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Zsolt Talloczy
Columbia University

Herbert W. Virgin IV
Washington University School of Medicine in St. Louis

Beth Levine
Columbia University

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Research Paper

PKR-Dependent Autophagic Degradation of Herpes Simplex Virus Type 1

Zsolt Tallóczy[1]
Herbert W. Virgin, IV[2]
Beth Levine[1,3,*]

[1]Department of Medicine; Columbia University College of Physicians & Surgeons; New York, New York USA

[2]Department of Pathology and Immunology; Washington University School of Medicine; St. Louis, Missouri USA

[3]Departments of Internal Medicine and Microbiology; University of Texas Southwestern Medical Center; Dallas, Texas USA

*Correspondence to: Beth Levine; Division of Infectious Diseases; Department of Internal Medicine; University of Texas Southwestern Medical Center at Dallas; 5323 Harry Hines Boulevard; Dallas, Texas 75390-9113 USA; Tel.: 214.648.0493; Fax: 214.648.0284; Email: beth.levine@utsouthwestern.edu

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KEY WORDS

autophagy, xenophagy, herpes simplex virus, PKR, eIF2α kinase

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ABSTRACT

The lysosomal pathway of autophagy is the major catabolic mechanism for degrading long-lived cellular proteins and cytoplasmic organelles. Recent studies have also shown that autophagy (xenophagy) may be used to degrade bacterial pathogens that invade intracellularly. However, it is not yet known whether xenophagy is a mechanism for degrading viruses. Previously, we showed that autophagy induction requires the antiviral eIF2α kinase signaling pathway (including PKR and eIF2α) and that this function of eIF2α kinase signaling is antagonized by the herpes simplex virus (HSV-1) neurovirulence gene product, ICP34.5. Here, we show quantitative morphologic evidence of PKR-dependent xenophagic degradation of herpes simplex virions and biochemical evidence of PKR and eIF2α-dependent degradation of HSV-1 proteins, both of which are blocked by ICP34.5. Together, these findings indicate that xenophagy degrades HSV-1 and that this cellular function is antagonized by the HSV-1 neurovirulence gene product, ICP34.5. Thus, autophagy-related pathways are involved in degrading not only cellular constituents and intracellular bacteria, but also viruses.

INTRODUCTION

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 factor 2 (eIF2α) and a subsequent shutdown of host and viral protein synthesis and viral replication (reviewed in Ref. 1). 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 refs. 2 and 3). 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. The HSV-1 neurovirulence protein, ICP34.5, binds to a protein phosphatase and causes it to dephosphorylate eIF2α, thereby negating the activity of PKR.[4-6] A neuroattenuated HSV-1 mutant lacking ICP34.5 exhibits wild-type replication and virulence in mice genetically lacking *pkr*,[3] proving that the ICP34.5 gene product mediates neurovirulence by antagonizing PKR-dependent functions.

Previously, we showed that PKR and eIF2α phosphorylation regulate another fundamental cellular process, the lysosomal degradation pathway of autophagy.[7] In yeast, we found that disruption of the eIF2α kinase, Gcn2, mutation of the Ser-51 phosphorylation site of eIF2α, and mutation of a downstream transcription factor, Gcn4, blocked starvation-induced autophagy. In mammalian cells, we found that disruption of PKR blocked virus-induced autophagy and that mutation of the Ser-51 phosphorylation site of eIF2α blocked starvation- and virus-induced autophagy. Similar to its effects on translational control regulated by the eIF2α kinase signaling pathway, we found that the HSV-1 neurovirulence gene product, ICP34.5, also antagonized eIF2α kinase-dependent autophagy. A mutant strain of HSV-1 lacking ICP34.5 but not wild-type HSV-1 was able to induce autophagy in virally-infected murine embryonic fibroblasts (MEFs). However, in this previous study, the effects of autophagy (and ICP34.5 antagonism of autophagy) on the HSV-1 life cycle were not examined; MEFs were pretreated with αIFN to optimize induction of PKR activity and consequently, they had very low levels of viral replication.

While the term "autophagy" denotes the degradation of self-constituents (reviewed in refs. 8–10) which primarily include long-lived proteins and obsolete or damaged organelles,

the term "xenophagy" was recently developed to denote the breakdown of foreign microbial invaders by an autophagy-like pathway.[11] Previous studies have suggested that xenophagy is involved in the degradation of *Mycobacterium tuberculosis*, *Shigella flexneri* and invading Group A *Streptococcus*.[12-14] In addition to bacteria, there is indirect evidence that xenophagy may also target viruses for lysosomal degradation. Enforced neuronal expression of the *beclin 1* autophagy gene decreases CNS alphavirus replication[15] and RNAi inactivation of the plant autophagy genes, *BECLIN 1*, *ATG3* and *ATG7* increases replication of tobacco mosaic virus.[16] However, it is not yet known whether these antiviral effects are mediated through direct degradation of viruses or indirect effects of autophagy on host cellular constituents involved in controlling viral replication.

To examine whether viruses are degraded by xenophagy, we used autophagy-competent and autophagy-deficient cells to compare the life cycle of wild-type HSV-1 and a mutant strain of HSV-1 lacking the autophagy inhibitory protein, ICP34.5. Our results indicate that xenophagy plays a role in degrading HSV-1 in both murine embryonic fibroblasts and sympathetic neurons and that this host cell function is antagonized by the HSV-1 neurovirulence protein, ICP34.5.

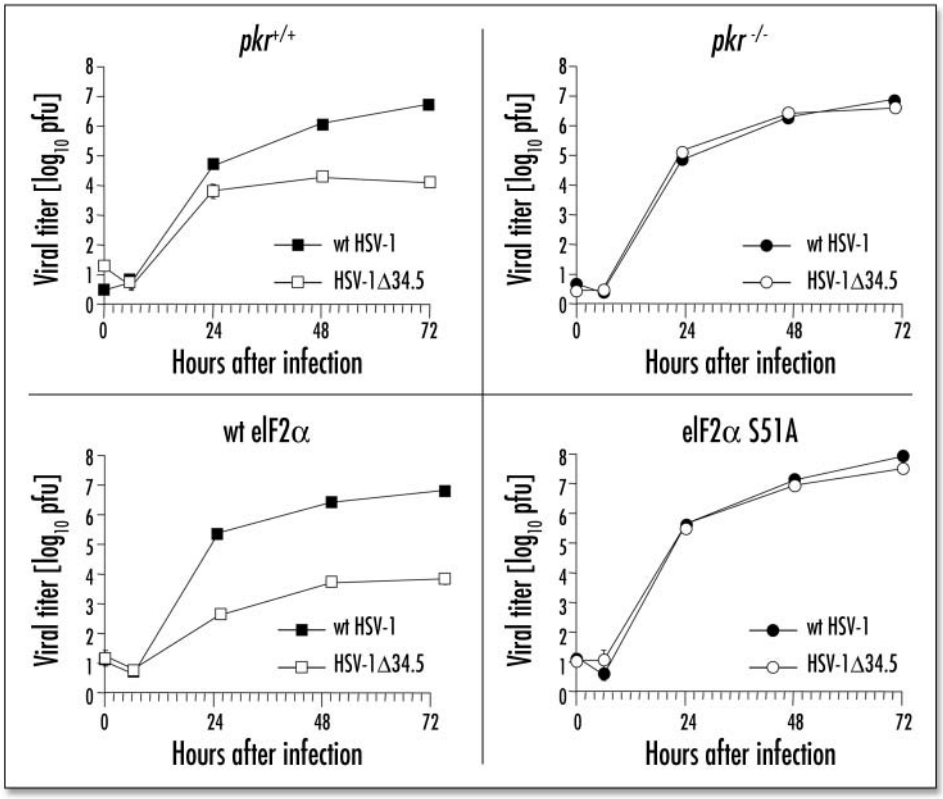

Figure 1. Viral growth of wild-type HSV-1 and mutant HSV-1Δ34.5 in *pkr*$^{-/-}$, S51A eIF2α mutant and isogenic control wild-type MEFs. Results shown represent mean ± SEM for triplicate samples. Similar results were obtained in five independent experiments.

MATERIALS AND METHODS

Cells. Murine embryonic fibroblasts (MEFs) were prepared and cultured using methods previously described[17] from *pkr*$^{-/-}$ and *pkr*$^{+/+}$ mouse embryos (mixed C57/129sv backgrounds).[18] MEFs were prepared and cultured from mutant eIF2α S51A and wild-type eIF2α isogenic control mouse embryos as described.[19] Primary cultures of sympathetic superior cervical ganglion neurons were prepared from postnatal day 2 mice and cultured as described.[20,21]

Virus strains and infections. The HSV-1 ICP34.5 mutant 17TermA (termed here HSV-1Δ34.5) and its marker-rescued virus 17TermA^{R} (termed here wt HSV-1) were made in the background strain 17 of HSV-1 and have been described elsewhere.[3,22] Virus stocks were grown and titered in Vero cells. For viral replication studies, MEFs were infected with HSV-1Δ34.5 or wt HSV-1 at a multiplicity of infection (MOI) of 0.01 plaque-forming unit (pfu) per cell. Cells were harvested at serial time points after infection and viral titers were determined by performing plaque assay titration on Vero cells. For electron microscopic analyses, MEFs and sympathetic neurons were infected at an MOI of 5 pfu/cell and fixed at 18 hours or 36 hours, respectively, after infection. For metabolic labeling experiments, MEFs were infected at an MOI of 5 pfu/cell and analyzed as described below.

Electron microscopic analyses. MEFs and sympathetic neurons were fixed with 2.5% glutaraldehyde, postfixed in 1% OsO_4, embedded in Epon, and randomly selected grid squares of ultrathin sections were examined. For each sample, approximately 50 cells with visible virions were examined by an observer blinded to experimental condition. For each cell, the number of virions inside the cytoplasm, inside viral vesicles, and inside autophagosomes was counted. Viral vesicles were defined as single membrane vesicles that contain intact HSV-1 virions and lack any cellular cytoplasmic contents. Autophagosomes were defined as membrane-bound 0.3–2.0 μm vacuoles with clearly recognizable cytoplasmic contents, and included both early autophagic vacuoles (that contain morphologically intact cytoplasm) and late autophagic vacuoles (that contain partially degraded but identifiable cytoplasmic material).

Viral protein degradation assays. Four hours after viral adsorption, cells were depleted of methionine and cysteine for one hour, and then metabolically labeled with 100 μCi/ml of L-[^{35}S]Met, Cys (ICN, Irvine, CA) for two hours. At the end of radiolabeling, cells were washed five times and media with excess unlabeled methionine and cysteine (2 mM each) was added. At serial time points after metabolic labeling, cells were lysed in Tris-HCl lysis buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) and cell lysates were immumoprecipitated with a rabbit anti-HSV-1 polyclonal antibody (DAKO, Carpinteria, CA). Radioimmunoprecipitated proteins were subjected to SDS-PAGE analysis and visualized by autoradiography.

RESULTS

HSV-1 is degraded in autophagosomes in murine embryonic fibroblasts (MEFs) and this degradation is inhibited by the HSV-1 neurovirulence gene product, ICP34.5. We hypothesized that xenophagy may be a cellular mechanism for degrading viruses. To investigate this hypothesis, we compared the ultrastructure of murine embryonic fibroblasts (MEFs) infected with wild-type HSV-1 (which encodes an inhibitor of autophagy, ICP34.5) with that of MEFs infected with a mutant strain of HSV-1 lacking ICP34.5 (HSV-1Δ34.5).[3,22] Previously, we showed that HSV-1Δ34.5, but not wild-type HSV-1 stimulated autophagy induction in virally-infected MEFs,[7] demonstrating that ICP34.5 functions as an inhibitor of virus-induced autophagy. Consistent with findings reported by Ward et al.,[23] we confirmed that the replication of HSV-1Δ34.5 as compared to wild-type HSV-1 is impaired in wild-type MEFs and that HSV-1Δ34.5 replicates to the same levels as wild-type HSV-1 in either *pkr*$^{-/-}$ MEFs or eIF2α S51A mutant

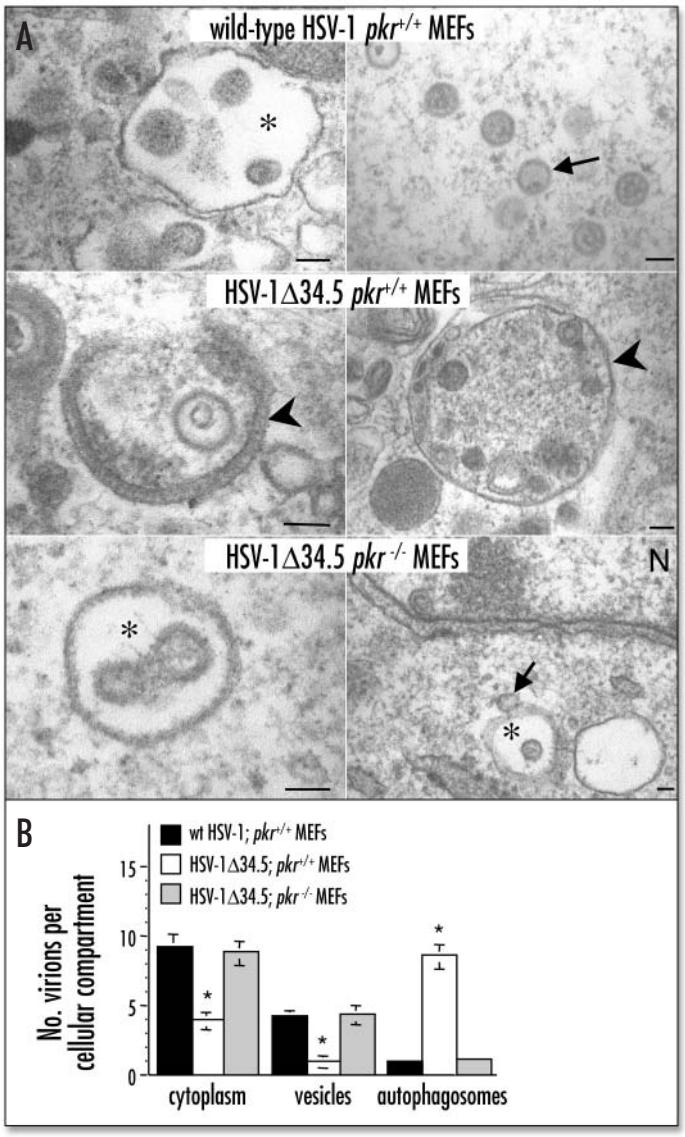

Figure 2. Ultrastructural analyses of HSV-1 and HSV-1Δ34.5 infected wild-type and *pkr*$^{-/-}$ MEFs. (A) Representative electron micrographs of *pkr*$^{+/+}$ MEFs infected with HSV-1 and HSV-1Δ34.5 and *pkr*$^{-/-}$ MEFs infected with HSV-1Δ34.5. Representative cytoplasmic viral vesicles are labeled by asterisks. Representative intracytoplasmic virions are labeled by arrows. Arrowhead in middle left panel shows an autophagic isolation membrane forming around an intracytoplasmic virion and arrowhead in middle right panel shows a late autophagosome with partially degraded virions and other cytoplasmic contents. N, nucleus. Scale bars, 100 nm. (B) Quantitation of subcellular localization of virions in *pkr*$^{+/+}$ MEFs infected with HSV-1 and HSV-1Δ34.5 and in *pkr*$^{-/-}$ MEFs infected with HSV-1Δ34.5. Data shown represents the mean ± SEM number of virions free inside the cytoplasm, inside cytoplasmic vesicles involved in viral egress (labeled vesicles), and inside autophagosomes for 50 cells per condition. Asterix denotes $p < 0.001$ versus other conditions, t-test.

MEFs (Fig. 1). While the requirement for ICP34.5 in viral replication in wild-type cells is thought to relate to its ability to block PKR-dependent host cell shutoff, we sought to examine whether ICP34.5 antagonism of PKR-dependent signaling may alter other stages in the viral life cycle besides translation.

At the ultrastructural level, no significant differences were observed in the nuclei of HSV-1 and HSV-1Δ34.5 infected MEFs, suggesting that the presence of autophagy (i.e., in HSV-1Δ34.5-infected cells) or absence of autophagy (i.e., in wild-type HSV-1-infected cells) did not visibly alter early stages of the viral life cycle. Aggregates of uniform granules, crystalline arrays composed of viral particles, and randomly dispersed viral capsids were observed in the nuclei of MEFs infected with both wild-type HSV-1 or HSV-1Δ34.5 (data not shown).

In contrast, marked differences were observed in the cytoplasm of HSV-1 and HSV-1Δ34.5-infected MEFs (Fig. 2A). We quantitated the number of virions freely dispersed inside the cytoplasm, inside single membrane vesicles that are intermediates in the egress of HSV-1 from the nucleus out of the cell[24] (referred to herein as cytoplasmic viral vesicles), and inside autophagosomes (Fig. 2B). In MEFs infected with wild-type HSV-1, the majority of intracytoplasmic virions were either randomly dispersed in the cytoplasm or found inside viral vesicles. In MEFs infected with HSV-1Δ34.5, there were fewer virus particles observed within the cytoplasm and fewer viral vesicles ($p < 0.001$, t-test). Rather, the majority of virus particles were localized inside autophagosomes, defined morphologically as double membrane vacuoles that (in contrast to viral vesicles that contain only virus particles) contain a mix of different cytoplasmic constituents. Virtually all stages of autophagy were observed in HSV-1Δ34.5-infected MEFs, including the formation of autophagosomes around virion-containing cytoplasm (which appears as a "cup-shaped" structure, see Fig. 2A, left center panel), early autophagosomes (data not shown) and late autophagosomes that have fused with the lysosome and contain partially degraded material, including virions (see Fig. 2A, right center panel). These observations demonstrate that HSV-1 is degraded by xenophagy and that HSV-1 ICP34.5 antagonizes cellular xenophagic degradation of HSV-1.

Xenophagic degradation of HSV-1 in MEFs requires *pkr*. Previously, we demonstrated a requirement for PKR in autophagy induced by HSV-1Δ34.5.[7] To examine whether PKR is required for the xenophagic degradation of HSV-1, we performed quantitative electron microscopy of pkr$^{-/-}$ MEFs infected with HSV-1Δ34.5 (Fig. 2). Unlike HSV-1Δ34.5-infected wild-type MEFs, HSV-1Δ34.5-infected *pkr*$^{-/-}$ MEFs had relatively few virions inside autophagosomes ($p < 0.001$; t-test). Instead, HSV-1Δ34.5-infected pkr$^{-/-}$ MEFs appeared similar to wild-type HSV-1 infected wild-type MEFs, with the majority of virus particles found dispersed inside the cytoplasm or inside cytoplasmic viral vesicles rather than inside autophagosomes. Thus, HSV-1 degradation via the autophagolysosomal pathway requires the antiviral molecule PKR.

PKR- and eIF2α kinase-dependent degradation of HSV-1 proteins that is blocked by HSV-1 ICP34.5. Our electron microscopy studies provide quantitative morphological evidence that HSV-1 is degraded by xenophagy and that ICP34.5 blocks PKR-dependent xenophagy. To extend these findings, we evaluated the kinetics of the degradation of radiolabeled HSV-1-encoded proteins in autophagy-competent and autophagy-deficient, *pkr*$^{-/-}$ and eIF2α S51A mutant MEFs infected with either wild-type HSV-1 or HSV-1Δ34.5 (Fig. 3). Using a polyclonal anti-HSV-1 antibody, we found that, as expected, the baseline amount of radiolabeled protein was greater in cells lacking a functional PKR signaling pathway (either due to infection with wild-type HSV-1 which contains the PKR inhibitor, ICP34.5, or due to loss-of-function mutations in PKR or eIF2α). This observation is consistent with the known effects of PKR signaling in translational control and the known effects of ICP34.5 in blocking PKR-dependent host cell shutoff.

Of note, in addition to these known effects of PKR and ICP34.5 in translational regulation, we also found that PKR signaling and ICP34.5 regulated the rate of viral protein degradation (Fig. 3). HSV-1 protein degradation was 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,[7] the rate of HSV-1 protein degradation was similar in HSV-1 and HSV-1Δ-34.5-infected cells, indicating that HSV-1 protein degradation is positively regulated by the PKR signaling pathway. Thus, the eIF2α kinase-dependent autophagy signaling pathway not only regulates the degradation of long-lived cellular proteins (as shown previously in ref. 7) but also regulates the degradation of viral proteins.

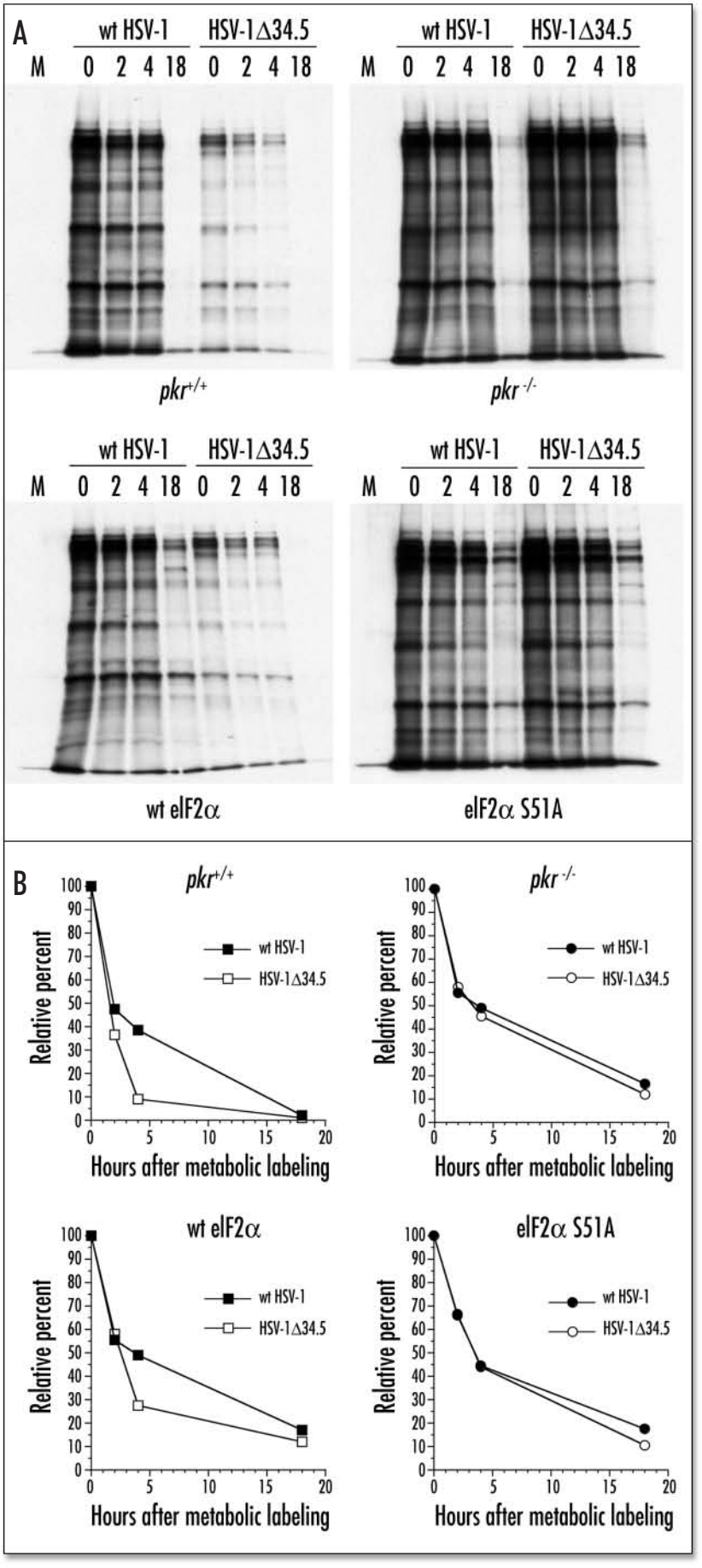

Figure 3. HSV-1 viral protein degradation in wild-type, *pkr*$^{-/-}$, and eIF2α S51A mutant MEFs infected with wt HSV-1 or HSV-1Δ34.5. (A) Autoradiographs of HSV-1 viral proteins. The number above each lane correspond to the number of hours after completion of metabolic labeling. M, mock-infected lysate. Results are shown of one representative experiment. Similar results were obtained in three independent experiments. (B) Quantitation of HSV-1 viral protein degradation in wild-type, *pkr*$^{-/-}$, and eIF2α S51A mutant MEFs infected with wt HSV-1 or HSV-1Δ34.5. The amounts of radioimmunoprecipitated HSV-1 viral proteins were quantitated by densitometric analysis using NIH Image 1.62 software. Y axis represents relative percent of the amount of radioimmunoprecipitated viral proteins present at the indicated time as compared to the baseline amount present at the completion of metabolic labeling (time 0). Results shown represent quantitation of the images shown in (A) and include quantitation of all radiolabeled viral proteins. Similar results were obtained in three independent experiments and similar patterns were observed for densitometric quantification of individual viral protein bands (data not shown).

PKR-dependent xenophagic degration of HSV-1 in mouse sympathetic neurons that is blocked by HSV-1 ICP34.5. The findings described above in MEFs demonstrate a role for PKR-dependent cellular xenophagy in the degradation of HSV-1 and a role for HSV-1 ICP34.5 in the antagonism of xenophagic degradation of HSV-1. However, since neurons are the primary cellular target of HSV-1 in vivo; HSV-1 ICP34.5 is required for neurovirulence;[4,22] and *pkr* deletion in mice restores neurovirulence of a mutant HSV-1 strain lacking ICP34.5,[3] we wished to evaluate whether similar virus-host interactions also occur in HSV-1-infected neurons. Therefore, we compared the ultrastructure of primary cultured sympathetic neurons from wild-type and *pkr*$^{-/-}$ mice infected with wild-type HSV-1 and HSV-1Δ34.5 (Fig. 4).

Similar to our observations in MEFs, the cytoplasmic, but not nuclear appearance of HSV-1 and HSV-1Δ34.5-infected wild-type neurons was strikingly different (Fig. 4A and B). In wild-type neurons infected with HSV-1, very few autophagosomes were present, and the majority of virus particles were freely dispersed within the cytoplasm or inside single membrane-bound cytoplasmic vesicles (Fig. 4C) (see Fig. 4B, left column for representative photomicrographs). In contrast, in HSV-1Δ34.5-infected wild-type neurons, there was a significant increase in the number of autophagosomes and the majority of cytoplasmic virus particles were localized in autophagosomes ($p < 0.001$; t-test). The autophagosomes contained a mix of different cytoplasmic constituents and numerous HSV-1 virions; the virions within the autophagosomes were intact in early autophagosomes (Fig. 4B, center middle panel) and were in different stages of degradation in late autophaghosomes (Fig. 4B, center lower panel). These findings demonstrate that HSV-1 is degraded in autophagosomes in primary neurons and that this host process is inhibited by HSV-1 ICP34.5. Furthermore, the xenophagic degradation of HSV-1Δ34.5 in neurons, like in MEFs, requires PKR since *pkr*$^{-/-}$ neurons infected with HSV-1Δ34.5 had very few autophagosomes and appeared similar to wild-type neurons infected with wild-type HSV-1 (Fig. 4A-C).

DISCUSSION

Previous observations have suggested a possible role for xenophagy in innate immunity against viral infections. First, enforced neuronal expression of the mammalian autophagy protein, Beclin 1, protects mice against

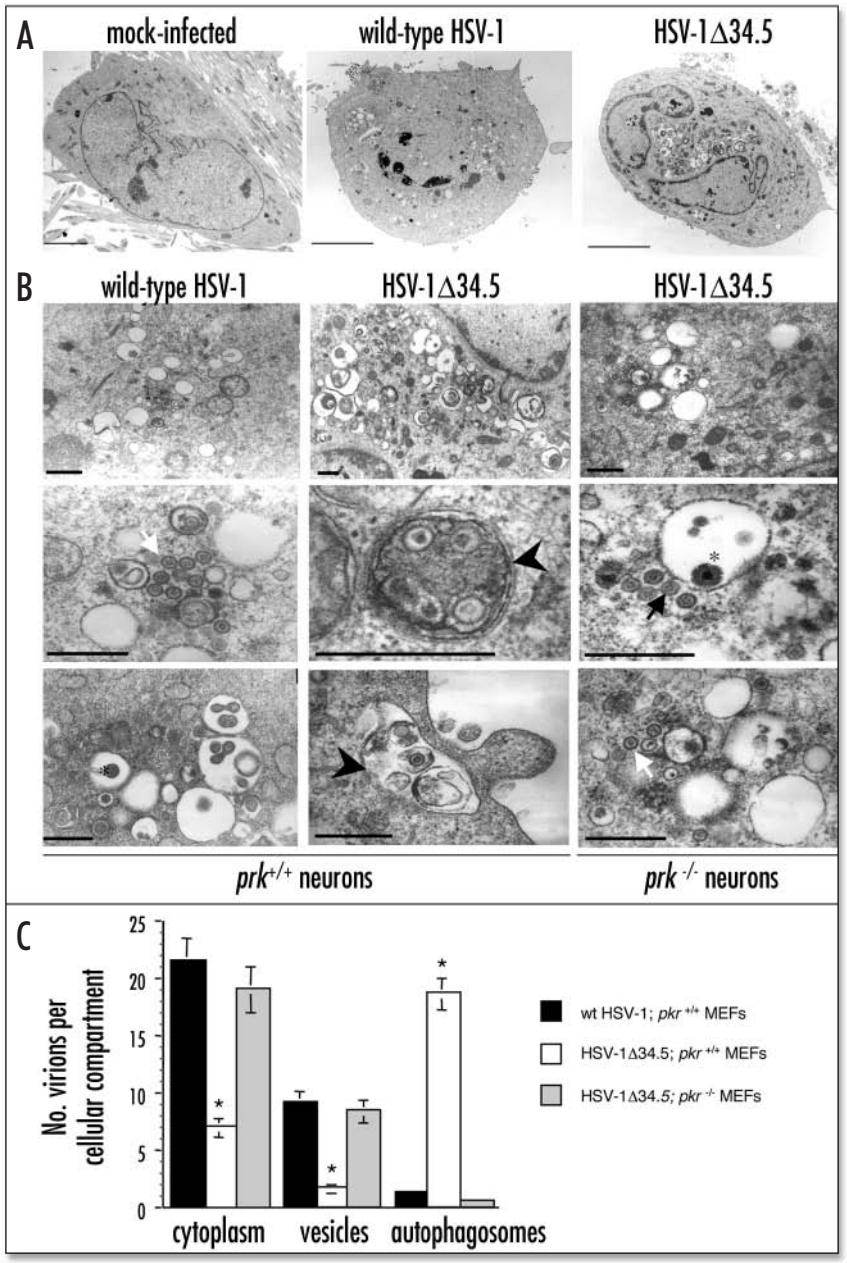

Figure 4. Ultrastructural analyses of HSV-1 and HSV-1Δ-34.5 infected wild-type and *pkr*$^{-/-}$ mouse sympathetic neurons. (A) Representative low power electron micrographs of HSV-1 and HSV-1Δ34.5 infected wild-type mouse sympathetic neurons. Scale bars, 2 μm. (B) Representative higher power electron micrographs of HSV-1 and HSV-1Δ-34.5 infected wild-type and *pkr*$^{-/-}$ mouse sympathetic neurons. Representative cytoplasmic viral vesicles are labeled by asterisks. Representative intracytoplasmic virions are labeled by arrows. Arrowheads in middle column show autophagosomes containing visible intact and partially degraded HSV-1 virions. Scale bars, 0.5 μm. (C) Quantitation of subcellular localization of virions in *pkr*$^{+/+}$ neurons infected with HSV-1 and HSV-1Δ34.5 and in *pkr*$^{-/-}$ neurons infected with HSV-1Δ34.5. Data shown represents the mean ± SEM number of virions free inside the cytoplasm, inside cytoplasmic vesicles involved in viral egress (labeled vesicles), and inside autophagosomes for 50 cells per condition. Asterix denotes $p < 0.001$ versus other conditions, t-test.

lethal Sindbis virus encephalitis.[15] Second, the interferon-inducible antiviral eIF2α kinase signaling pathway positively regulates autophagy.[7] Third, the viral virulence gene product, HSV-1 ICP34.5, antagonizes host autophagy.[7] Fourth, several different plant autophagy genes have been shown to limit tobacco mosaic virus replication.[16] Although these findings are consistent with a protective role of xenophagy in the host response to viral infection, direct evidence that autophagy degrades cytoplasmic virus particles has been lacking.

Our findings demonstrate that HSV-1 virions are degraded in autophagosomes, that HSV-1ΔICP34.5 inhibits the xenophagic degradation of HSV-1, and that the xenophagic degradation of HSV-1 requires cellular PKR. To the best of our knowledge, our data provide the first evidence that viruses can be degraded by the cellular xenophagy pathway. Other studies with RNA viruses have suggested that viruses may coopt components of the autophagy pathway to promote their own intracellular replication (reviewed in ref. 25). For example, poliovirus, equine arterivirus and mouse hepatitis virus RNA replication complexes form on membranes that share some similarities with autophagosomes;[26-30] poliovirus replication is enhanced by treatment with autophagy-inducing agents, rapamycin and tamoxifen[25] and decreased by RNA interference with autophagy genes,[30] and murine hepatitis virus replication is decreased in transformed MEFs lacking the autophagy gene, *atg5*.[31] However, at the ultrastructural level, the double membrane vacuoles associated with the RNA replication complexes of these viruses do not contain normal cellular constituents and do not contain visible evidence of degradation of the vacuolar contents. Thus, it appears that the autophagic machinery can both be exploited by viruses to establish replication "niches" and be used by the host cell to degrade viruses inside autophagolysosomes. HSV-1 (and possibly other viruses that also encode PKR inhibitors) possesses strategies to block autophagy signaling and xenophagic degradation of cytoplasmic virus particles.

Our findings also demonstrate a newly described function of the PKR signaling pathway which is the regulation of the degradation of viral proteins. We found that viral protein degradation is accelerated in infected cells with intact PKR function (e.g., HSV-1Δ34.5-infected cells) as compared to cells in which PKR function is blocked by expression of HSV-1 ICP34.5, a null mutation in *pkr*, or a non-phosphorylatable mutation in eIF2α. In our experiments, we cannot assess 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 HSV-1 replication. 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. Nonetheless, it seems logical to speculate that PKR-dependent xenophagic degradation of viruses might be an antiviral host defense mechanism.

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