Maintenance of Gammaherpesvirus Latency Requires Viral Cyclin in the Absence of B Lymphocytes

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Gammaherpesviruses establish a life-long chronic infection that is tightly controlled by the host immune response. We previously demonstrated that viruses lacking the gammaherpesvirus 68 (γHV68) viral cyclin (v-cyclin) exhibited a severe defect in reactivation from latency and persistent replication. In this analysis of chronic infection, we demonstrate that the v-cyclin is required for γHV68-associated mortality in B-cell-deficient mice. Furthermore, we identify the v-cyclin as the first gene product required for maintenance of gammaherpesvirus latency in vivo in the absence of B lymphocytes. While the v-cyclin was necessary for maintenance of latency in the absence of B cells, maintenance of v-cyclin-deficient viruses was equivalent to that of wild-type γHV68 in the presence of B cells. These results support a model in which maintenance of chronic γHV68 infection requires v-cyclin-dependent reactivation and reseeding of non-B-cell latency reservoirs in the absence of B cells and raise the possibility that B cells represent a long-lived latency reservoir maintained independently of reactivation. These results highlight distinct mechanisms for the maintenance of chronic infection in immunocompetent and B-cell-deficient mice and suggest that the different latency reservoirs have distinct gene requirements for the maintenance of latency.

The gammaherpesviruses are large, double-stranded DNA viruses that include the human pathogens Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8), as well as murine gammaherpesvirus 68 (γHV68), a closely related gammaherpesvirus that serves as a small-animal model. These viruses establish a lifelong infection in the host and encode many genes that manipulate the host cell machinery. A hallmark of chronic gammaherpesvirus infection is the ability of these viruses to establish a quiescent infection in host lymphocytes termed latent infection, which is characterized by maintenance of the viral genome as an episome, limited gene expression, and the absence of virus replication (25). Upon appropriate stimulation, latently infected cells can reactivate from latency, resulting in virus replication and production of infectious virus. During chronic infection, there may also be production of infectious virus at sites of persistent infection, allowing transmission to new hosts. EBV and KSHV persistently replicate at mucosal sites, including the oropharynx and genital tract (1, 25). γHV68 has been demonstrated to persistently replicate in the lungs, aorta, and peritoneal cells of immunocompromised mice (7, 16, 27, 30). Evidence of long-term T-cell stimulation in infected normal mice further supports the existence of ongoing persistent replication during chronic gammaherpesvirus infection (2, 3).

While gammaherpesvirus infection of immunocompetent individuals is typically controlled with few pathogenic outcomes, immune suppression can result in impaired control of chronic infection and lead to disease. Immune suppression and chronic infection with both EBV and KSHV results not only in well-recognized classical monoclonal lymphomas (8) but also in malignancies of mixed cell types, with inflammatory infiltrates and detectable reactivation within some cells including nasal pharyngeal carcinoma and Kaposi’s sarcoma (1, 25). In addition, KSHV (24) and γHV68 (6, 31) have been associated with pneumonia during chronic infection of immunocompromised hosts. Chronic γHV68 infection of immunocompromised mice has also been demonstrated to result in large-vessel arteritis (42) and splenic fibrosis (12).

To characterize basic aspects of gammaherpesvirus pathogenesis, γHV68 infection of mice has been developed as a tractable small-animal model (26, 27). γHV68 is a gammaherpesvirus of rodents that can infect both inbred and outbred strains of mice and is genetically related to EBV and KSHV on the basis of shared genomic organization, conserved genes, and associated pathologies (39). Several of the gammaherpesviruses, including γHV68, KSHV, and the primate viruses herpesvirus saimiri and rhesus rhadinovirus, encode a viral cyclin that is homologous to host D-type cyclins (39). Previously, we demonstrated that transgenic expression of the γHV68-encoded cyclin homolog (v-cyclin) transforms primary cells (37). During virus infection of the host, the v-cyclin is essential for efficient reactivation from latency when cultured ex vivo. Cells latently infected with v-cyclin-deficient viruses have an at least 100-fold-decreased ability to reactivate from latency (38). This specific requirement for the v-cyclin in reactivation is even more striking given that the v-cyclin is dispensable for lytic replication, virulence in acute infection, and establishment of latency (38). We have also demonstrated that the v-cyclin is required for persistent infection in gamma interferon (IFN-γ)-
deficient mice (17), likely as a consequence of its requirement for efficient reactivation from latency (38).

The host immune response plays an essential role in controlling the extent of both latent and persistent infections (26, 27). γHV68 can establish a latent infection in multiple cell types, including B cells, macrophages, and dendritic cells (13, 32, 44). The viral genes involved in this process are not known. Previous analyses of γHV68 have demonstrated a dynamic interplay between viral infection and the host immune response. A striking example of this is revealed by γHV68 infection of B-cell-deficient mice, which lack both B cells as a major latency reservoir (13, 32) and B-cell-dependent immune responses. Analysis of these mice and other antibody-deficient mice has demonstrated that antibody can regulate the levels of reactivation and latency in these mice (16, 20). On the basis of these observations, it has been proposed that in certain antibody-deficient hosts, ongoing reactivation and reseeding are critical for the maintenance of high levels of latently infected cells (16). Notably, different immunodeficiencies can result in a failure to control chronic infection. For example, both CD8 T-cell-deficient (CD8−/−) and B-cell-deficient (MuMT) mice fail to control γHV68 latency and reactivation to similar extents (36, 41, 43).

Given the dynamic nature of chronic infection and its regulation by the host, reactivation from latency is likely to play an important role in successful chronic infection. To test this, we have analyzed the role of the v-cyclin in the following parameters of chronic γHV68 infection: reactivation from latency, maintenance of latent infection, persistent infection, and long-term latency. We have performed analyses with both CD8 T-cell-deficient (CD8−/−) and B-cell-deficient (MuMT) mice. A significant difference between these mice is the absence of B cells, a major latency reservoir, in B-cell-deficient mice. By comparison of these different hosts, these studies further establish that v-cyclin and v-cyclin-dependent reactivation are critical for maintenance of gammaherpesvirus latency and chronic disease in the absence of B cells.

MATERIALS AND METHODS

Viruses and tissue culture. γHV68 clone WUMS (ATCC VR1465) and v-cyclin-deficient viruses were passaged and grown and their titers were determined on NIH 3T12 cells as previously described (39). This study used two γHV68 virus strains containing the wild-type (wt) v-cyclin gene: (i) parental wt γHV68 (strain WUMS) from which the v-cyclin-deficient viruses were generated, and (ii) the v-cyclin marker rescue virus (v-cyclin.MR), in which wt v-cyclin sequences were reintroduced into the v-cyclin.LacZ mutant (38). Wt γHV68 and v-cyclin.MR have been phenotypically identical in all of the assays tested to date (38). The v-cyclin-deficient viruses include (i) the v-cyclin.LacZ mutant, in which the v-cyclin gene was disrupted by insertion of a LacZ expression cassette under control of the cytomegalovirus immediate-early promoter, and (ii) the v-cyclin .Stop mutant, in which the v-cyclin gene was disrupted by insertion of a translation stop and frasemesh linker cassette (38).

Mice, infections, and organ harvests. MuMT (B-cell-deficient) mice (21, 41) backcrossed onto a C57BL/6 background and C57BL/6 (BL/6) mice deficient in the CD8 α chain (CD8−/−) (15) (Jackson Laboratory catalog no. 002665) were bred and maintained at Washington University School of Medicine, St. Louis, Mo., in accordance with all federal and university policies. BL/6 mice were purchased from The Jackson Laboratory (catalog no. 000664). Unless otherwise stated, mice were age and sex matched and infected at 7 to 10 weeks of age with 106 PFU of γHV68 by intraperitoneal injection in 0.5 ml of complete Dulbecco modified Eagle medium (38). Peritoneal cells and splenocytes were harvested and processed as previously described (38) from groups of five mice per experiment.

RESULTS

The v-cyclin is not required for long-term maintenance of latency following infection of immunocompetent BL/6 mice. We have previously demonstrated that the v-cyclin is required for efficient reactivation from latency but not for the establishment of latency at 42 days p.i. (38). Given the potential role of reactivation in chronic infection (16, 20), we initiated a detailed analysis of long-term infection with viruses sufficient or deficient in v-cyclin function. BL/6 mice were infected and analyzed for (i) the frequency of cells that reactivate the virus ex vivo and (ii) the frequency of cells that harbor the viral genome. At 6 months p.i., two viruses containing wt v-cyclin, wt γHV68 and v-cyclin.MR, reactivated equivalently from latently infected peritoneal cells when the cells were cultured ex vivo (wt reactivation frequency, 1 in 81,900; Fig. 1A). Consistent with previous studies that analyzed latency at earlier times p.i. (18, 38), two v-cyclin-deficient viruses, v-cyclin.LacZ and v-cyclin .Stop, failed to reactivate from infected peritoneal cells (Fig. 1A). Despite the severe defect of v-cyclin-deficient viruses in reactivation from latency, the frequencies of viral genome-containing peritoneal cells were very similar between wt and v-cyclin-deficient viruses (wt γHV68, 1 in 12,200; v-
cyclin.MR, 1 in 10,100; v-cyclin.Stop, 1 in 10,300; v-cyclin.LacZ, extrapolated to be ca. 1 in 25,800; Fig. 1B). Similar results were obtained with splenocytes harvested from these mice (Fig. 1C and D), although, as previously shown, little reactivation was observed in splenocytes infected with either wt γHV68 or v-cyclin-deficient viruses at earlier times p.i. (38).

Thus, although the lack of v-cyclin function leads to a severe impairment in virus reactivation from latency, this did not have a significant impact on the overall maintenance of latently infected cells in either the peritoneum or spleen at 6 months p.i. On the basis of these data, in immunocompetent BL/6 mice, the v-cyclin, and potentially v-cyclin-dependent reactiva-

FIG. 1. The v-cyclin is required for reactivation from latency but not for maintenance of latency at 6 months to 1 year p.i. of C57BL/6 mice. BL/6 mice were infected with wt γHV68 (□), v-cyclin.MR (○), v-cyclin.LacZ (△), or v-cyclin.Stop (▽) for quantification of the frequency of cells reactivating virus (A and C) and the frequency of viral genome-positive cells (B and D) in either peritoneal cells (A and B) or splenocytes (C and D). For reactivation analyses, closed symbols denote mechanically disrupted samples (uniformly negative here and coincident with open triangles and the x axis). The data shown represent independent experiments, as indicated (wt γHV68, n = 2; v-cyclin.MR, n = 3; v-cyclin.LacZ, n = 2; v-cyclin.Stop, n = 1). Curve fit lines were derived by nonlinear-regression analysis, and symbols represent the mean ± the standard error of the mean (error bars) of data. The dashed line at 63% is the value used to calculate the frequency of reactivating cells or viral genome-positive cells by the Poisson distribution. Statistically significant differences: A, \( P < 0.0001 \) for both v-cyclin.LacZ and v-cyclin.Stop compared to wt γHV68; B, \( P < 0.001 \) for v-cyclin.LacZ compared to wt γHV68.LacZ compared to wt γHV68. CPE, cytopathic effect.
tion, is not essential for long-term (6 months) maintenance of \( \gamma \text{HV68} \) latency.

The v-cyclin is critical for long-term maintenance of latency in B-cell-deficient mice. The outcome of gammaherpesvirus infection is heavily influenced by the immune status of the host, and therefore the dynamics and regulation of chronic infection may vary significantly between immunocompetent and immunocompromised hosts. Chronic infection of immunocompromised MuMT mice differs from that of normal immunocompetent mice, since MuMT mice fail to efficiently control latent infection and succumb to long-term infection (43). MuMT mice also lack B cells, which serve as a major latency reservoir for \( \gamma \text{HV68} \) (13, 32). \( \gamma \text{HV68} \) has the capacity to establish a latent infection in other cell types, including macrophages, dendritic cells, and, potentially, lung epithelium cells (13, 30, 44).

We therefore analyzed latency and reactivation in MuMT mice. By 6 months to 1 year p.i., wt \( \gamma \text{HV68} \) was capable of...
reactivating from peritoneal cells (1 in 2,300 cells; Fig. 2A) whereas v-cyclin-deficient viruses demonstrated a severe defect in reactivation from peritoneal cells (<1 in 100,000, Fig. 2A). Strikingly, however, analysis of the frequency of latently infected cells in MuMT mice demonstrated that v-cyclin-deficient viruses also have a significant defect in the maintenance of latently infected cells. This defect resulted in a greater-than-100-fold decrease in the frequency of v-cyclin-deficient latently infected cells by 6 months p.i. compared to that of latently wt γHV68-infected cells (wt γHV68, 1 in 1,300; v-cyclin-deficient viruses, <1 in 100,000; Fig. 2B). Splenocytes harvested from these mice revealed a similar defect of v-cyclin-deficient viruses both in reactivation from latency and in maintenance of latently infected cells (Fig. 2C and D). This is the first example of a herpesvirus mutant that can establish a latent infection in all of the mice tested to date (Fig. 1 and 3) (38) but is unable...
to efficiently maintain a latent infection (Fig. 2) in B-cell-deficient mice. The failure to maintain latency in MuMT mice infected with v-cyclin-deficient virus could be due to the lack of B cells or to a general feature of immunocompromised hosts, that is, the failure to control latent infection.

**The v-cyclin is not required for long-term maintenance of latency following infection of immunocompromised CD8-deficient mice.** To test whether failure of immunocompromised mice to control latent infection results in a common failure to maintain latency, we analyzed γHV68 latency and reactivation in CD8−/− mice. Previous analyses of CD8−/− and MuMT mice infected with wt γHV68 have shown that both CD8−/− and MuMT mice fail to appropriately control γHV68 latency and reactivation, resulting in similar increases in the frequency of latently infected cells and hyperactivation from latency (36, 43). Previously, we have found that, at 42 days p.i., the v-cyclin is required for ongoing virus replication in the lungs of immunocompromised mice (36, 43). These data demonstrate that v-cyclin-deficient virus could be due to the lack of B cells or to a general feature of immunocompromised hosts, that is, the failure to control latent infection.

**The v-cyclin is required for ongoing virus replication in the lungs of immunocompromised mice.** Another important parameter of chronic gammaherpesvirus infection is the establishment of a persistent infection at certain sites in the host. We have previously demonstrated that γHV68 mutants impaired in reactivation from latency (v-cyclin- or v-bcl-2-deficient viruses) are also defective in persistent virus replication in the peritoneum of IFN-γ−/− mice (17). In addition, persistent virus replication in these mice correlated with increased mortality and viruses lacking v-cyclin or v-bcl-2 expression showed improved long-term survival (17). On the basis of these observations, there appears to be an intimate link between reactivation and persistent infection.

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To further understand the requirements for v-cyclin in persistent infection, we quantified persistent infection by using mechanically disrupted cells from both the peritoneum and lungs of infected BL/6, CD8−/−, and MuMT mice. Notably, we detected no persistent production of virus in the peritoneum of BL/6, CD8−/−, or MuMT mice at 6 months p.i. (see disrupted samples, as indicated by closed symbols in Fig. 1A, 2A, and 3A), despite ready detection of cells carrying viral DNA (Fig. 1B, 2B, and 3B). Latently infected cells carry episomal viral
Mice as follows: BL/6, wt 0.003 at 1 year p.i.). The fact that mortality is decreased significantly, but not eliminated, is consistent with a significant decrease in the mortality of MuMT mice infected with v-cyclin-deficient virus (Fig. 5A). Interestingly, although CD8<sup>−/−</sup> mice are immunocompromised and fail to effectively control both reactivation from latency and persistent lung infection, neither wt γHV68-infected nor v-cyclin-deficient virus-infected CD8<sup>−/−</sup> mice exhibited significant mortality over 6 months of infection (Fig. 5C). In contrast, more than 60% of MuMT mice infected with wt γHV68 succumbed to chronic infection (Fig. 5B). Remarkably, the mortality of MuMT mice infected with v-cyclin-deficient virus was significantly decreased, with less than 20% of the animals dying by 1 year p.i. (Fig. 5B; P < 0.008 at 6 months p.i., P < 0.003 at 1 year p.i.). The fact that mortality is decreased rather than eliminated is consistent with a significant, but not essential, role for the v-cyclin in reactivation from latency, as previously reported (38, 17). On the basis of these data, the v-cyclin is critical for lethality in a model of gammaherpesvirus-induced mortality in immunocompromised animals. These data further suggest that ongoing reactivation from latency is critical for lethality in the MuMT model of chronic infection and that ongoing reactivation and its resolution are inherently different in CD8<sup>−/−</sup> mice.

**DISCUSSION**

Chronic gammaherpesvirus infection exists in a delicate balance between host and viral factors. One of the most important mechanisms of gammaherpesvirus regulation is the host immune system. While multiple cell types and effector mechanisms are responsible for controlling gammaherpesvirus infec-
tion (5, 11, 40), there is an emerging appreciation that different mechanisms of immunity regulate different stages and sites of chronic infection (e.g., see reference 36). This is nicely illustrated by the common inability of CD8\(^{-/-}\) and MuMT mice to control latency, reactivation, and persistence, yet only MuMT mice display significant virus-associated mortality. While the basis of this differential mortality is unknown, it is clear that viral persistence is not strictly correlated with mortality, as even v-cyclin-infected animals with virtually no detectable persistence succumb to infection. One possibility is that there is viral persistence at an undetected site or at an extremely low level that predisposes to mortality. Interestingly, MuMT mice are also susceptible to large-vessel arteritis (42) in chronic infection with wt \(\gammaHV68\) but with a decreased incidence following infection with v-cyclin-deficient viruses (17). Induction of arteritis in CD8\(^{-/-}\) mice has not been described to date. Chronic infection may also result in immune-mediated pathology involving CD8\(^{+}\) T cells, as has been shown in \(\gammaHV68\)-infected spleens of IFN-\(\gamma\)-receptor\(^{-/-}\) mice at early times (12). Alternatively, antibody may be important for quenching of reactivation events and for prevention of systemic spread and infection of distant tissues in vivo. In contrast, CD8\(^{+}\) T cells may primarily provide local control of virus reactivation by killing infected cells. Consistent with this hypothesis, recent reports have demonstrated that passive transfer of immune antisera restores an increased level of immunological control on reactivation from latency and persistence in mice lacking protective antibodies (16, 20).

Notably, the dynamics of chronic infection appear to be highly regulated by the host immune response. While immune status regulates reactivation and persistence, it may also contribute to the establishment of qualitatively different forms of latency, as has been demonstrated by the distinct transcriptional programs of EBV infection in humans (25). Failure to control chronic infection is exemplified in MuMT mice, which exhibit hyperreactivation from latency and an apparent ongoing reseeding and replenishment of the latency pool derived from spontaneous reactivation in vivo. This model is supported by three pieces of data: (i) v-cyclin-deficient viruses that are defective in reactivation fail to be maintained efficiently in MuMT mice, (ii) passive transfer of neutralizing antibodies to virion proteins results in decreased reactivation and latency in MuMT mice (16, 20), and (iii) treatment of infected MuMT mice with an antiviral inhibitor of lytic replication results in a decreased frequency of latently infected cells (16).

These observations suggest a model in which B cells have a critical role as a long-lived reservoir of \(\gammaHV68\) latency exempt from the requirements of v-cyclin-mediated reactivation and reseeding and that these cells are unique relative to other latently infected cell types, including macrophages, dendritic cells, and lung epithelial cells. Previous work by our group has established that macrophages are a major latency reservoir in the peritoneum of both normal BL/6 mice and B-cell-deficient mice (44). The requirement for the v-cyclin in B-cell-deficient mice suggests that \(\gammaHV68\) genes may have cell type-specific roles and that the B-cell latency reservoir may be independent of v-cyclin and/or v-cyclin-dependent reactivation. In support of this concept, recent work has demonstrated that \(\gammaHV68\) latency is found predominantly in germinal-center B cells (14) and memory B cells (D. Willer and S. H. Speck, unpublished data) (14). Memory B cells represent a long-lived cell type that may allow long-term maintenance of latency independent of reactivation. This is consistent with EBV, a human gammaherpesvirus in which long-term latency reservoirs have also been identified in memory B cells (34). The requirement for the v-cyclin in long-term maintenance of latency in the absence of B cells is consistent with either a gradual accumulation of latency exclusively within B cells in normal mice infected with v-cyclin-deficient virus or with ongoing latent infection of multiple cell types with reseeding from the B-cell compartment. While the v-cyclin and v-cyclin-dependent reactivation are not required for long-term latency in the presence of B cells, the v-cyclin is, in fact, expressed in these latently infected B cells (14) and may have a function that has yet to be described.

Vaccination against gammaherpesviruses has been a major goal in preventing gammaherpesvirus-associated morbidity and mortality (22, 28, 29); however, it has not yet proven efficient in preventing chronic disease (4). Recent studies have demonstrated effective protection from wt \(\gammaHV68\) latency (35) by vaccination with a v-cyclin-deficient live attenuated virus; this promising strategy has been predicted to result in prevention of chronic disease. However, it is critical to continue to consider alternative targets and approaches to control established chronic gammaherpesvirus infection. This study establishes that the v-cyclin and v-cyclin-dependent reactivation are critical for chronic gammaherpesvirus-associated disease in MuMT mice. Together with the previous demonstration that the v-cyclin and v-cyclin-dependent reactivation are critical to chronic gammaherpesvirus-associated disease in IFN-\(\gamma\)-deficient mice, this work suggests that the v-cyclin and v-cyclin-dependent reactivation are potential points of intervention in chronic gammaherpesvirus disease. These data are consistent with chronic gammaherpesvirus diseases associated with ongoing persistent infection or reactivation that have been effectively treated with antiviral drugs known to inhibit viral replication (1, 10, 23). The gammaherpesvirus v-cyclins have unique biochemical features (19, 33) distinct from host cyclins that may allow the development of highly specific antiviral therapies for chronic gammaherpesvirus disease associated with reactivation and persistent infection.

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