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Coronavirus Replication Complex Formation Utilizes Components of Cellular Autophagy*

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The coronavirus mouse hepatitis virus (MHV) performs RNA replication on double membrane vesicles (DMVs) in the cytoplasm of the host cell. However, the mechanism by which these DMVs form has not been determined. Using genetic, biochemical, and cell imaging approaches, the role of autophagy in DMV formation and MHV replication was investigated. The results demonstrated that replication complexes co-localize with the autophagy proteins, microtubule-associated protein light-chain 3 and Apg12. MHV infection induces autophagy by a mechanism that is resistant to 3-methyladenine inhibition. MHV replication is impaired in autophagy knockout, Apg5−/−, embryonic stem cell lines, but wild-type levels of MHV replication are restored by expression of Apg5 in the Apg5−/− cells. In MHV-infected Apg5−/− cells, DMVs were not detected; rather, the rough endoplasmic reticulum was dramatically swollen. The results of this study suggest that autophagy is required for formation of double membrane-bound MHV replication complexes and that DMV formation significantly enhances the efficiency of replication. Furthermore, the rough endoplasmic reticulum is implicated as the possible source of membranes for replication complexes.

Autophagy is a cellular stress response that functions to recycle proteins and organelles (1, 2). The mechanism of autophagy has been extensively studied in yeast, with more than 15 genes identified that are required for functional autophagy (1, 2). Autophagy has been most widely studied as a response to amino acid starvation; however, the role that autophagy may play in development, disease pathogenesis, and microbial infections is only beginning to be examined. Studies with Sindbis virus and herpes simplex virus-1 have demonstrated that autophagy may be an important defense mechanism against infection with those viruses (3, 4).

Cellular autophagy has been proposed to be a mechanism of replication complex formation for the positive sense RNA viruses, poliovirus, equine arteritis virus, and mouse hepatitis virus (MHV) 5 (5–8). For all of these viruses, replication complexes have been shown to form as double membrane vesicles (DMVs) in the cytoplasm, which is suggestive of an autophagic origin. For poliovirus and MHV, multiple organelle markers have been reported to co-localize or co-fractionate with replication complexes, also consistent with an autophagy-like process (6, 7, 9, 10). In addition, poliovirus and MHV replication complexes have been reported to acquire lysosomal markers over the course of infection similar to the maturation of autophagosomes. These studies have demonstrated that replication complexes formed by these viruses share features of autophagosomes; however, it is not known whether cellular autophagy is required for the formation of replication complexes. Recently, markers for autophagic vacuoles in mammalian cells have been described: microtubule-associated protein light chain 3 (LC3) and Apg12 (14, 15). In addition, murine embryonic stem (ES) cells lacking a critical gene product in the pathway of cellular autophagy (Apg5) have been established (12). Together, these advances provide new approaches to investigating the role of cellular autophagy in viral infections.

Coronaviruses are enveloped positive sense RNA viruses that replicate entirely in the cytoplasm of cells. Coronaviruses are important causes of disease in many domesticated animals and are responsible for up to 30% of human colds. In addition, a newly recognized human coronavirus has recently been identified as the causative agent of severe acute respiratory syndrome (SARS) (13–15). Coronaviruses and arteriviruses are the two families within the order Nidovirales. Viruses in this order have similar genome organization and express the proteins required for RNA replication as polyproteins (5, 9, 16–23). Thus, studies of the mechanism of coronavirus replication complex formation may be critical to understanding the pathogenesis, treatment, and prevention of coronavirus infections.

MHV is the prototype coronavirus for studies of replication complex formation and function. MHV replication complexes

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2 The abbreviations used are: MHV, mouse hepatitis virus; DMV, double membrane vesicles; 3-MA, 3-methyladenine; LC3, light chain 3 (microtubule-associated protein); ES, embryonic stem; SARS, severe acute respiratory syndrome; CoV, coronavirus; p.i., postinfection; DBT, delayed brain tumor; N, nucleocapsid; M, membrane; hel, helicase; pfu, plaque-forming unit; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum.
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Observations were performed in triplicate ± S.D. Briefly, DBT cells were labeled for 72 h in medium containing 65 μM concentrations of unlabeled leucine and [3H]leucine (1 μCi/ml). The cells were then washed and incubated in medium containing excess cold leucine (2 mM) for 24 h to allow for degradation of short-lived proteins. These labeled cells were then infected, amino acid starved, or main-}
been shown previously to correspond to association of LC3 with autophagic vacuoles (29). When MHV-infected cells were probed at 6 h.p.i. for LC3 and MHV proteins known to localize to replication complexes (p22 and N), both p22 and N co-localized with a subset of LC3-positive foci (Fig. 1A). Quantitative analysis of co-localization was performed for the indicated protein pairs. p22 and LC3 (triangles) were 85% co-localized throughout infection. hel and N (stars) were 85% co-localized throughout infection. N and LC3 co-localization (diamonds) decreased over the course of infection from 75% at 5 and 6 h.p.i. to 18% at 8 and 9 h.p.i. hel and M co-localization (squares) increased from 12% at 5 h.p.i. to 60% by 8 h.p.i. M and LC3 (circles) were ~18% co-localized throughout infection.

To determine whether the co-localization with autophagosome markers was specific to replication complexes, the localization of LC3 was compared with the replication complex-associated LC3 were associated with microtubules, we compared the localization of MHV-p22 to β-tubulin in MHV-infected cells. β-Tubulin was only 18% co-localized with p22, indicating that the majority of replication complexes and LC3 were not associated with microtubules (Fig. 1).

LC3 Remains Associated with Replication Complexes throughout Infection—Having shown that replication complexes co-localized with LC3, we next determined if LC3 remained associated with replication complexes throughout the course of infection. The co-localization of LC3, hel, N, p22, and M was determined quantitatively at 5, 6, 7, 8, 9, and 10 h.p.i. At 5 and 6 h.p.i., hel and M were 12 and 13% co-localized, but by 7 h.p.i., the two proteins were 40% co-localized, and by 8 h.p.i., hel and M were 64% co-localized. When the localization of the hel and N proteins were compared, hel and N were >85% co-localized throughout the infection (Fig. 2). Together, these results provided quantitative confirmation of previous observations demonstrating that the hel and N proteins translocate away from replication complexes to sites of viral assembly, possibly to deliver newly synthesized RNA nucleocapsids for integration into newly forming virions (25).

When LC3 localization was compared with N and M at 5 and 6 h.p.i., N and LC3 were 74% co-localized, but by 7 h.p.i., N and LC3 were only 55% co-localized and by 8 and 9 h.p.i. were only 17% co-localized. When the localization of LC3 and M were compared, LC3 and M were <18% co-localized throughout the course of the infection (Fig. 2). Thus, LC3 did not translocate with hel and N to sites of assembly. To confirm that LC3 remained associated with replication complexes, the localization of LC3 was compared with the replication complex-associated protein, p22 (25). LC3 and p22 remained 85% co-localized throughout the course of infection (Fig. 2). These experiments demonstrated that LC3 did not change localization over time significantly associated with membranes derived from Golgi or ER-Golgi intermediate compartment or with sites of virus assembly (Fig. 1). LC3 has also been reported to be associated with microtubules. However, not all of the LC3 in an infected cell co-localized with replication complexes. Thus, to determine whether replication complexes and the replication complex-associated LC3 were associated with microtubules, we compared the localization of MHV-p22 to β-tubulin in MHV-infected cells. β-Tubulin was only 18% co-localized with p22, indicating that the majority of replication complexes and LC3 were not associated with microtubules (Fig. 1).

Fig. 1. Replicase proteins co-localize with markers for autophagy. A, DBT cells were infected with MHV for 6 h and then fixed and processed for immunofluorescence. Cells were probed with antibodies against LC3, Apg12, β-tubulin, N, p22, and M. The localization of LC3 and β-tubulin (red, all panels) was compared with N, p22, and M (green, as labeled in panels), with yellow pixels indicating areas of co-localization. B, quantitation of fluorescence co-localization. The percent co-localization of the indicated proteins was determined as described under “Experimental Procedures.”

Fig. 2. The hel and N proteins translocate away from LC3-positive membranes to sites of assembly over the course of infection. MHV-infected DBT cells were harvested from 5 to 9 h.p.i. Quantitative analysis of co-localization was performed for the indicated protein pairs. p22 and LC3 (triangles) were 85% co-localized throughout infection. hel and N (stars) were 85% co-localized throughout infection. N and LC3 co-localization (diamonds) decreased over the course of infection from 75% at 5 and 6 h.p.i. to 18% at 8 and 9 h.p.i. hel and M co-localization (squares) increased from 12% at 5 h.p.i. to 60% by 8 h.p.i. M and LC3 (circles) were ~18% co-localized throughout infection.
but remained associated with proteins in viral replication complexes.

**Autophagy Is Induced by MHV Infection**—The co-localization of replicase proteins with LC3 suggested that replication complexes were forming on autophagic vacuoles. A possible mechanism for targeting of replicase proteins to autophagic vacuoles would be the specific induction of cellular autophagy by MHV. The rate of degradation of long-lived proteins is a well-characterized method shown to provide an accurate measure of the level of autophagy in cells (1, 3, 12). To determine whether MHV infection induced autophagy, the rate of long-lived protein degradation was determined in DBT cells that were either amino acid-starved, MHV-infected, starved and MHV-infected, or incubated with normal media (Fig. 3). DBT cells that were starved increased their long-lived protein degradation from 1.2 to 2.9% \((p < 0.0001, \text{Student's} \ t \ \text{test})\) compared with DBT cells incubated with normal media. In MHV-infected cells, protein degradation was significantly increased to 2.1% \((p < 0.0006, \text{Student's} \ t \ \text{test})\) over control cells, and when cells were both starved and infected, protein degradation was increased to 2.9% \((p < 0.0001, \text{Student's} \ t \ \text{test})\) compared with control cells.

When cells were starved in the presence of the autophagy inhibitor 3-methyladenine (3-MA), the increase in protein degradation was blocked. In contrast, long-lived protein degradation increased to 2.6% \((p < 0.0001, \text{Student's} \ t \ \text{test})\) in 3-MA-treated infected cells, demonstrating that MHV-induced protein degradation was 3-MA insensitive (Fig. 3). 3-MA did inhibit MHV growth in a dose-dependent manner (data not shown). However, 3-MA is a nucleoside analog that may interfere with viral RNA replication and transcription. Nevertheless, our results suggest that MHV induced autophagy via a unique pathway or was able to complement the 3-MA-inhibited steps resulting in functional autophagy.

To determine whether an intact autophagic pathway was required for the MHV-induced stimulation of long-lived protein degradation, analysis of long-lived protein degradation was performed in murine embryonic stem cell lines expressing Apg5 (R1) or in Apg5 knockout (A11) cells under the conditions used for DBT cells. The knockout of Apg5 has been shown to prevent the formation of autophagic vacuoles (12). Furthermore, these cells were shown to have reduced rates of degradation of long-lived proteins (12, 31, 32).

When R1 cells were starved or infected with MHV, long-lived protein degradation rates were 7.5% and 7.1%, respectively (Fig. 4). In contrast, when A11 cells were starved, infected, or starved and infected, there was no change in levels of protein degradation compared with mock-infected cells (Fig. 4). When 3-MA was added to starved or infected A11 cells, no change in protein degradation was observed compared with mock-infected cells (Fig. 4). Thus, an intact autophagic signaling pathway appears to be required for MHV-induced protein degradation.

**MHV Replication Is Decreased in the Absence of Apg5**—Having demonstrated that MHV infection induced autophagy and that MHV replication complexes contained markers for autophagic vacuoles, we next determined whether autophagy was required for MHV replication. MHV growth was compared in ES cell lines that were Apg5+/+ (R1) and Apg5−/− (A11, B22) and in ES cells that were Apg5−/− but reconstituted by Apg5 expression from stably integrated Apg5 plasmid (WT13). To demonstrate that Apg5 was expressed in R1 and WT13 but not in the A11 or B22 cell lines, Western blot analysis with Apg12 antibodies, and Apg5 antibodies was performed. It has been shown previously that nearly all Apg5 and Apg12 is detected as a 56-kDa Apg12-Apg5 conjugate, and that detection of a 56-kDa band is an accurate representation of the level of Apg5 expression in a given cell line (12). Western blotting with antibodies to Apg12 revealed the presence of the 56-kDa Apg5-Apg12 conjugate in the R1 and WT13 cells but not in the A11 or B22 cells (Fig. 5A). When the membrane was stripped and reprobed with antibodies against Apg5, 56-kDa proteins were again detected in the R1 and WT13 lines, but not in the A11 or B22 lines (Fig. 5B). The levels of Apg5 expression in wild-type R1 and Apg5-reconstituted WT13 cells were equivalent. Having experimentally confirmed that Apg5 was expressed in R1 and WT13 cells and not expressed in A11 and B22 cells, these cells were then used to determine whether defects in autophagy impacted the ability of MHV to grow. Viral yield following MHV infection of in R1 cells was 6.35 ± 0.10 log pfu/ml at 24 h p.i. (Fig. 5C). MHV grew to 2.15 ± 0.96 log pfu/ml and 2.62 ± 0.19 log pfu/ml at 24 h p.i. in the B22 and A11 Apg5−/− cell lines, respectively, a decrease of >99.99%. Reconstitution of the Apg5 knockout by plasmid expression of Apg5 increased viral yield to 5.90 ± 0.07 log pfu/ml, demonstrating that the inhibition of MHV replication seen in the A11 and B22 cell lines was because of the lack of Apg5 expression and that the defect could be complemented by expression of Apg5.

**MHV-induced DMV Formation Requires Autophagy**—The above results demonstrated that functional autophagy was critical for wild-type levels of MHV replication. To determine whether formation of DMVs was linked to autophagy in MHV-infected cells, electron micrographs were obtained of MHV-infected autophagy-incompetent A11 cells. In mock-infected A11 cells, a minor swelling of rough endoplasmic reticulum (RER) was seen, but normal membrane morphology was otherwise observed (Fig. 6). In the autophagy-competent R1 cells,
MHV infection resulted in the development of DMVs similar to those seen in other MHV-infected cells (Fig. 6). In contrast, despite the demonstrated ability of MHV to replicate in A11 cells, DMVs were not detected in the MHV-infected A11 cells. Instead, the morphology of the membranes in MHV-infected autophagy-incompetent A11 cells was impressively deranged, with hyper-swollen membranes detected throughout the majority of cells (Fig. 6). These membranes had continuity with the nuclear envelope and decoration with ribosomes, strongly suggesting an origin in the RER. When viewed in sagittal section, multiple large vesicles appeared to be surrounded by the swollen ER (white arrow) occasionally detected. MHV-infected A11 cells contained extremely swollen interconnected areas of RER that appear to surround cytoplasmic regions to form vesicles (bottom left panel). The lower right panel shows a higher magnification view of swollen RER (black arrow points to same vesicle in both panels). The black arrowheads indicate an area of membrane confluence with the outer nuclear membrane (bottom right panel). Mi, mitochondrion; Nuc, nucleus.

MHV infection resulted in the development of DMVs similar to those seen in other MHV-infected cells (Fig. 6). In contrast, despite the demonstrated ability of MHV to replicate in A11 cells, DMVs were not detected in the MHV-infected A11 cells. Instead, the morphology of the membranes in MHV-infected autophagy-incompetent A11 cells was impressively deranged, with hyper-swollen membranes detected throughout the majority of cells (Fig. 6). These membranes had continuity with the nuclear envelope and decoration with ribosomes, strongly suggesting an origin in the RER. When viewed in sagittal section, multiple large vesicles appeared to be surrounded by the swollen ER (white arrow) occasionally detected. MHV-infected A11 cells contained extremely swollen interconnected areas of RER that appear to surround cytoplasmic regions to form vesicles (bottom left panel). The lower right panel shows a higher magnification view of swollen RER (black arrow points to same vesicle in both panels). The black arrowheads indicate an area of membrane confluence with the outer nuclear membrane (bottom right panel). Mi, mitochondrion; Nuc, nucleus.

**DISCUSSION**

The findings in this report demonstrate that cellular autophagy plays an important role in MHV replication and that infection with MHV induces cellular autophagy. To our knowledge, this is the first report to demonstrate that components of the autophagic pathway are required for formation of a viral replication complex and for efficient viral growth. The autophagy induced by MHV infection was not susceptible to 3-MA treatment, despite 3-MA inhibition of viral yield by up to 86% (data not shown), suggesting that either an alternative pathway of autophagy induction is activated by MHV infection or that viral proteins, insensitive to 3-MA treatment, may replace or complement autophagic pathways. The mechanism by which MHV induces autophagy remains to be determined. Protein kinase R activation has been shown to be essential for autophagic activity (3). Because double-stranded RNA is known to be a potent stimulator of protein kinase R, it is possible that double-stranded RNA generated during viral replication may activate protein kinase R and stimulate autophagy. An alternative possibility is that binding of MHV to the cellular receptor, CEACAM-1, may induce signaling events that result in autophagy.

Viral infection was not able to induce autophagy in APG5−/− (A11) cells, indicating that the proteins necessary for autophagic vacuole formation are required for MHV-induced autophagy. The lack of double membrane vesicles in MHV-infected A11 cells was particularly interesting in light of the ability of MHV to replicate in these cells, albeit to reduced levels. This result strongly suggests that DMV formation may not be required for viral replication. However, viral growth was decreased by >99% in the APG5−/− cell lines, A11 and B22,
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