Interaction of ICP34.5 with Beclin 1 Modulates Herpes Simplex Virus Type 1 Pathogenesis through Control of CD4⁺ T-Cell Responses[▽]

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Received 10 August 2009/Accepted 9 September 2009

Autophagy is an important component of host innate and adaptive immunity to viruses. It is critical for the degradation of intracellular pathogens and for promoting antigen presentation. Herpes simplex virus type 1 (HSV-1) infection induces an autophagy response, but this response is antagonized by the HSV-1 neurovirulence gene product, ICP34.5. This is due, in part, to its interaction with the essential autophagy protein Beclin 1 (Atg6) via the Beclin-binding domain (BBD) of ICP34.5. Using a recombinant virus lacking the BBD, we examined pathogenesis and immune responses using mouse models of infection. The BBD-deficient virus (Δ 68H) replicated equivalently to its marker-rescued counterpart (Δ 68HR) at early times but was cleared more rapidly than Δ 68HR from all tissues at late times following corneal infection. In addition, the infection of the cornea with Δ 68H induced less ocular disease than Δ 68HR. These results suggested that Δ 68H was attenuated due to its failure to control adaptive rather than innate immunity. In support of this idea, Δ 68H stimulated a significantly stronger CD4⁺ T-cell-mediated delayed-type hypersensitivity response and resulted in significantly more production of gamma interferon and interleukin-2 from HSV-specific CD4⁺ T cells than Δ 68HR. Taken together, these data suggest a role for the BBD of ICP34.5 in precluding autophagy-mediated class II antigen presentation, thereby enhancing the virulence and pathogenesis of HSV-1.

Autophagy is a conserved cellular pathway that eliminates defective proteins and organelles, prevents abnormal protein aggregate accumulation, and removes intracellular pathogens (11, 22, 32, 56). This process begins with the formation and elongation of a double membrane that fuses to form an autophagosome. The cytoplasmic contents are nonspecifically sequestered inside the autophagosome and then are degraded once the autophagosome fuses with the lysosome. Autophagy is upregulated during starvation, growth factor withdrawal, hypoxia, and infection (10). Following metabolic stress, autophagy can generate metabolic precursors that can be recycled for the de novo synthesis of proteins. The autophagic pathway has important roles in development, immune defense, apoptosis, tumor suppression, and the prevention of neuronal degeneration (reviewed in references 21 and 31).

Autophagy is not limited to the degradation of self proteins; it also can engulf and break down invading microorganisms in a process termed xenophagy (26). Xenophagy can limit the replication of pathogens (3, 4, 29, 34, 42), but some infectious agents can exploit autophagy to enhance their replication (2, 18, 38). There are pathogens that actively inhibit autophagy through interaction with the essential autophagy-promoting protein, Beclin 1 (24, 35). Beclin 1 is the mammalian homolog of yeast Atg6 and is required for the formation of the autophagosome membrane through its interaction with VPS34, a class III phosphatidylinositol 3-kinase (19, 27). Autophagy/xenophagy is also an important process for the adaptive im-

mune response to infection through the delivery of antigens for major histocompatibility complex class I and II (MHC-I and -II) presentation (7, 9, 12, 36, 45). The inhibition of autophagy by pathogens therefore would serve to block CD4⁺ and CD8⁺ cell responses and allow pathogens to remain underrecognized by the adaptive immune response.

The interferon (IFN)-inducible double-strand RNA-inducible protein kinase (PKR) pathway is required for virus- and starvation-induced autophagy (50). The PKR-mediated induction of autophagy requires the phosphorylation of the translation initiation factor eIF2 α (50). Herpes simplex virus type 1 (HSV-1) inhibits autophagy through at least two domains and the activities of the late protein ICP34.5, its C-terminal mediation of dephosphorylation of eIF2 α , and its N-terminal binding to Beclin 1 (5, 16, 35). HSV-1 strains lacking ICP34.5 show significant attenuation in vivo, increased sensitivity to IFN (33), an inability to counteract the PKR-induced phosphorylation of eIF2 α , and the induction of the generalized shutoff of protein synthesis in infected cells. These activities originally were ascribed solely to ICP34.5's ability to recruit PP1 α and redirect its activity to dephosphorylate eIF2 α to counteract the general shutoff of protein synthesis mediated by PKR (17). This function is mediated by the C-terminal domain of ICP34.5 that contains homology to the growth arrest and DNA damage 34 (GADD34) gene (6). Independently of the role of ICP34.5 in countering the PKR-induced antiviral state, viruses lacking ICP34.5 also exhibit altered patterns of autophagy. This manifests as increased long-lived protein degradation, the increased formation of autophagosomes, increased autophagic vacuole volume density, and the enhanced xenophagic degradation of virions (35, 50, 51). Such alterations in the autophagy pathway now can be ascribed to ICP34.5's ability to bind Beclin 1 in addition to its mediation of eIF2 α dephosphorylation (35).

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[▽] Published ahead of print on 14 October 2009.

The deletion of the Beclin 1-binding domain (BBD) of ICP34.5 renders HSV-1 less able to regulate autophagosome formation, and viruses lacking this domain are neuroattenuated (35).

To determine the impact of Beclin 1-ICP34.5 interactions on the pathogenesis of HSV-1 during infection, we examined the ability of BBD-deficient virus to replicate, cause disease, and stimulate an immune response in mice. We determined that mice infected with HSV-1 lacking the ICP34.5 BBD were able to clear virus more efficiently than mice infected with wild-type virus. We observed the significantly enhanced stimulation of CD4⁺ T cells by the virus lacking the BBD compared to that of mice infected with wild-type virus. These data suggest that Beclin 1 binding and the inhibition of autophagy by ICP34.5 are important for HSV-1 pathogenesis through its ability to suppress autophagy and to dampen the activation of CD4⁺ T cells.

MATERIALS AND METHODS

Cells and viruses. African green monkey kidney (Vero) cells were propagated as previously described (40). Viruses used in this study were in the background of strain 17syn+. The ICP34.5 Beclin 1-binding mutant (Δ 68H) and its marker-rescued virus (Δ 68HR) have been described previously (1).

Animal procedures. In all experiments, male and female 6- to 8-week-old strain 129 Ev/Sv mice and C57BL/6 (Charles River Laboratories, Wilmington, MA) or congenic *Rag1*^{-/-} and *Ir3*^{-/-} mice (kindly provided by Michael Diamond with permission from Tadatsugu Tanaguchi) were used (43, 47). For neurovirulence experiments, anesthetized mice were infected intracranially with 20 μ l of virus at the indicated dose (49). These mice were monitored daily for survival for 21 days. For corneal infections, corneas were bilaterally scarified with a 25-gauge needle and inoculated with 2×10^6 PFU of virus in a volume of 5 μ l as previously described (40). Eye swab material was collected and assayed for virus by standard plaque assay as previously described (25). Trigeminal ganglia and 6-mm biopsy specimen punches of periocular skin were removed and placed in 1.5-ml tubes containing 1-mm-diameter glass beads and 1 ml of medium. Biopsy punches of periocular skin also were taken for hematoxylin and eosin staining for histopathology. Trigeminal ganglia and periocular skin homogenates were prepared by freezing and thawing samples, mechanically disrupting them in a Mini-Beadbeater-8 (Biospec Products, Bartlesville, OK), and sonicating them. Brain homogenates were prepared by freezing and thawing samples, shaking them in 4 ml of medium with 3-mm-diameter glass beads, and clarifying them at $5,000 \times g$ for 5 min. The titers of homogenates were determined by plaque assay on Vero cells, and the amount of virus was expressed as the PFU per milliliter of tissue homogenate. Mice also were observed and scored for disease and weighed for the indicated time period. Scoring was done in a semiquantitative fashion on a scale of 0 to 4, with a score of 0 being no disease and 4 being death (46). Disease scores represent a composite score reflecting keratitis, blepharitis, neurological symptoms, and general health, such as fur ruffling and lethargy.

Latency establishment and reactivation assays. Ocular infections were performed as described above, except with 2×10^4 PFU/eye of the indicated virus. To assay for the establishment of latency, mice were sacrificed 28 days postinfection and their trigeminal ganglia were removed. DNA was isolated using a DNeasy tissue preparation kit (Qiagen, Valencia, CA). The number of latent genomes per trigeminal ganglia was determined by real-time PCR essentially as described previously (48). Briefly, a 70-bp fragment of the thymidine kinase (tk) gene was amplified from trigeminal ganglia DNA and 10-fold dilutions of HSV-1 bacterial artificial chromosome (BAC) DNA (17-49 BAC) (14). BAC DNA was used simply to generate a standard curve to determine the number of genome copies per trigeminal ganglion, since BAC DNA models the episomal, latent genome. To control for the total DNA content of each sample, the single-copy mouse adipin gene was amplified in each sample along with dilutions of mouse genomic DNA to generate a standard curve. The values for the tk copy number were normalized to the lowest value of the mouse adipin copy number to yield the normalized genome copy per ganglion, which was expressed on a log scale. For the reactivation assay, mice were sacrificed 28 days postinfection, and their trigeminal ganglia were removed, bisected, and explanted onto Vero cell monolayers essentially as described previously (25). For 7 days after explant, 100- μ l samples of culture supernatant were sampled and replated on a fresh monolayer of Vero cells and monitored for the presence of infectious virus.

Measurement of CD4 T-cell responses, T-cell depletion, and cytokine expression. For the measurement of delayed-type hypersensitivity (DTH), mice were infected on the cornea with Δ 68H or Δ 68HR as described above. Seven days later, recipient mice were challenged in the right footpad with 1×10^6 PFU of UV-inactivated Δ 68HR (15) and in the left foot with culture medium (0.033 ml per injection). Twenty-four hours later, footpad thickness was determined with a micrometer by a masked observer. DTH responses were recorded as the difference between the right and left footpads and expressed as micrometers $\pm$ standard errors of the means (SEM). The depletion of CD4⁺ and CD8⁺ T cells was performed using anti-CD4 (GK1.5) and anti-CD8 (clone 2.43) antibodies purified from hybridoma supernatants. Mice were depleted of CD4⁺ or CD8⁺ cells by three daily 100- μ g doses of anti-CD8 beginning 4 days prior to viral infection. The efficiency of depletion was determined to be >99.9% for both CD4⁺ and CD8⁺ T cells by flow cytometry on isolated spleen cells at the end of the experiment.

For the measurement of CD4⁺ T-cell IFN- γ and interleukin-2 (IL-2) production, mice were infected with Δ 68H or Δ 68HR via the cornea as described above. Seven days later spleen and draining lymph nodes were harvested, and the CD4⁺ T cells were purified using a CD4⁺ T-cell enrichment kit (Stem Cell Technologies, Vancouver, British Columbia, Canada). CD4⁺ T cells (4×10^6 /ml) were mixed with an equal volume of 4×10^5 /ml dendritic cells (DC) in complete medium (RPMI 1640, 10% FBS, 5×10^{-5} M 2-mercaptoethanol), and 0.2 ml of the mixture was added per well to a 96-well tissue culture plate for a final ratio of 10:1 (T cell:DC). Each well then received 2 μ l Δ 68HR antigens. Antigens for the T-cell cytokine assays were prepared as described previously (57). Briefly, antigens were extracted from Vero cells 10 h after Δ 68HR infection at a multiplicity of infection of 4. Extracts underwent three freeze-thaw cycles, two 2-min homogenization cycles using UV inactivation, and clarification by centrifugation at $200 \times g$. Supernatants were harvested at 48 h, and IFN- γ and IL-2 concentrations were determined by using a Bio-Rad Bioplex 200.

RESULTS

Interaction between ICP34.5 and Beclin 1 is important for efficient viral replication in peripheral tissues at late times following corneal infection. Previous studies showed that the BBD of ICP34.5 was necessary for wild-type neurovirulence following intracerebral infection (35), but it was completely dispensable for wild-type replication in primary MEFs (1). Furthermore, the ability of ICP34.5 lacking the BBD to block eIF2 α phosphorylation was equivalent to that of the wild type (D. E. Alexander and D. A. Leib, unpublished data). This demonstrated that the dephosphorylation-mediating activity of the C-terminal GADD34 homologous domain of ICP34.5 was unaffected by the deletion of the BBD. The role of the BBD in promoting viral replication and pathogenesis following peripheral infection, however, has not been tested. We therefore examined the growth and pathogenesis of the BBD-deleted virus, Δ 68H, and its marker-rescued virus, Δ 68HR, following corneal infection. Viral titers in eye swab material (cornea), trigeminal ganglia, periocular skin, and brain were determined at various times postinfection (Fig. 1). The replication of both viruses was comparable in all tissues at days 1 and 3 postinfection, but by days 5 and 7 the titers of Δ 68H were significantly lower than those of Δ 68HR. This was especially true in the periocular skin, perhaps reflecting a higher efficacy of inflammation-mediated immune clearance from this tissue compared to that of corneas and the nervous system. The replication of Δ 68HR in peripheral tissues was not significantly different from that of wild-type strain 17 at any time postinfection (data not shown). Thus, Beclin 1 binding by ICP34.5 contributes to the persistence of HSV-1 replication following corneal inoculation at late times postinfection.

Interaction between ICP34.5 and Beclin 1 results in greater virulence and disease following corneal infection. Replication

FIG. 1. Beclin 1 binding is important for efficient HSV-1 growth following corneal infection. 129 Ev/Sv mice were infected with 2×10^6 PFU/eye of $\Delta 68H$ or $\Delta 68HR$ on bilaterally scarified corneas. Mice were sacrificed, and tear film (cornea), trigeminal ganglia, periocular skin, and brains were harvested on days 1, 3, 5, and 7 postinfection. Data are expressed as the geometric mean PFU/milliliter of homogenized tissue $\pm$ SEM for six samples per experimental group in each of two independent experiments. The limit of detection of these assay was 10 PFU/ml for corneas, trigeminal ganglia, and periocular skin and 100 PFU/ml, as indicated by the horizontal dashed line, for the assay of brain homogenates. Asterisks indicate $P < 0.05$ (Student's t test).

differences between $\Delta 68H$ and $\Delta 68HR$ were reflected in the course of clinical disease and the overall health of the infected animals. Clinical disease and weight loss were greater for $\Delta 68HR$ than $\Delta 68H$ at days 5 and 6 postinfection (Fig. 2A). Compared to the status of mock-infected mice (Fig. 2B) on day 5, severe inflammation was observed in the periocular skin in mice infected with $\Delta 68HR$ (Fig. 2C). The skin had multiple foci of ulcerative dermatitis and the epidermis was either necrotic or lost, with associated serum exudate and cellular debris containing degenerate neutrophils. In the underlying dermis there was infiltration by neutrophils and macrophages. In the deeper portion of the dermis, there was diffuse infiltration by neutrophils, lymphocytes, and macrophages, with moderate edema. In general, while qualitatively similar pathology was seen following infection with $\Delta 68H$, it was much less severe (Fig. 2D). In addition, none of the mice infected with $\Delta 68HR$ survived beyond 7 days, while 91% of mice infected with $\Delta 68H$ survived (data not shown). Taken together, these data provide further evidence that interaction with Beclin 1 contributes to the efficient replication and pathogenesis of HSV-1.

Establishment of latency and reactivation does not require interaction of ICP34.5 with Beclin 1. Decreased growth in the cornea observed for $\Delta 68H$ may limit the amount of virus that can traffic to the trigeminal ganglia and establish a latent infection. Decreased growth in the trigeminal ganglia may further limit the ability of the virus to establish latency and to reactivate. Consequently, we evaluated the ability of $\Delta 68H$ and $\Delta 68HR$ to establish a latent infection by harvesting trigeminal ganglia 28 days following corneal infection and quantifying the number of latent genomes via real-time PCR. Despite growth

defects in the cornea and trigeminal ganglia at times beyond day 5, $\Delta 68H$ and $\Delta 68HR$ established latency equivalently (Fig. 3A). In addition, following the explant cocultivation of latently infected trigeminal ganglia, both the ICP34.5 Beclin 1-binding mutant ($\Delta 68H$) and its marker-rescued virus ($\Delta 68HR$) reactivated comparably, as determined by the presence of infectious virus (Fig. 3B). Thus, the BBD of ICP34.5 is dispensable for the establishment of, and reactivation from, latency.

IRF3 deficiency in mice does not restore neurovirulence to $\Delta 68H$. Viruses lacking the ICP34.5 BBD are neuroattenuated in terms of lethality and replication following the intracranial infection of mice (35 and data not shown). It also has been shown that a domain of ICP34.5 that partially overlaps with the BBD is necessary for the disruption of the interaction between IFN regulatory factor 3 (IRF3) and TANK-binding kinase 1 (TBK1) (55). This TBK1-interacting domain of ICP34.5 thereby is able to prevent the induction of IFN-stimulated antiviral genes. We wished, therefore, to address the possibility that the neuroattenuation of $\Delta 68H$ is due to its inability to control TBK1-mediated signaling rather than its inability to control autophagy and autophagy-related pathways. To accomplish this, we intracerebrally infected mice lacking IRF3 (*Irf3*^{-/-}) and mice lacking MyD88 (Fig. 4 and data not shown). The prediction was that if ablated TBK1 binding was the explanation for the attenuation of $\Delta 68H$, then virulence should be completely restored in mice lacking these innate response mediators.

Wild-type C57BL/6 mice and congenic *Irf3*^{-/-} mice were intracranially infected with $\Delta 68H$ and $\Delta 68HR$. Similar to our findings with wild-type 129 Ev/Sv mice (35), the infection of

FIG. 2. Decreased disease and weight loss are observed in mice infected with $\Delta 68H$ compared to those of mice infected with $\Delta 68HR$. 129 Ev/Sv mice were infected with 2×10^6 PFU/eye of either $\Delta 68H$ or $\Delta 68HR$ on bilaterally scarified corneas. (A) Animals were scored for periocular disease and overall clinical disease from days 2 to 10 postinfection, along with a change in weight. Disease data are expressed as the geometric mean disease score, or percent change in body weight, $\pm$ SEM for five mice per experimental group in two independent experiments. Asterisks indicate $P < 0.05$ by Student's t test. (B to D) Representative hematoxylin- and eosin-stained histology sections of the periocular skin of mice 5 days following mock infection (B) or infection with $\Delta 68HR$ (C) or $\Delta 68H$ (D). Magnification, $\times 200$.

wild-type C56BL/6 mice with $\Delta 68H$ resulted in significantly less mortality than that of mice infected with $\Delta 68HR$ ($P < 0.0001$ by log-rank test) (Fig. 4A). Consistently with previously described mouse strain differences in susceptibility to HSV-1 infection, C57BL/6 mice are more resistant to fatal HSV-1 infection than 129 Ev/Sv mice (20). Interestingly, the neurovirulence of $\Delta 68H$ was not restored to that of $\Delta 68HR$ following the infection of IRF3-deficient mice, with differences between the survival of *Irf3*^{-/-} and control mice remaining significant ($P = 0.0046$, log-rank test) (Fig. 4B). In fact, there also was no significant increase in the mortality of *Irf3*^{-/-} mice compared to that of wild-type mice infected with either $\Delta 68H$ or $\Delta 68HR$. Consistent with these findings, the infection of *Myd88*^{-/-} mice also revealed no difference in pathogenesis between $\Delta 68H$ and $\Delta 68HR$ (data not shown). Taken together, these data suggest that innate immunity regulated by the MyD88-independent (IRF3) and MyD88-dependent pathways do not account for the difference in neurovirulence between $\Delta 68H$ and $\Delta 68HR$. Furthermore, an inability to interfere with IRF3-mediated signaling does not account for the attenuation of $\Delta 68H$.

Virulence of $\Delta 68H$ is restored in *Rag1*^{-/-} mice. The difference in the replication between $\Delta 68H$ and $\Delta 68HR$ is observed at later time points (5 to 7 days), suggesting that the control of the immune response to HSV-1 by the BBD involves adaptive immunity. The inability of IRF3 and Myd88 deficiency to restore virulence to $\Delta 68H$ further supported this hypothesis (Fig. 4 and data not shown). We addressed this initially by examining *Rag1*^{-/-} mice, which lack functional T and B cells (44, 47). Mice were infected intracerebrally with $\Delta 68H$ and $\Delta 68HR$, and

lethality was monitored (Fig. 5). These studies show that the neurovirulence of $\Delta 68H$ was completely restored following the infection of *Rag1*^{-/-} mice, with no significant difference between the lethality of mice infected with $\Delta 68H$ and $\Delta 68HR$ ($P = 0.1147$ by log-rank test) (Fig. 6). This suggests that the attenuation of $\Delta 68H$ is due to its inability to overcome the adaptive, rather than the innate, immune response.

$\Delta 68H$ stimulates stronger CD4⁺ T-cell responses than $\Delta 68HR$. The restoration of virulence to $\Delta 68H$ in *Rag1*^{-/-} mice (Fig. 5), coupled with observations by others that autophagy plays a pivotal role in promoting the endogenous MHC-II processing of antigens for presentation to CD4⁺ T cells (7, 9, 36), provided a rationale for further examining BBD and adaptive immunity. Recent work has demonstrated a role for ICP34.5 in the control of autophagy and of MHC-I-restricted antigen presentation (12), but its role in controlling MHC-II-restricted responses has not been addressed. Given that CD4⁺ T-cell responses are important for immunity to HSV-1 (23, 41, 52), we wished to address this directly by measuring the T-cell responses in DTH assays following corneal infection with $\Delta 68H$ and $\Delta 68HR$ (Fig. 6A). $\Delta 68H$ -infected mice exhibited a significantly greater ($P < 0.001$) DTH response to HSV antigens than $\Delta 68HR$ -infected mice. The depletion of CD4⁺ T cells ($-CD4$) eliminated this response in $\Delta 68H$ -infected mice, while the removal of CD8⁺ T cells ($-CD8$) had no effect. In addition, CD4⁺ T cells from mice infected with $\Delta 68H$ produced significantly more IFN- γ (Fig. 6B) and interleukin-2 (IL-2) (Fig. 6C) when stimulated in vitro with HSV-1 antigens than CD4⁺ T cells from $\Delta 68HR$ -infected mice. Taken to-

FIG. 3. $\Delta 68H$ and $\Delta 68HR$ establish latency and reactivate equally. 129 Ev/Sv mice were infected with 2×10^4 PFU/eye of $\Delta 68H$ or $\Delta 68HR$ on bilaterally scarified corneas. Following the establishment of latency, mice were sacrificed and trigeminal ganglia were harvested. (A) Real-time PCR for the HSV-1 tk gene was performed on DNA isolated from trigeminal ganglia of latently infected mice. All samples were normalized to the single-copy mouse adipsin gene. Data are reported as the geometric means of the number of genome copies per trigeminal ganglion $\pm$ SEM for 12 or 14 trigeminal ganglia per experimental group. (B) Trigeminal ganglia were bisected and cocultured on a monolayer of Vero cells. Supernatants were removed daily for 7 days after explant and added to fresh Vero monolayers. The results were recorded as the percentage of wells positive for reactivated virus for 12 or 14 trigeminal ganglia per experimental group in two independent experiments.

gether, these data suggest that the BBD of ICP34.5 is important for HSV-1 pathogenesis through its suppression of autophagy and subsequent dampening of the MHC-II-restricted stimulation of CD4⁺ T cells.

DISCUSSION

In this study, we have further dissected the role of ICP34.5's BBD in the pathogenesis of HSV-1. Our previous work determined that this domain was important for neurovirulence following intracerebral infection. From the standpoint of pathogenesis and immunity, however, the corneal model of HSV-1 infection is both more physiologically relevant to pathogenesis in humans and revealing of mechanisms of immunity. The reductions of $\Delta 68H$ replication in the cornea, trigeminal ganglia, and periocular skin were consistent with previously observed reductions in brain titers (35). The unexpected observation in this study was the inability of BBD-deficient $\Delta 68H$ to control the adaptive immune response. This is likely to be true for ICP34.5-null viruses and explain the mechanism behind the previous observation of increased MHC-II expression in cultured cells infected with an ICP34.5 null virus relative to that

FIG. 4. Deletion of IRF-3 does not restore neurovirulence to an ICP34.5 Beclin 1-binding mutant. (A and B) Survival of C57BL/6 wild-type (A) and congenic *lrf3*^{-/-} (B) mice infected intracranially with 1×10^2 PFU of the indicated virus. The number of animals was $n = 10$ for each experimental group in two independent experiments.

of wild-type virus (53). Our observations also lend further rationale to the deletion of ICP34.5 from live-attenuated HSV vaccines, not only to reduce neurovirulence but also to promote antigenicity and the stimulation of a stronger adaptive response. Interestingly, however, there was no alteration of the establishment of or reactivation from the latency of $\Delta 68H$ compared to that of $\Delta 68HR$. This demonstrates that the BBD and its control of autophagy do not significantly impact retrograde or anterograde spread or the reactivation of HSV-1.

Previous work has demonstrated the activity of a specific domain of ICP34.5 (amino acids 72 to 104) that binds to TBK1 and disrupts its functional interaction with IRF3 (55). This domain serves to prevent the induction of IFN and IFN-stimulated genes and overlaps partially with the BBD that spans residues 68 to 87 (35). Data in this study show that the virulence of $\Delta 68H$ is not increased in *lrf3*^{-/-} mice relative to that of control mice. Thus, the BBD-deleted ICP34.5 still may be able to interact with TBK1, and the residues between 87 and 104 are necessary and sufficient for this interaction. Experiments are in progress to test this idea, but importantly, our data show that the attenuation of $\Delta 68H$ is due to its inability to bind Beclin 1 rather than its inability to bind TBK1. IRF3 is a pivotal component in the development and amplification of the type 1 IFN response, and mice lacking IRF3 show increased susceptibility to virus infections (8). Our experiments with *lrf3*^{-/-} mice also provide evidence that innate immunity is not a major component in the attenuation of $\Delta 68H$.

Previous work showed that BBD is dispensable for wild-type

FIG. 5. Deletion of *Rag1* restores neurovirulence to an ICP34.5 Beclin 1-binding mutant. (A and B) Survival of C57BL/6 wild-type (A) and congenic *Rag1*^{-/-} (B) mice infected intracranially with 1×10^2 PFU of the indicated virus. The number of animals was $n = 20$ for each experimental group in two independent experiments.

replication in cultured murine fibroblasts and neurons (1). The current work, however, shows that BBD mutants are compromised in their ability to grow in epithelial and neuronal tissues in vivo. This supports the hypothesis that the BBD-mediated inhibition of autophagy impacts viral replication only in the context of an intact adaptive immune system. Alternatively, this may reflect other differences between cultured cells and in vivo infection. The relatively late clearance of Δ68H from infected mice and its attenuation in *Irf3*^{-/-} mice, however, are consistent with a role for adaptive immunity rather than direct xenophagic degradation in the enhanced clearance of Δ68H. Autophagy plays a role in the adaptive immune response by delivering antigens for MHC-II presentation (7, 9). Since it has been shown that viral proteins from Epstein-Barr virus and influenza virus can be presented on MHC-II molecules via autophagy, it is likely that HSV-1 also presents antigens to CD4⁺ T cells via the autophagy pathway (36, 45). The significantly increased CD4⁺ T-cell activation observed in Δ68H-infected mice is strong evidence that a major role of BBD is to downregulate antigen presentation to CD4⁺ T cells. Further evidence for this is that the virulence of Δ68H was restored in *Rag1*^{-/-} mice, which lack B and T cells. The restoration of virulence to Δ68H also has been observed in mice lacking PKR (35). We previously had ascribed this to the autophagic degradation of HSV-1 being PKR dependent (51) and that the *Pkr*^{-/-} mice therefore were unable to mount a direct xenophagic attack. This conclusion currently is being reevaluated to

FIG. 6. CD4⁺ T-cell responses to Δ68H and Δ68HR. (A) For the measurement of DTH response, C57BL/6 mice were infected via the cornea with Δ68H or Δ68HR. Seven days later, recipient mice were challenged in the right footpad with 1×10^6 PFU of UV-inactivated Δ68HR and in the left foot with cell culture medium. Twenty-four hours later, footpad thickness was determined with a micrometer by a masked observer. DTH responses were recorded as the difference between the right and left footpads and are expressed as micrometers $\pm$ SEM. For the measurement of cytokine production, C57BL/6 mice were infected on the cornea with Δ68H or Δ68HR. Seven days later, spleen and draining lymph nodes were harvested, and the CD4⁺ T cells were purified. CD4⁺ T cells were mixed with DC at a ratio of 10:1 and stimulated with HSV-1 antigens. Supernatants were harvested at 48 h, and IFN-γ (B) and IL-2 (C) concentrations were determined by Bioplex 200 (Bio-Rad).

test whether T-cell responses are diminished in HSV-infected PKR-deficient mice. We also currently are clarifying whether other components of the adaptive immune system can be countered by BBD, since autophagy plays a role in other aspects of the adaptive immune response, such as T-cell survival and proliferation and B lymphocyte development (30, 39). Recent work also has shown a novel process of membrane-derived autophagy to be important for delivering antigens for presentation on MHC-I molecules, and that this process also is inhibited by ICP34.5 (12). This work was performed using an ICP34.5-null virus, and it will be of interest to study the specific role of BBD in inhibiting MHC-I-restricted T-cell activation.

The mechanism by which ICP34.5 inhibits Beclin 1-mediated autophagy is unknown, but the interaction between these proteins may provide some clues. ICP34.5 does not interact with the N-terminal domain of Beclin 1 (35). This region of Beclin 1 contains a *Bcl-2*-interacting domain, suggesting that HSV-1 and gammaherpesviruses have evolved different mechanisms to target Beclin 1-mediated autophagy (28, 37). Second, given that ICP34.5 binds the C-terminal region of Beclin 1, which contains the evolutionarily conserved VPS34 binding domain, ICP34.5 may block or compete for VPS34 binding to Beclin 1. The conserved domain of Beclin 1 is required for autophagy function; therefore, ICP34.5 interaction with this region may prevent or limit Beclin 1-mediated autophagy (13).

It is clear that ICP34.5 has multiple functions that promote efficient viral replication and pathogenesis. However, PP1 α binding and the blockade of translational arrest is the dominant function. This idea is supported by previous work showing that ICP34.5-null viruses remain attenuated in SCID mice (54). Thus, counteracting the innate immune response may be a critical function of ICP34.5 independent of BBD. This is exemplified further by an ICP34.5 mutant that is unable to bind PP1 α but retains the BBD (D. Alexander, unpublished results). Following infection, this mutant undergoes the complete shutoff of protein synthesis, and in vitro and in vivo growth phenotypes are indistinguishable from those of an ICP34.5-null virus. Furthermore, ICP34.5-null mutants are attenuated to a much greater degree than that of the ICP34.5 Beclin 1-binding mutant. Notwithstanding the dominance of the PP1 α binding function of ICP34.5, it is clear that Beclin 1 binding is sufficient to control autophagy and to modulate host immunity, and that this domain significantly impacts the fitness of HSV-1. Our future studies will elucidate the precise mechanisms by which BBD interfaces with the cell and, in turn, with the immune system.

ACKNOWLEDGMENTS

This work was supported by NIH grants to D. Leib (EY09083), to T. A. Ferguson (EY06765 and EY015570), and to the Department of Ophthalmology and Visual Sciences Core Grant (EY02687) from the National Eye Institute. D. Alexander also was supported by a research training grant in the visual sciences (NIH EY013360). Support from Research to Prevent Blindness to the Department of Ophthalmology and Visual Sciences, a Macular Vision Research Foundation award to T. A. Ferguson, and a Senior Scientific Investigator Award to D. A. Leib are gratefully acknowledged.

We thank Belinda McMahon and Suellen Greco for assistance with the histopathology and Michael Diamond and Tadatsugu Tanaguchi for the provision of the *Irf3*^{-/-} mice.

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