AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Virology®

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A Small Viral PPxY Peptide Motif To Control Antiviral Autophagy

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ABSTRACT Autophagy is an essential metabolic program that is also used for clearing intracellular pathogens. This mechanism, also termed selective autophagy, is well characterized for invasive bacteria but remains poorly documented for viral infections. Here we highlight our recent work showing that endosomolytic adenoviruses trigger autophagy when entering cells. Our study revealed how adenoviruses exploit a capsid-associated small PPxY peptide motif to manipulate the autophagic machinery to prevent autophagic degradation and to promote endosomal escape and nuclear trafficking.

KEYWORDS adenoviruses, autophagy, innate immunity, virus entry

Accepted manuscript posted online 28 June 2017

Citation Montespan C, Wiethoff CM, Wodrich H. 2017. A small viral PPxY peptide motif to control antiviral autophagy. J Virol 91:e00581-17. https://doi.org/10.1128/JVI.00581-17.

Editor Billy Tsai, University of Michigan Medical School

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To initiate productive infection, most DNA viruses deliver their genomes into the nucleus to activate viral gene expression and to begin the replication cycle. To reach the nucleus, viruses first have to overcome several barriers, including the limiting cellular membrane, movement in the crowded cytoplasm, and transport past the nuclear envelope. Once in the cytosol, viruses often access motor proteins conducive for net retrograde transport toward the nuclear periphery and find a way to efficiently translocate their genomes into the nucleus.

In this respect, adenovirus (AdV), a nonenveloped double-stranded DNA virus, is a prime example of rapid and efficient viral nuclear genome delivery. After cell attachment, AdV virions are rapidly endocytosed, which prompts structural changes in the 90-nm capsid, allowing release of the internal membrane lytic capsid protein VI (PVI). This protein contains an N-terminal amphipathic helix responsible for endosomal membrane fragmentation (1, 2). Endosome fragmentation via PVI allows the virus to access the cytosol and to separate from the membrane remnants in a process called endosomal escape. Escaped virions engage in microtubule-dependent transport to reach the nucleus. Employing fluorescently labeled AdV and mCherry-tagged galectin-3 (Gal3) to mark the membrane rupture event in living cells, we were recently able to show that endosomal membrane fragmentation by PVI and endosomal escape of the virions are two distinct steps that can be separated in time and space (3, 4). Crossing the endosomal membrane via PVI release is a key step during the entry of AdV, as it was shown that a thermosensitive AdV mutant (AdV-TS1), which displays hyperstable capsids when produced at the nonpermissive temperature, fails to release PVI (5). As a consequence, no membrane penetration can occur and the respective virions are degraded through the endocytic pathway (5). We showed previously that, next to the membrane lytic amphipathic helix, PVI contains a conserved PPxY motif (where x can be any amino acid), which is exposed upon membrane lysis and plays a crucial role during AdV entry. PPxY motifs interact with WW domains in host proteins, including ubiquitin ligases of the Nedd4 family of Hect-E3-ubiquitin ligases (6). We showed that wild-type (WT) PVI has a binding preference for Nedd4.1 and Nedd4.2 in the Nedd4 ubiquitin ligase family and mutating the motif to PGAA (M1) abrogated the ligase interaction (6). Incorporating the mutation into virions (AdV-M1) or depleting individual

Nedd4 ligases from cells suggested that the recruitment of Nedd4.2 specifically supports AdV entry (6). Interestingly, an absence of Nedd4.2 recruitment by either means did not impair PVI release or membrane rupture efficiency but had an impact on endosomal escape and subsequent viral trafficking toward the microtubule-organizing center (MTOC), resulting in up to 20-fold reduced infectivity (6). In our most recent work, we found that AdV-induced membrane damage triggered an autophagic cellular response, and we showed that the virus uses the conserved PPxY motif in PVI to escape autophagic degradation (7). Moreover, our data suggest that AdV requires parts of the autophagic response for endosomal escape and for accelerated nuclear transport for efficient genome delivery (7).

SELECTIVE AUTOPHAGY TARGETS INVADING PATHOGENS

Due to their size, invading pathogens often damage membranes to enter the host cell. Cells have put surveillance systems in place to monitor membrane integrity, which incidentally also target those pathogens. In this system, cytosolic galectins act as sensing molecules capable of detecting sudden cytosolic exposure of extracellular or intralumenal glycans upon membrane damage, due to their carbohydrate-binding domains (8, 9). Galectin recruitment to membranes with damage caused by invading pathogens permits the cell to respond rapidly by mounting an autophagic response to clear the damaged membrane and its potential pathogen contents (8, 9).

Autophagy is an evolutionarily conserved cytosolic degradation pathway that maintains cellular homeostasis by regulating the turnover of cytosol, accumulating proteins or protein assemblies, and (dysfunctional) organelles by forming double-membrane vesicles (i.e., autophagosomes) around the cargo (10). Cargo-containing autophagosomes then migrate to the perinuclear region where they fuse with lysosomes to form autolysosomes, resulting in content degradation (11), which supplies the cell with nutrients during periods of starvation or other types of stress (12). The initiation of autophagy begins with the isolation of a piece of membrane called a phagophore. The onset of the phagophore and its growth into an autophagosome are highly regulated and controlled by ATG (autophagy-related gene) proteins. A key member of this family is LC3 (microtubule-associated protein 1A/1B light chain; ATG8 in yeast). Upon autophagy induction, cytosolic LC3 becomes conjugated with phosphatidylethanolamine through an ubiquitinylation-like E1-E2-E3-conjugation system. Conjugated LC3 is incorporated into the growing autophagosomal membrane (13), a process that is thought to be essential for membrane elongation and that is implicated in autophagosome closure and trafficking. LC3 is also essential for cargo selection, because it provides a physical link between autophagosomal membranes and specific cargo adapters tagging the cargo (14).

Autophagy is an important part of cellular innate immunity, targeting and degrading incoming pathogens such as bacteria or viruses (15). Initial recognition of invasive bacteria for antimicrobial autophagy occurs either through bacterium-associated ubiquitin moieties or through galectins, which mark pathogen-mediated membrane damage (9, 14). Several galectins were found to label bacterium-induced membrane damage, but so far only Gal8 has been shown to restrict bacterial replication (9). Gal8 specifically binds the adapter protein NDP52, which permits recruitment of the autophagic machinery toward the bacterium-containing vacuole (9). In addition to Gal8, NDP52 targets bacterium-associated ubiquitin moieties, which for other autophagy receptors (such as p62, optineurin, and TAX1BP1) seem to be the exclusive approach for cargo recognition (14). In addition to the recognition domains for modified cargo, all receptors include LC3-interacting region (LIR) domains with which they bind LC3, linking the cargo into the forming autophagosome.

ENDOSOMOLYTIC ADENOVIRUS TRIGGERS SELECTIVE AUTOPHAGY

Not surprisingly, pathogens have evolved mechanisms to control antimicrobial autophagy. Autophagic degradation threatens the invading pathogen by limiting its replication; in addition, pathogen degradation via autophagy leads to the production

of microbial peptides, which are presented through major histocompatibility complex (MHC) molecules to initiate an adaptive immune response (16).

We initially set out to ask whether virus-induced membrane damage would provoke responses in cells similar to those shown for invasive bacteria. We used AdV for this and showed that endosome rupture upon AdV infection is detected by Gal3, Gal8, and Gal9 and triggers the formation of autophagosomal structures at the site of membrane damage (7). Interestingly, our results showed that, despite triggering autophagy, AdV infectivity was not sensitive to autophagy inhibition. The case was different when we used a mutant virus lacking an essential PPxY motif in capsid protein PVI (AdV-M1), which efficiently lysed the endosomal membrane but was defective in endosomal escape (7). By inhibiting autophagy, we were able to restore the infectivity of the M1 mutant to close to wild-type levels, showing an important role for the PPxY motif in overcoming restriction by autophagy for the wild-type virus. M1 infectivity was also enhanced when we depleted Gal8 (but not Gal3 or Gal9), showing that Gal8 was responsible for linking AdV-damaged membranes containing M1 viruses to the autophagic machinery, thereby degrading all viruses unable to escape in time. Interestingly, depleting Gal8 rescued the M1 virus by preventing its recognition as an autophagy substrate but it did not prevent autophagy induction itself. Thus, it seems that AdV-caused membrane damage generates a currently unknown signal that triggers autophagosome formation independent of Gal8 (7).

Another striking difference from bacteria was that none of the three classic autophagy receptors (p62, NDP52, and OPTN) was essential for the degradation of AdV when the escape-defective mutant was used. This was in spite of finding Gal8, ubiquitin, NDP52, and p62 at the site of virus membrane penetration. This observation may indicate the existence of alternative or additive pathways for recruitment of the autophagic machinery to membrane-penetrating viruses. Possible candidates include the autophagy receptor TAX1BP1, which was recently shown to play a role in the selective clearance of *Salmonella enterica* serotype Typhimurium by autophagy (17), or tripartite motif (TRIM) proteins. TRIM family proteins are known to regulate several biological processes, including autophagy (18). Recent studies have shown that galectins and TRIM proteins can cooperate to trigger selective autophagy upon endomembrane damage, by enhancing the recruitment of factors required for autophagosome formation (e.g., ULK1, beclin-1, and ATG16L [19, 20]). It will be interesting to see whether either TAXBP1 or TRIMs are involved in the antiviral response against AdV.

Our work using the endosomal-escape-defective mutant has shown that AdV uses a virion-encoded factor, PVI, to dissociate from damaged endosomes, which is sufficient to bypass viral degradation via autophagy (7). Other membrane-penetrating viruses appear to use cellular factors to avoid selective autophagy; this is the case for picornaviruses, which manipulate the lipid-modifying enzyme PLA2G16 to facilitate viral genome dislocation from Gal8-positive ruptured membranes, to avoid degradation through selective autophagy (21). Other viruses were shown to interfere more directly with essential factors of the autophagic machinery, such as enteroviruses, which encode a protease that is able to cleave the adaptor protein p62 to impair its function in selective autophagy and subsequently in host defense signaling (22).

ADENOVIRUS USES A VIRAL PPxY MOTIF TO ESCAPE ANTIVIRAL AUTOPHAGY

While rapid endosomal escape clearly protects AdVs from autophagic degradation, we also showed that AdVs are able to limit autophagy by preventing efficient autophagosome maturation even after they have escaped (7). This viral ability may further reduce autophagy-mediated antigen presentation of viral peptides or delay the autophagic response, leaving more time for escape. Interestingly, this ability of AdVs is lost when the PPxY motif in PVI is mutated. Thus, PVI harbors at least two domains that are crucial for AdV entry, namely, the amphipathic helix, which is required for membrane penetration, and the PPxY motif, which is necessary for rapid endosomal escape and also prevention of autophagosome maturation (7).

PPxY motifs are widely present in the viral proteome and were originally described

as playing an important role during the budding of enveloped viruses (23, 24). We were the first to show that PPxY motifs may also be important during virus entry, at least for nonenveloped viruses such as AdVs (6, 7).

Based on our recent study, we think that Nedd4.2 recruitment through the PPxY of PVI upon AdV entry could serve as a viral strategy to divert the ligase from its physiological role in the regulation of autophagy, which appears to be central to AdV autophagy evasion. Because autophagy also feeds the antigen-presenting pathway, limiting autophagy may constitute the earliest adenoviral countermeasure against immune detection (7). Targeting Nedd4.2 would be a sensible approach, because two recent studies reported that Nedd4.2 is involved in autophagy regulation. Nedd4.2 mediates the autophagic response upon endoplasmic reticulum stress (25), probably by downregulating the kinase ULK1 through ubiquitylation to limit autophagy (26). We showed that Nedd4.2 sequestration upon AdV infection, via the PPxY motif, prevents the efficient formation of autolysosomes. By depleting Nedd4.2 from cells, the ability of the virus to prevent autophagosome maturation was removed, which incidentally highlighted the direct involvement of Nedd4.2 in autophagy control (7). Whether this occurs through ULK1 or other cellular substrates of Nedd4.2 remains to be tested. We favor the idea that, by diverting Nedd4.2, AdV interferes with the elongation process of the autophagosomal membrane to prevent vesicle closure, a step needed for the fusion between autophagosomes and lysosomes.

Another important regulator of autophagy is the closely related ubiquitin ligase Nedd4.1, which regulates beclin-1, the kinase that provides a phosphatidylinositol 3-phosphate (Pi3P) platform to assemble the LC3 conjugation machinery (27, 28). While in our case only depletion of Nedd4.2 showed a biological effect on AdV entry (6), Japanese encephalitis virus was shown to use Nedd4.1 to promote viral replication by suppressing virus-induced autophagy (29). In that context, recruitment of the ligase via a viral PPxY motif was not addressed. Other viral proteins with late domains have recently been shown to recruit WW-domain-containing host proteins known to regulate autophagy. For example, the PPxY motif of the VP40 proteins of Ebola virus and Marburg virus interacts with the WW domain of the chaperone-mediated autophagy protein BAG3 (30), while the PPxY motif present in the M proteins of vesicular stomatitis virus and rabies virus was shown to interact with the Yes-kinase-associated protein (YAP), which was reported to regulate autophagy in response to nutrient deprivation (31, 32). Given the widespread occurrence of viral PPxY motifs, it seems possible that such motifs could constitute a more general viral strategy to interfere with (antiviral) autophagy.

ADENOVIRUS REDIRECTS AUTOPHAGY TO PROMOTE VIRAL TRAFFICKING

Several studies have reported that autophagy can restrict viral infections. For instance, autophagy protects mice against Sindbis virus pathogenesis by clearing viral proteins (33). Similarly, autophagy limits vesicular stomatitis virus replication in a *Drosophila* model (34). In contrast, it was shown that Epstein-Barr virus uses ATG8/LC3-coupled membranes for cytosolic envelopment (35), while blocking autophagic processes decreases the production of infectious progeny virions for dengue virus or influenza virus (36, 37) and affects the maturation of poliovirus (38). These examples show how viruses exploit autophagy, especially in the late phases of infection, to elicit proviral effects.

One of the most striking results of our recent study was the observation that, in the case of wild-type AdV, autophagy promotes the early phase of infection and supports viral trafficking to the MTOC and the nucleus (7). We showed that an absence of functional autophagy (in cells depleted of the LC3-conjugating factor ATG5) prevents efficient dissociation of capsids from ruptured endosomes, as well as their subsequent transport toward the MTOC, and as a consequence slows nuclear genome delivery (7). This observation is supported by a previous study suggesting that autophagosome-endosome fusion facilitates AdV endosomal escape (39). An attractive explanation for our observations would be that the ATG5-ATG12-LC3 conjugation system aids in

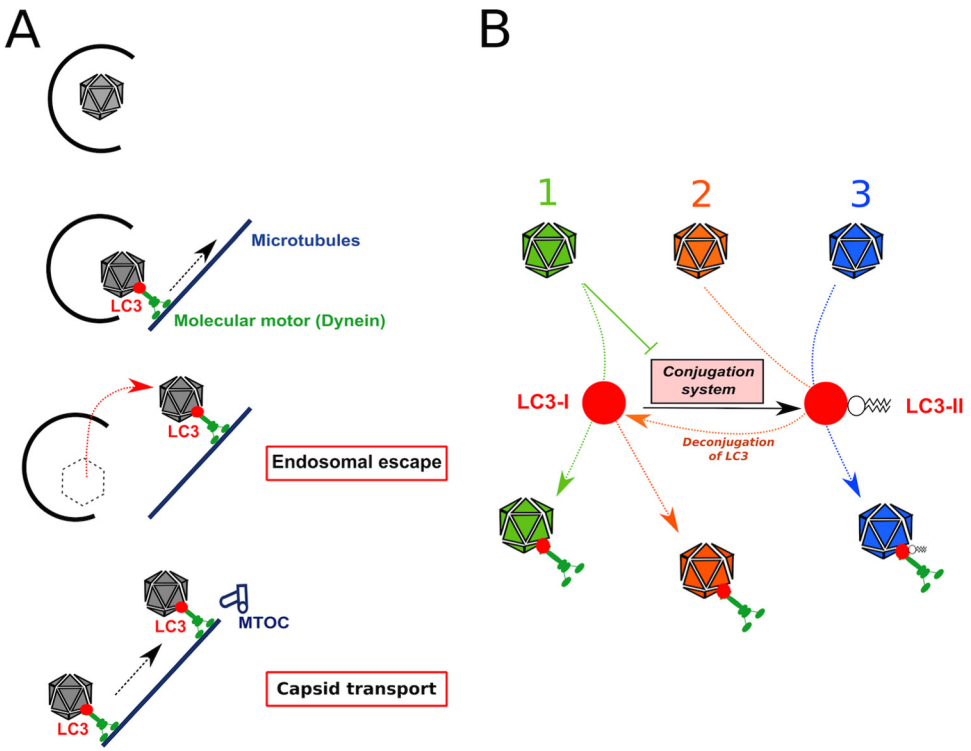

FIG 1 Putative role for the autophagy factor LC3 in AdV endosomal escape and cytosolic capsid transport. (A) AdV ruptures the endosomal membrane, via the lytic capsid PVI, to access the cytosol for motor protein recruitment to engage in subsequent transport toward the MTOC. We hypothesize that capsid-motor interactions are stabilized through the autophagy protein LC3, to accelerate retrograde transport of the capsid. This stabilization may take place at any of three steps, i.e., in the initial motor recruitment (top), during extraction from the endosome (middle), or in facilitating retrograde transport (bottom). (B) LC3 recruitment toward trafficking viruses may occur via recruitment of unconjugated LC3 (1) (LC3-I), virus-mediated deconjugation of already conjugated LC3 (2) (LC3-II to LC3-I), or recruitment of conjugated LC3 (3) (LC3-II) following autophagy triggered by membrane damage.

molecular motor recruitment toward viral particles entrapped in ruptured endosomes and enhances capsid extraction from the damaged vesicles (Fig. 1A). Our pharmacological inhibition experiments with dynein, which traps AdV in ruptured endosomes (7), and our previous live cell imaging studies (4) support the idea that endosomal escape is a motor-driven process. If parts of the autophagic machinery aid in this process, then Nedd4.2 interactions with PVI may be able to stall the formation of autophagosomes around Gal8-decorated membrane fragments until the virus has managed to escape. More direct involvement in motor recruitment could also be possible. Recently, LC3 has been described as an essential factor during microtubule-dependent trafficking of autophagosomal vesicles toward the MTOC (40). This MTOC-directed movement depends on dynein motor proteins and is enhanced by the interactions between LC3 and the scaffolding protein JIP1 and FYCO1 (41, 42). Interestingly, LC3 binding to the chaperone JIP1 promotes autophagosome retrograde trafficking by preventing JIP1-mediated kinesin-1 activation, a function that would be most welcome to viruses in their quest for net movement toward the nucleus. Since AdV also uses microtubules and dynein-dependent transport to reach the MTOC, we think it is possible that AdV acquires LC3 as part of its own retrograde transport to the nucleus (Fig. 1B). This idea is supported by our *in vivo* imaging analysis showing AdV moving from the cell surface to the perinuclear area in association with LC3 (7). It remains unclear whether the LC3 associated with trafficking viruses is recruited in a conjugated or nonconjugated form (Fig. 1B). Electron microscopic analysis of wild-type AdV provides no indication of membrane-associated transport, suggesting that LC3 associated with cytosolic virus is unconjugated. On the other hand, we showed that depletion of ATG5, which is part of the conjugation machinery, traps wild-type viruses in ruptured endosomes, suggesting

that LC3 conjugation is necessary for endosomal escape (7). Therefore, one alternative possibility is that AdV promotes the deconjugation of LC3, by an unknown mechanism, after the autophagic machinery has been functionally recruited to the site of membrane damage and has started to incorporate LC3 into the growing phagophore (Fig. 1B). Future experiments using nonconjugatable LC3 should provide an answer to this exciting question. Interestingly, two very recent studies have shown that Nedd4 ubiquitin ligases also bind directly to LC3 through a ligase LIR motif (27, 43) and this motif is essential for autophagy regulation, suggesting that Nedd4 recruitment through PVI could also have a much more direct role in LC3 recruitment to the virus.

Like AdV, many other viruses use microtubules and molecular motors to reach the MTOC during the entry process (44). It is an attractive but purely speculative thought that additional viruses could also use a combination of autophagy induction and subsequent inhibition of the process to recruit LC3 (or other parts of the machinery) as a means to stabilize the interactions between viral particles and molecular motors, to have access to a probably evolutionarily conserved efficient retrograde transport pathway, which would make a good viral strategy to distort antiviral autophagy for the advantage of the viruses.

ACKNOWLEDGMENTS

We acknowledge COST action BM1307 Proteostasis and COST action CA15138 Transautophagy for support. H.W. is an INSERM fellow.

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