Human Rhinovirus 2 Induces the Autophagic Pathway and Replicates More Efficiently in Autophagic Cells[✓]

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Picornaviruses rearrange cellular membranes to form cytosolic replication sites. In the case of poliovirus and several other picornaviruses, these membranes are derived from subversion of the cellular autophagy pathway. We also reported observation of autophagosome-like structures during infection by two human rhinoviruses (HRVs), HRV-2 and HRV-14 (W. T. Jackson et al., *PLoS Biol.* 3:e156, 2005). Another group reported that HRV-2 does not induce autophagosomes or respond to changes in cellular autophagy (M. Brabec-Zaruba, U. Berka, D. Blaas, and R. Fuchs, *J. Virol.* 81:10815–10817, 2007). In this study, we tested HRV-2-infected cells for activation of autophagic signaling and changes in virus growth in response to changes in autophagy levels. Our data indicate that HRV-2 induces and subverts the autophagic machinery to promote its own replication.

Autophagy is a pathway of cellular homeostasis in which double-membraned vesicles take up cytoplasmic content and target it for degradation (29). In times of stress, such as starvation, autophagy is upregulated to generate new amino acid pools from degraded cellular contents (15). During infection, autophagy is often deployed as an innate immune response to remove infectious agents from the cytoplasm (18).

The relationship between autophagy and bacterial and viral pathogens varies widely, from inhibiting to promoting pathogen growth (8, 16, 21). For example, autophagy is part of the antiviral defense against the negative-strand RNA virus vesicular stomatitis virus (25). However, autophagy is subverted to maximize replication by influenza A virus, the M2 protein of which inhibits the maturation of autophagosomes into degradative autolysosomes (6). There are some viruses, such as vaccinia virus, which have no apparent relationship to the autophagic pathway (30).

Many positive-sense RNA viruses, including hepatitis C virus, encephalomyocarditis virus, dengue virus, and foot-and-mouth disease virus, subvert parts of the cellular autophagy machinery to promote their own replication (3, 7, 13, 17, 20). Certain picornaviruses, including poliovirus (PV), coxsackievirus, and enterovirus 71 subvert the autophagic pathway to generate double-membraned vesicles, which are thought to serve as substrates for viral genome replication (9, 10, 12, 28). Increased autophagy has a positive effect on PV replication, and PV, human rhinovirus 2 (HRV-2), and HRV-14 induce autophagosome formation, based on a single assay analyzing colocalization of LC3 and the lysosome/endosome marker LAMP1 (10). A subsequent study found that HRV-2 replication neither induced autophagosome formation nor responded to autophagic stimuli and concluded that the virus is indifferent

to the presence of autophagy (1). No alternate source of viral replication membranes was demonstrated or postulated.

HRV-14 is a major-group rhinovirus that uses ICAM-1 as a receptor for cellular entry. HRV-1A and HRV-2 are both minor-group rhinoviruses, which use the low-density-lipoprotein receptor (LDL-R) for cellular entry (22). If HRV-14 induces autophagy and HRV-1A and HRV-2 do not, this would suggest that major-group rhinoviruses induce autophagy, while minor group rhinoviruses may not. In this study, we tested this hypothesis by reanalyzing the autophagic response induced by HRV-2, as well as replication of HRV-2 in response to autophagic stimuli.

Autophagosome formation in HRV-2-infected cells. The primary assay for autophagy activation is modification of the cellular protein LC3 from a cytosolic form to a membrane-associated form. This can be assayed in several ways, but one of the easiest is to visualize the induction of punctate LC3 structures (14). We previously demonstrated that in HRV-2-infected H1-HeLa cells, a punctate green fluorescent protein (GFP)-LC3 signal colocalized with LAMP1 staining, indicating autophagosome formation (10). LAMP1 colocalization was necessary because the high background autophagy in HeLa cells and their clonal derivatives induces LC3 puncta in cells without autophagic stimulation (27). 293T human embryonic kidney cells have a much lower background level of autophagy; therefore, LC3 puncta do not form in these cells until autophagy is induced, and formation of puncta can be used to assay for generation of autophagosomes (14, 27).

We transiently transfected 293T cells with an enhanced GFP (EGFP)-LC3-expressing vector as described previously and, 24 h later, infected cells for 6 h with PV, HRV-1A, or HRV-2 at a multiplicity of infection (MOI) of 50 (23). For controls, we mock infected cells or treated cells for 6 h with either 10 μ M rapamycin or an equivalent volume of dimethyl sulfoxide (DMSO) carrier. Rapamycin, an inhibitor of mTOR, is a well-established stimulator of autophagy (14). Images of these cells are shown in Fig. 1A. In unstimulated cells (with DMSO or mock infected), few puncta are visible, and the GFP signal in

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FIG. 1. Induction of autophagosomes by HRV-2. 293T cells were transfected with a GFP-LC3 expression plasmid. Twenty-four hours later, cells were infected with virus at an MOI of 50, mock infected, or treated with 10 μ M rapamycin (RAPA) or DMSO carrier. After 6 h of infection or treatment, cells were fixed for imaging and analysis. (A) Sample images of GFP-LC3 in cells. Arrows indicate a representative cell scored as punctate. (B) Quantification of cells displaying GFP-LC3 puncta. Three random fields, each containing at least 100 cells, were counted. Results from one representative experiment are shown.

transfected cells is diffuse and cytoplasmic. PV infection or rapamycin treatment induced formation of puncta; arrows indicate a representative cell in each field with visible puncta. HRV-1A infection did not induce formation of puncta, in agreement with our previous work, indicating that formation of puncta is not a general response to picornavirus infection (23). Infection by HRV-2, however, induces formation of puncta similar to that seen during PV infection.

Figure 1B shows the quantification of formation of LC3 puncta. Three fields of at least 100 cells each were counted at a single depth of focus. Cells were scored as punctate or non-punctate, and counts are graphically depicted. Rapamycin induction caused approximately 70% of cells in the population to display visible puncta, while PV and HRV-2 induced about half of the cells to display visible puncta. These numbers are in line with previous data for PV, indicating that HRV-2 and PV score equivalently in this assay (23). Therefore, by assaying forma-

FIG. 2. LC3-II formation in response to HRV-2 infection. 293T cells were infected with PV, HRV-2, or HRV-1A at an MOI of 50. For controls, cells were mock infected or treated with 100 nM bafA for 6 h. At the designated hpi, cell lysates were collected and run on a 13% SDS-PAGE gel, which was probed for LC3 and actin or GAPDH. Unmodified LC3-I and lipid-modified LC3-II are labeled. Quantitation of lipid modification is below each band; intensities were normalized to loading controls, and then the ratio of LC3-II to LC3-I was expressed in arbitrary units.

tion of punctate LC3 structures, we observed that HRV-2 appears to stimulate autophagosome formation.

LC3 modification during HRV-2 infection. LC3 associates with autophagic membranes through conjugation to phosphatidylethanolamine. This modification can be identified by a change in migration on an SDS-PAGE gel, making Western blotting against LC3 a simple assay for monitoring increased flux through the autophagic pathway (14, 27). Bafilomycin A1 (bafA), an inhibitor of the vacuolar H^+ ATPase, prevents lysosomal acidification and thus blocks turnover of modified LC3 by the autolysosome (4). 293T cells were mock infected or treated with 100 nM bafA for 6 h. Parallel cultures were infected with PV, HRV-2, or HRV-1A for 0, 2, 4, or 6 h, all at an MOI of 50. Lysates were collected, run on a 13% SDS-PAGE gel, and blotted for endogenous LC3 and actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading controls. Unmodified LC3-I, which has an apparent molecular mass of approximately 18 kDa, and modified LC3-II, with an apparent molecular mass of 16 kDa, are labeled in Fig. 2. Below each band is the ratio of LC3-II to LC3-I. These data have been normalized to the loading control in each lane and represent the best-established way of monitoring the level of autophagic induction (14, 27).

As expected, background levels of autophagy (i.e., from mock infection) are low in 293T cells, while bafA treatment causes a buildup of LC3-II. PV infection stimulates autophagic induction, as can be seen by the increase in the ratio of LC3-II to LC3-I. HRV-1A does not induce LC3 modification, in agreement with Fig. 1. HRV-2 infection results in increases in LC3-II, which can be seen at 4 and 6 h postinfection (hpi). These data are in agreement with the formation of LC3 puncta seen in Fig. 1, and they provide verification that HRV-2 infection stimulates autophagic induction as seen through LC3 modification.

HRV-2 replication correlates with autophagic stimulation. Although autophagy is induced by infection with HRV-2, it is unclear what role the autophagic machinery plays in replication of the virus. It is possible that autophagy is activated as an

FIG. 3. HRV-2 replication correlates with autophagic activation. (A) H1-HeLa cells were treated with 10 μ M rapamycin for 5 h, then infected with PV or HRV-2 at an MOI of 0.1, and fed with EMEM containing 10 μ M rapamycin or an equivalent volume of DMSO carrier. At 6 hpi, cells were collected and cell-associated virus was measured by plaque assay. (B) H1-HeLa cells were infected with PV, HRV-1A, or HRV-2 at an MOI of 0.1 and fed with EMEM containing 10 mM 3-MA. Virus was collected at 0, 2, 4, and 6 h and assayed as described for panel A. Three replicate infections were assayed in each experiment; asterisks indicate significant differences from infections without 3-MA (Student's *t* test *P* values of <0.05).

innate immune response against HRV-2. However, given our data on PV and the data from other groups about positive-strand RNA viruses and autophagy, we hypothesized that the autophagy machinery was acting to promote HRV-2 replication. Rapamycin stimulates autophagic induction, as seen in Fig. 1, so if our hypothesis is correct, rapamycin would be expected to stimulate HRV-2 replication. 3-Methyladenine (3-MA), an established inhibitor of autophagy, would be expected to inhibit HRV-2 replication.

For these assays, we used H1-HeLa cells, which have higher baseline autophagy levels than 293T and therefore are more amenable to reduction in autophagy (27). Cells were treated with rapamycin as in Fig. 1 and collected at 6 hpi. For 3-MA treatment, cells were infected for 30 min then fed with Eagle's minimal essential medium (EMEM) containing 10 mM 3-MA. We have found that dissolving 3-MA directly into the medium is more effective than dissolving 3-MA in carrier and then diluting it into medium. Cell-associated virus was collected at 0, 2, 4, and 6 hpi and counted by plaque assay. The results of one representative experiment (of three) are shown (Fig. 3).

We found that PV (used as a control) responded to rapamycin

and 3-MA, as expected from our previous work, increasing viral yield in response to stimulation with rapamycin and decreasing in response to 3-MA inhibition of autophagy (10). Also as expected, the yield of HRV-1A was unaffected by drug treatment (23). We found that the HRV-2 yield paralleled that of PV closely, increasing 2- to 3-fold with rapamycin treatment and decreasing by >1 log with 3-MA treatment. The results of one representative experiment are shown (Fig. 3); three replicate infections were assayed for each condition. The differences between treated and untreated cells are significant for PV and HRV-2, as tested by Student's *t* test (*P* < 0.05). We interpret these data to indicate that the HRV-2 yield increases with induction of autophagy and decreases with suppression of autophagy. HRV-2 parallels PV in stimulation of and response to autophagy, indicating that subversion of the autophagic pathway to promote virus replication is a common trait between these two viruses.

Our analysis of HRV-2 found that the virus both induces autophagy and replicates more efficiently under autophagic stimulation. We have considered several possible reasons for the difference between our results and the 2007 data indicating that HRV-2 does not activate autophagy (1). One is the assays used; in the time since the HRV-2 analyses were published, assays for autophagy have become better understood and the field has defined acceptable criteria for analysis of the pathway (14). In the 2007 paper, LAMP2 colocalization with LC3 was used to mark autophagosome formation, although we are not aware of data indicating that LAMP2 is present in immature autophagosomes. LAMP1 is thought to be delivered to immature autophagosomes by endosomes (11, 26). However, LAMP2 is present in autolysosomes, in which LC3 is degraded, so colocalization of these two markers is expected to be rare (5, 24). In addition, the 2007 study used H1-HeLa cells, which have high background autophagy, for visual punctum assays. We have found that visual assays using HeLa derivatives are difficult to interpret. The previous work also infected cells with 100 to 300 50% tissue culture infective dose (TCID₅₀) units, whereas we used MOIs of 50 for Western blotting and immunofluorescence experiments and 0.1 for growth assays. It is difficult to compare PFU and TCID₅₀ units, but it is possible that the amounts of virus differ between the two studies (1). Finally, the concentrations of pharmacological agents, such as 3-MA and tamoxifen, used in the 2007 study, are lower than the concentrations we (and others in the field) have used (2, 19). We have also found that the potency of rapamycin varies greatly between lots and suppliers, and we now use higher concentrations to ensure consistent activation of autophagy (23).

In summary, we have found confirmation of our original result that HRV-2 induces autophagosome formation. In addition, we found that HRV-2 responds to autophagic stimuli. Clearly, subversion of the autophagy pathway is not a universal feature of picornaviruses, since in our hands another rhinovirus serotype, HRV-1A, neither induces autophagy nor responds to autophagic stimuli (23). HRV-2 and HRV-1A are both minor-group viruses which use LDL-R for cellular entry. Therefore, our data indicate that the relationship of a given rhinovirus to autophagy is not predicted by its choice of cellular receptor. Our data highlight the complexity of the relationship between viruses and autophagy.

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