numbers of membranous vesicles that were postulated to develop by an autophagic-like mechanism[121]. More recently, work by Kirkegaard et al, has extended these findings to further show that poliovirus replication complexes are associated with double-membrane vesicles that resemble autophagosomes, in that they (1) have

similar double membrane-bound morphology; (2) have low buoyant density; and (3) label with the autophagosome marker, GFP-LC3, and the lysosome marker, LAMP1[13,122]. Unlike classical autophagosomes, these autophagic-like vesicles do not appear to have a destructive role or mature into degradative compartments. In support of this, treatment with autophagy inducers, rapamycin or tamoxifen, both increase, rather than decrease poliovirus growth[13]. Furthermore, these double-membrane vesicles are also different from classical autophagosomes in that they contain Sec13 and Sec31, components of the anterograde transport system that bud from the ER[123].

Therefore, it is possible that poliovirus-induced vesicles arise from an alternate source rather than the PAS, but still share some of the same characteristics of classical autophagosomes (e.g. GFP-LC3 and labeling, augmentation with rapamycin treatment). Similar to the replication vacuoles that are associated with certain intracellular bacterial pathogens (e.g. *Legionella pneumophila*)[124], the poliovirus-induced double-membrane vesicles likely originate from the ER[123]. Furthermore, these poliovirus-induced vesicles seem to have an alternate function than autophagosomes (i.e. they are pro-replicative, rather than degradative compartments). These observations suggest that poliovirus may promote its own replication by inducing dynamic membrane rearrangements that share in common certain features of the autophagy pathway (e.g. formation of sequestering double-membrane vesicles, presence of overlapping markers) but avoid, other unwanted features of the autophagy pathway (e.g. maturation into degradation compartments). Of note, specific poliovirus proteins, including 2BC and 3A, have been identified that are sufficient for the induction of these "autophagic-like" double-membrane bound vesicles[122]. However, the mechanisms by which these proteins induce the formation of such vesicles are not yet known.

A recent study with the coronavirus, murine hepatitis virus, has provided more direct evidence that components of the autophagic machinery can be utilized for RNA virus replication[51]. MHV replication complexes localize to double-membrane vesicles (that are also thought to arise from the ER) (Figure 5A) and they co-localize with certain autophagy proteins, including LC3 and Atg12. In MHV-infected *atg5*^{-/-}ES cells, double-membrane vesicles are not detected (Figure 5B), and viral replication is dramatically reduced. These observations provide the first genetic demonstration that proteins necessary for autophagic vacuole formation are also required for maximal levels of viral replication. Thus, MHV, and potentially other viruses that replicate in association with double-membrane vesicles (e.g. poliovirus, equine arterivirus), utilize components of cellular autophagy to foster their own growth. Presumably, the Atg protein conjugation system (involving

Atg5) that plays a role in autophagic vesicle expansion and completion also plays a role in the formation of double-membrane vesicles involved in viral replication. It is not yet known whether the entire autophagic machinery or only selective components of the autophagic machinery are used for the formation of double-membrane vesicles that are associated with viral replication complexes.

These observations with poliovirus and MHV represent two examples of how viruses can "subvert" elements of the host autophagy pathway to promote their own intracellular growth. In these infections, RNA replication complexes are observed in association with "autophagic-like" double-membrane vesicles but not in association with degradative autophagosomes. It is not clear whether this represents fundamental differences in the host pathways leading to the formation of "autophagic-like" double-membrane vesicles and classical degradative autophagosomes, the diversion of the autophagic machinery towards the formation of "autophagic-like" double-membrane vesicles from the formation of classical degradative autophagosomes, or specific viral mechanisms to antagonize the maturation of "autophagic-like" double-membrane vesicles into mature degradative autophagosomes. However, interestingly, MHV infection does lead to the induction of *atg-5*-dependent long-lived cellular protein degradation, ruling out the hypothesis that the autophagic machinery is entirely diverted to form membranes required for viral replication complexes. Perhaps MHV possesses as-of-yet defined mechanisms to shield its replication complexes from autophagic degradation.

7.   CONCLUSIONS

Although research in this area is still in a stage of infancy, it seems likely that the lysosomal degradation pathway of autophagy plays an evolutionarily conserved role in antiviral immunity. The interferon-inducible, antiviral PKR signaling pathway positively regulates autophagy, and both mammalian and plant autophagy genes restrict viral replication and protect against virus-induced cell death. Given this role of autophagy in innate immunity, it is not surprising that viruses have evolved numerous strategies to inhibit host autophagy. Different viral gene products can either modulate autophagy regulatory signals or directly interact with components of the autophagy execution machinery. Moreover, certain RNA viruses have managed to "co-apt" the autophagy pathway, selectively utilizing certain components of the dynamic membrane rearrangement system to promote their own replication inside the host cytoplasm.

In addition to this newly emerging role of autophagy in innate immunity, autophagy plays an important role in many other fundamental biological processes, including tissue homeostasis, differentiation and development, cell growth control, and the prevention of aging. Accordingly, the inhibition of host autophagy by viral gene products has important implications not only for understanding mechanisms of immune evasion, but also for understanding novel mechanisms of viral pathogenesis. It will be interesting to dissect the role of viral inhibition of autophagy in acute, persistent, and latent viral replication, as well as in the pathogenesis of cancer and other medical diseases.

ACKNOWLEDGEMENTS

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