Identification of the In Vivo Role of a Viral bcl-2

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Abstract

Many γ-herpesviruses encode candidate oncogenes including homologues of host bcl-2 and cyclin proteins (*v-bcl-2*, *v-cyclin*), but the physiologic roles of these genes during infection are not known. We show for the first time in any virus system the physiologic role of *v-bcl-2*. A γ-herpesvirus *v-bcl-2* was essential for efficient ex vivo reactivation from latent infection, and for both persistent replication and virulence during chronic infection of immunocompromised (interferon [IFN]-$\gamma^{-/-}$) mice. The *v-cyclin* was also critical for the same stages in pathogenesis. Strikingly, while the *v-bcl-2* and *v-cyclin* were important for chronic infection, these genes were not essential for viral replication in cell culture, viral replication during acute infection in vivo, establishment of latent infection, or virulence during acute infection. We conclude that *v-bcl-2* and *v-cyclin* have important roles during latent and persistent γ-herpesvirus infection and that herpesviruses encode genes with specific roles during chronic infection and disease, but not acute infection and disease. As γ-herpesviruses primarily cause human disease during chronic infection, these chronic disease genes may be important targets for therapeutic intervention.

Key words: viral genes • viral latency • reactivation • persistent replication • chronic infection

Introduction

Regulation of both cell cycle progression and apoptosis is critical for many aspects of virus infection, particularly for oncogenic DNA viruses such as adenoviruses, polyomaviruses, papillomaviruses, and γ-herpesviruses. Adenoviruses, polyomaviruses, and papillomaviruses target the functions of pRb and p53 to regulate cellular functions such as cell cycle and apoptosis with significant consequences including oncogenesis (1–4). Oncogenic γ-herpesviruses manipulate the same aspects of cell function via expression of multiple genes including *v-bcl-2* and *v-cyclin*. While these genes clearly regulate important aspects of cellular physiology, the physiologic role of these genes during infection is unknown.

v-bcl-2 genes are encoded by oncogenic γ-herpesviruses including the human γ-herpesviruses EBV and Kaposi's sarcoma herpesvirus (KSHV),* the primate γ-herpesvirus herpesvirus saimiri (HVS), and the closely related murine virus γ-herpesvirus 68 (γHV68) (5–10). Adenovirus and African swine fever virus also encode anti-apoptotic v-bcl-2 proteins (11–16). The in vivo role of *v-bcl-2* genes has never been defined. However, the capacity of these proteins to inhibit apoptosis is clear since transient expression of γ-herpesvirus v-bcl-2 proteins is anti-apoptotic for cells in culture (6–10). An EBV *v-bcl-2* mutant has been constructed, but had no detectable phenotype in immortalization of primary human B cells (17). This lack of phenotype suggests that the *v-bcl-2* gene may play a role in infection that can only be detected by in vivo experimentation.

Consistent with a role for regulation of cell cycle progression in γ-herpesvirus infection and disease, KSHV, HVS, and γHV68 encode a *v-cyclin,* and EBV regulates the expression of host cyclin molecules (5, 18–25). To determine the in vivo role for *v-cyclin* we characterized γHV68 *v-cyclin* mutants. We found that the γHV68 *v-cyclin* is onco-

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**Abbreviations used in this paper:* CPE, cytopathic effect; γHV68, γ-herpesvirus 68; HVS, herpesvirus saimiri; KSHV, Kaposi's sarcoma herpesvirus; MEF, mouse embryonic fibroblast; RAG, recombination activating gene; wt, wild-type.

genic when expressed in transgenic mice (26), and is required for efficient ex vivo reactivation from viral latency, but not for viral replication in wild type mice during acute infection (18, 27). The role of the *v-cyclin* in persistent replication and chronic disease has not been assessed.

In this study we use loss of function mutagenesis to evaluate the role of the *v-bcl-2* gene in γHV68 infection in vivo. Because regulation of cell cycle and apoptosis is intimately intertwined during infection with papillomaviruses, polyomaviruses, and adenoviruses, we compared properties of *v-bcl-2* and *v-cyclin* mutants. We found that neither of these genes has an important role during acute infection, but that both are important for ex vivo reactivation from latency, persistent replication, and disease during chronic infection.

Materials and Methods

Viruses and Tissue Culture. γHV68 clone WUMS (ATCC VR1465) was passaged, grown, and titered in NIH 3T12 cells or BALB/c or C57Bl/6 murine embryonic fibroblasts (MEFs) as described (18, 28). Viral stocks were generated from NIH 3T12 cells infected at multiplicity of infection (MOI) = 0.05 and harvested at 50% cytopathic effect (CPE) (18).

Generation of Mutant γHV68 by Homologous Recombination. The parental genomic subclone for the targeting plasmids contains the 3,723 bp BamHI/BsrGI fragment of γHV68 from genomic coordinates 101,654 to 105,377 in Litmus 38 (pL3700; references 5 and 18). The pL3700-v-bcl-2.Stop1 mutant targeting plasmid was generated using PCR and primers that excise 70 bp (from 103,641 to 103,711) and insert the 9 bp, 5′ CTCGAG-T**AG** 3′, which includes an XhoI site (underlined) and an in frame stop codon (bold). For pL3700-v-bcl-2.Stop2, the primers insert 7 bp, 5′ AGC**TAG**C 3′, which includes an NheI site (underlined) and a stop codon (bold) at genomic coordinate 103,450. The *v-bcl-2* mutant viruses were generated by transfection of NIH 3T12 cells with v-cyclin.LacZ virus genomic DNA (1.5 μg) and pL3700-v-bcl-2 mutant plasmid (1.5 μg) (18, 29). Recombinant virus was purified by white plaque morphology after staining with X-Gal (5-bromo-4-chloro-3-indole-β-D-galactoside) (18). The *v-cyclin* marker rescue virus (v-cyclin.MR) has been described previously (18). Plaque purified (three rounds) viral stocks were characterized by Southern blot and Western blot analyses (18, 29).

Mice, Infections, and Organ Harvests. Recombination activating gene (RAG)-$1^{-/-}$ (The Jackson Laboratory; 002096) and IFN-$\gamma^{-/-}$ mice (The Jackson Laboratory; 002287) on a C57Bl/6 background (B6) were bred and maintained at Washington University School of Medicine in accordance with all federal and university policies. C57Bl/6J (B6) mice were purchased from The Jackson Laboratory. γ interferon receptor-deficient (IFNγ$R^{-/-}$) mice on a 129 background were obtained from Michel Aguet, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland (30). Unless otherwise stated, mice were age and sex matched, used between 7 to 10 wk of age, and infected with 10, 10^3, or 10^6 PFU of γHV68 by intraperitoneal injection in 0.5 ml of complete DMEM (18, 29). CD-1 outbred lactating mice with pups (12 d old) were obtained from Charles River Laboratories, and the pups were infected intracerebrally with virus diluted in 10 μl of complete DMEM. Organs for titer were frozen at −80°C in 1 ml DMEM before plaque assay (18, 29). Peritoneal cells were harvested by peritoneal lavage with 10 ml DMEM (31). To assess inflammatory disease in the great elastic arteries, the heart and attached aortic base were harvested at the time of death or sacrifice from IFNγ$R^{-/-}$ mice and analyzed for incidence and severity of arteritic lesions (32, 33).

Quantitation of Cells Harboring γHV68 Genome. The frequency of cells harboring the γHV68 genome was determined by a limiting dilution nested PCR assay that amplifies γHV68 gene 50 sequences with single copy sensitivity (31, 34). Briefly, peritoneal cells and splenocytes were frozen in 10% DMSO at −80°C, thawed, counted, resuspended in isotonic buffer, and serially diluted into 96-well PCR plates. Uninfected NIH 3T12 cells were added so that each well contained 10^4 cells. Cells were then lysed in proteinase K and nested PCR performed (31, 34). 10 PCR reactions were analyzed for each cell dilution, with six dilutions per sample per experiment. Control reactions in each experiment included uninfected cells alone (6 reactions/plate) or cells with 10, 1, or 0.1 copies of plasmid DNA (p*Bam* HIN) containing target sequence (six reactions/plate each). There were no false-positive PCR reactions in assays reported here, and all assays demonstrated approximately one-copy sensitivity for plasmid DNA, with reactions containing 10, 1, or 0.1 copies of plasmid DNA positive in 89, 29, and 3% of all reactions.

Ex Vivo Reactivation from Latency. The frequency of cells reactivating from latency ex vivo was determined using a limiting dilution reactivation assay (28, 34). Briefly, peritoneal cells and splenocytes were harvested 16, 28, or 42 d after infection, and plated in serial twofold dilution (starting at 4×10^4 peritoneal cells and 10^5 splenocytes per well) onto MEF monolayers in 96-well plates. Wells were scored microscopically 21 d later for viral CPE. When CPE was difficult to discern at high cell numbers, wells were replated onto fresh MEFs and CPE assessed. 24 wells were plated per dilution and 12 dilutions were plated per sample. Preformed virus in tissues was detected by plating parallel cell samples that had been subjected to mechanical disruption. Mechanical disruption does not inactivate virus but kills >99% of cells, and thus samples treated in this way detect preformed virus rather than virus reactivating from latency (28, 31, 34).

Statistical Methods. All data was analyzed using GraphPad Prism software (GraphPad Software). Titer data were statistically analyzed with the nonparametric, Mann-Whitney *t* test. Frequencies were obtained from the cell number at which 63% of the wells scored positive for either reactivating virus or the presence of viral genome based on Poisson distribution. Data were subjected to nonlinear regression analysis to obtain single-cell frequency for each limiting dilution analysis. The frequencies of reactivation and genome-positive cells were statistically analyzed by paired *t* test. Alterations in the incidence of arteritis were compared using χ-square test and Fisher's exact test (which gave comparable results).

Results

Generation of Viruses Containing Mutations in the γHV68 v-bcl-2 Gene. To determine the role of *v-bcl-2* in viral infection, we constructed four γHV68 *v-bcl-2* mutants. The γHV68 v-bcl-2 ORF (M11 original designation) spans 513 bp located between the *v-cyclin* gene (ORF72) and ORF73 (Fig. 1 A). Of the four recognized bcl-2 homology domains (BH domains; BH1-BH4) that are conserved between different bcl-2 family members, the predicted γHV68-v-bcl-2 protein has a recognizable BH1-like do-

main, but lacks clear BH2, BH3, or BH4 domains (5–7). The γHV68 v-bcl-2 also shares with bcl-2 and v-bcl-2 proteins a hydrophobic COOH-terminal domain (5–10). Presuming the functional importance of conserved regions of the protein, we constructed two γHV68 mutants with stop codons and frame shift mutations in the v-bcl-2 ORF (v-bcl-2.Stop1, v-bcl-2.Stop2) in which neither the BH1 domain nor the COOH-terminal hydrophobic domain should be translated (Fig. 1). The Stop1 mutation deletes the predicted BH1 domain and inserts a stop codon after 76 amino acids of the predicted 171 amino acid v-bcl-2 (Fig. 1 B). v-bcl-2.Stop2 contains a mutation that inserts a stop codon and frame shift mutation after 10 amino acids of the v-bcl-2 (Fig. 1 B). We generated two independent clones of each construct, indicated as A and B. The genomic structure of v-bcl-2.Stop1A and 1B, and v-bcl-2.Stop2A and 2B were confirmed by Southern blot analyses (Fig. 1 C). Western blot analysis showed that v-bcl-2.Stop viruses express v-cyclin normally in lytically infected 3T12 fibroblasts (Fig. 1 D).

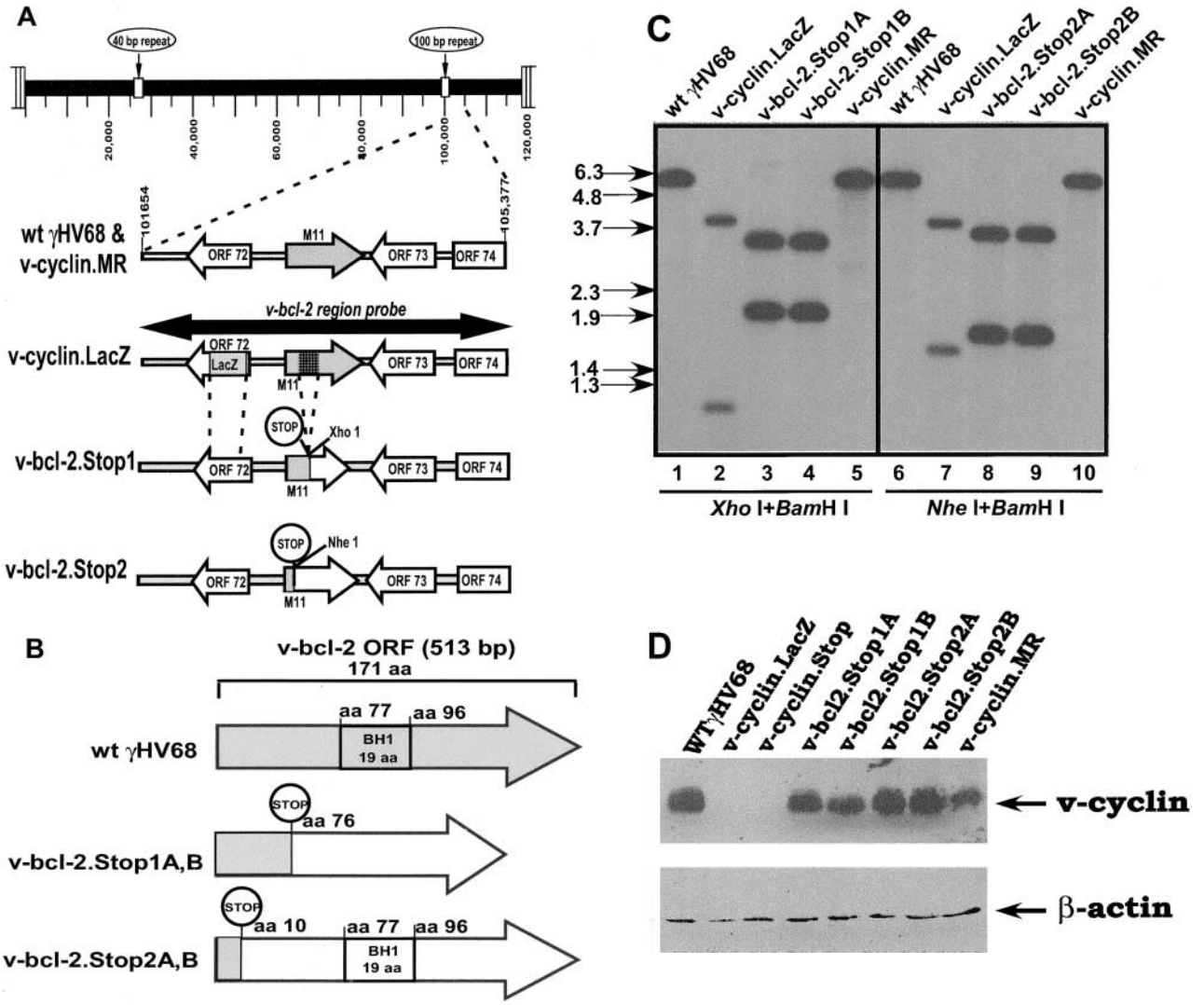

Figure 1. Genomic structure of v-bcl-2.Stop mutant viruses. (A) Schematic representation of the region of the γHV68 genome encoding the *v-bcl-2* gene (M11 ORF; genomic coordinates 103,418–103,930) (reference 5), the structure of *v-bcl-2* mutant viruses and the *v-bcl-2* region Southern blot probe (genomic coordinates 101,654–105,377) (reference 5). The shaded region within ORF72 in the v-cyclin.LacZ virus represents a LacZ cassette insertion between 102,704 bp and 103,179 bp (reference 18). The crosshatched region in the M11 ORF in v-cyclin.LacZ signifies the part of the M11 ORF deleted in v-bcl-2.Stop1. (B) Schematic representation of the v-bcl-2 ORF showing the position of the conserved BH1 domain and the sites of Stop1 and Stop2 mutations in the v-bcl-2 ORF. Shaded regions are the portions of the ORF predicted to be translated. (C) Southern analysis of wt γHV68, v-bcl-2.Stop1 and Stop2 mutants, v-cyclin.LacZ, and v-cyclin.MR viruses. Purified genomic viral DNA (1 μg) from the indicated viruses was digested with XhoI and BamHI (lanes 1–5) or NheI and BamHI (lanes 6–10) and Southern analysis performed with the *v- bcl-2* region probe (A; references 18 and 29). Expected fragment sizes for XhoI and BamHI digests: lane 1/wt γHV68, 5.2 kb; lane 2/v-cyclin.LacZ mutant, 3.8 kb and 1.1 kb; lane 3/v-bcl-2.Stop1A mutant, 3.3 and 1.9 kb; lane 4/v-bcl-2.Stop1B mutant, 3.3 and 1.9 kb; lane 5/v-cyclin.MR, 5.2 kb. Expected fragment sizes for NheI and BamHI digests: lane 6/wt γHV68, 5.2 kb; lane 7/v-cyclin-LacZ mutant, 3.8 kb and 1.5 kb; lane 8/v-bcl-2.Stop2A mutant, 3.5 and 1.7 kb; lane 9/v-bcl-2.Stop2B mutant, 3.5 and 1.7 kb; lane 10/v-cyclin.MR; 5.2 kb. (D) Immunoblot analysis of wt γHV68, v-bcl-2.Stop1 and Stop2 mutants, v-cyclin.LacZ, or v-cyclin.MR virus infected NIH 3T12 cell lysates for v-cyclin and β-actin protein expression.

As v-bcl-2.Stop viruses were derived from the γHV68 mutant v-cyclin.LacZ, we compared the mutant viruses to both wild-type γHV68 (wt γHV68) and v-cyclin.MR, a virus in which the v-cyclin.LacZ mutation has been rescued using wt viral sequences (18). To definitively map the phenotypes we report to the v-bcl-2 gene, we characterized two independently generated viral mutants containing each of two different mutations in the v-bcl-2 gene. The use of two independent isolates to map a phenotype to a herpesvirus gene is an accepted approach (35–37).

No Role for v-bcl-2 in Acute Replication. We first tested whether the γHV68 *v-bcl-2* was required for viral replication in cultured cells or in acutely infected wt B6 or immunocompromised IFN-$\gamma^{-/-}$ mice on the B6 background. As predicted from studies of an EBV *v-bcl-2* mutant (17), γHV68 v-bcl-2.Stop1 and v-bcl-2.Stop2 grew normally in cultured cells under both single step and multi-step growth conditions (Fig. 2, A and B). *v-bcl-2* mutants also replicated as well as wt γHV68 in the spleen and liver of wt B6 mice 4 or 9 d after infection as measured by plaque assay (Fig. 2 C, left panel). Similarly, growth of *v-bcl-2* mutants during acute infection of IFN-$\gamma^{-/-}$ was indistinguishable from growth of wt γHV68 (Fig. 2 C, right panel). These data show that *v-bcl-2* is not essential for replication during acute infection in either wt B6 or immunocompromised IFN-$\gamma^{-/-}$ mice. We have previously demonstrated that the *v-cyclin* is not required for replication during acute infection of wild type mice (18). We confirmed this finding and additionally showed that the v-cyclin, similar to the *v-bcl-2*, is not essential for replication in IFN-$\gamma^{-/-}$ mice (Fig. 2 C, right panel). While a 1.8-fold difference in titer between the wt γHV68 and v-cyclin.MR at day 9 in B6 mice, and a 2.4-fold difference between v-cyclin.MR and v-cyclin. Stop at day 4 in IFN-$\gamma^{-/-}$ mice reached statistical difference for spleen, the biological significance of such small differences in titer are unclear and do not influence our conclusions regarding v-bcl-2 mutants.

v-bcl-2 Is Important for Ex Vivo Reactivation from Latency, but Not Establishment of Latency. Given the lack of a role for *v-bcl-2* during acute infection, we turned our attention to parameters of chronic infection. We examined the role of *v-bcl-2* in establishment of latency (stable carriage of the viral genome in a cell without active replication) and ex vivo reactivation from latency. By 16 d after infection, infectious γHV68 is cleared from the spleen and peritoneal cells of wt B6 mice and latency is established (18, 28, 29, 32, 34). The γHV68 *v-bcl-2* is not required for establishment of latency since splenocytes (data not shown) and peritoneal cells harvested 16 or 42 d after infection of B6 mice with wt γHV68, v-cyclin.MR, or v-bcl-2.Stop mutants contained equivalent frequencies of viral genome bearing cells (Fig. 3 A). Despite normal establishment of latency and absence of productive infection as shown by the disrupted samples in Fig. 3 B, v-bcl-2.Stop mutants reactivated 4–5-fold less efficiently than either wt γHV68 or v-cyclin.MR virus in the peritoneal exudate cells (PECs) but not in the spleen (data not shown) both day 16 and day 42 after infection (Fig. 3 B). This viral phenotype is similar to *v-cyclin* mutants which also fail to efficiently reactivate from latency (18, 27), suggesting that regulation of both apoptosis and cell cycle is key to ex vivo reactivation from the latent state.

The γHV68 v-bcl-2 and v-cyclin Are Necessary for Persistent Replication of Virus in Immunocompromised Hosts. Persistent replication of γ-herpesviruses likely contributes to both disease in immunocompromised hosts and spread of

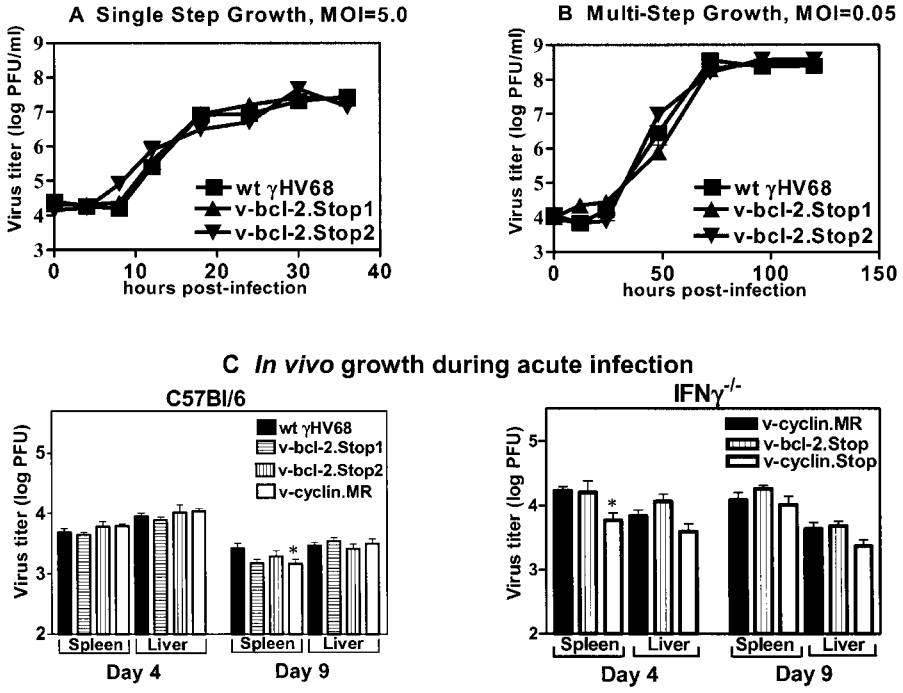

Figure 2. γHV68 *v-bcl-2* mutant viruses grow like wt γHV68 in cultured cells and during acute infection of mice. NIH 3T12 cells were infected with 5.0 (A) or 0.05 (B) PFU of virus per cell and viral titer determined at the times indicated. Data is pooled from two experiments (mean ± SEM). (C) Viral titers in spleen and liver of B6 (left panel) and IFN-$\gamma^{-/-}$ (right panel) mice 4 and 9 d after intraperitoneal infection with 10^6 PFU of virus. Data is pooled from two separate experiments with five mice total per time point/experiment (mean ± SEM). The virus titer in the spleen were statistically significant (*) as follows. For B6 mice, day 9 spleen; wt γHV68 versus v-cyclin.MR, 1.8-fold difference, $P = 0.03$. For IFN-$\gamma^{-/-}$ mice, day 4 spleen; v-cyclin.MR versus v-cyclin.Stop, 2.4-fold difference, $P = 0.001$.

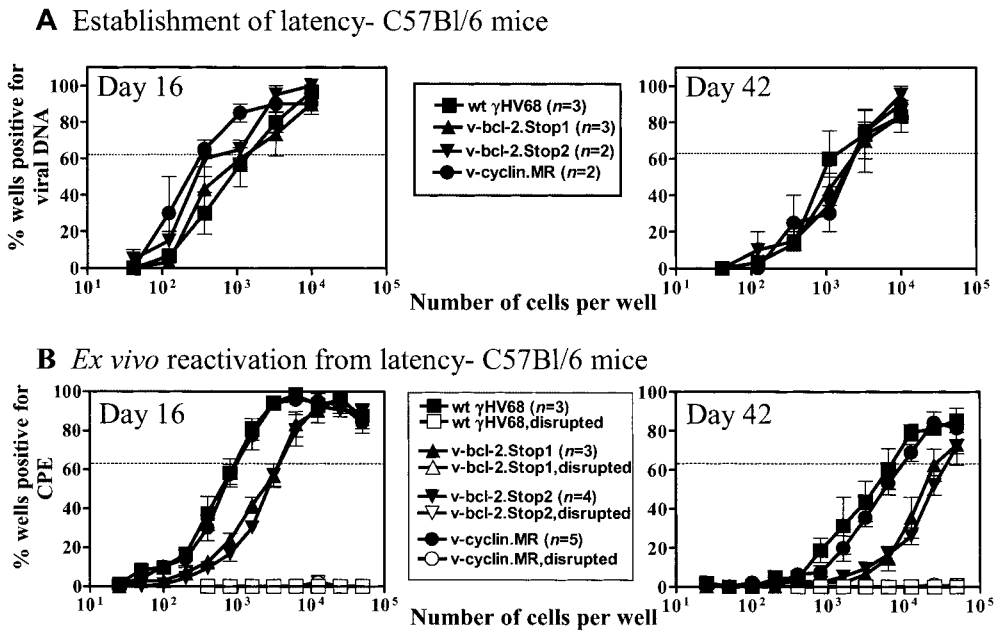

Figure 3. *v-bcl-2* mutants establish latency normally but fail to reactivate efficiently during ex vivo culture. B6 mice were infected with 10^6 PFU of virus and peritoneal cells were harvested at 16 or 42 d after infection for quantitation of the frequency of latently infected cells (A) and the frequency of cells reactivating from latency (B). Data represent results of two to five separate experiments using two independent mutant isolates (mean ± SEM), with each experiment containing cells pooled from five mice. The difference in frequency of genome positive cells at day 16 and day 42 were not statistically significant between the wt γHV68 versus v-bcl-2.Stop1 and v-cyclin.MR versus v-bcl-2.Stop2. At 16 d (A and B, left panels), the decreased frequencies of ex vivo reactivation of both v-bcl-2.Stop mutants compared with wt γHV68 and v-cyclin.MR were statistically significant as follows: v-bcl-2.Stop1 versus wt γHV68, $P = 0.006$; v-bcl-2.Stop2 versus wt γHV68, $P = 0.005$; v-bcl-2.Stop1 versus v-cyclin.MR, $P = 0.013$; v-bcl-2.Stop2 versus v-cyclin.MR, $P = 0.010$. At 42 d (A and B, right panels), the decreased frequencies of ex vivo reactivation of both v-bcl-2.Stop mutants compared with wt γHV68 and v-cyclin.MR were statistically significant as follows: v-bcl-2.Stop1 versus wt γHV68, $P = 0.004$; v-bcl-2.Stop2 versus wt γHV68, $P = 0.005$; v-bcl-2.Stop1 versus v-cyclin.MR, $P = 0.006$; v-bcl-2.Stop2 versus v-cyclin.MR, $P = 0.009$.

the viruses between hosts. Consistent with prior reports (18, 29), we did not detect persistent replication of either wt γHV68 or v-bcl-2.Stop viruses in the spleen (data not shown) or peritoneal cells of normal mice 16 and 42 d after infection (Fig. 3 B, open symbols). However, wt γHV68 persistently replicates in peritoneal cells of IFN-$\gamma^{-/-}$ mice for at least 6 wk after infection (unpublished data), providing a model for determining whether the *v-bcl-2* has a role in persistent replication. One scenario is that persistent replication is due to reactivation from latency. Because γHV68 *v-cyclin* mutants share with *v-bcl-2* mutants (above) the phenotype of decreased reactivation from latency despite normal establishment of latency (18), we analyzed *v-bcl-2* and *v-cyclin* mutants for their capacity to persistently replicate in IFN-$\gamma^{-/-}$ mice.

Consistent with findings in immunocompetent B6 mice (Fig. 3), v-bcl-2.Stop1 and v-bcl-2.Stop2 showed 4–5-fold decreased frequency of reactivation from peritoneal cells of IFN-$\gamma^{-/-}$ mice compared with wt γHV68 and v-cyclin.MR virus (Fig. 4, left panels). This decrease in ex vivo reactivation was not due to a decrease in the number of cells carrying the viral genome because PECs from IFN-$\gamma^{-/-}$ mice infected with v-cyclin.MR, v-bcl-2.Stop, or v-cyclin.Stop mutant contained comparable frequency of genome positive cells (Fig. 4 D). The *v-cyclin* is also required for efficient ex vivo reactivation from latency in cells from IFN-$\gamma^{-/-}$ mice (Fig. 4, A–C, left panels).

Interestingly, neither v-bcl-2.Stop nor v-cyclin.Stop mutants showed significant persistent replication in IFN-$\gamma^{-/-}$ mice (Fig. 4, A–C, right panels). Persistent replication was detected for both wt γHV68 and control virus v-cyclin.MR. These results demonstrated the requirement for both *v-bcl-2* and *v-cyclin* genes for persistent replication in immunocompromised hosts. This is in striking contrast to the lack of a role for these genes in acute replication in either wt or IFN-$\gamma^{-/-}$ mice (Fig. 2). This demonstrates that different viral genes are required for acute replication and persistent replication, strongly arguing that acute and persistent γ-herpesvirus replication are genetically distinct processes.

Requirement for v-bcl-2 and v-cyclin for Lethal Chronic Disease but Not Lethal Acute Disease. The data presented so far argues for a specific role of *v-bcl-2* and *v-cyclin* in chronic but not acute infection. If this distinction is physiologically important, these genes should be required for virulence in models of chronic disease but not for virulence during acute infection. Chronically infected IFN-$\gamma^{-/-}$ and IFNγR$^{-/-}$ mice develop lethal persistent infection and severe large vessel vasculitis (32). The vasculitis is due to persistent replication in smooth muscle cells of the immunoprivileged media of the great elastic arteries (33). Consistent with the data above demonstrating a critical role for *v-cyclin* and *v-bcl-2* in persistent replication in IFN-$\gamma^{-/-}$ mice, *v-bcl-2* mutant and *v-cyclin* mutant viruses killed IFNγR$^{-/-}$ mice less efficiently than wt virus during chronic infection (Fig. 5 A). Evaluation of pathology of arteritic lesions revealed significant change in incidence of lesions (v-cyclin.MR [14/19 = 74%] versus v-bcl-2.Stop [10/35 = 28%], $P = 0.0002$; v-cyclin.MR versus v-cyclin.Stop [7/20 = 35%], $P = 0.001$) but not in the severity of aortic lesions between

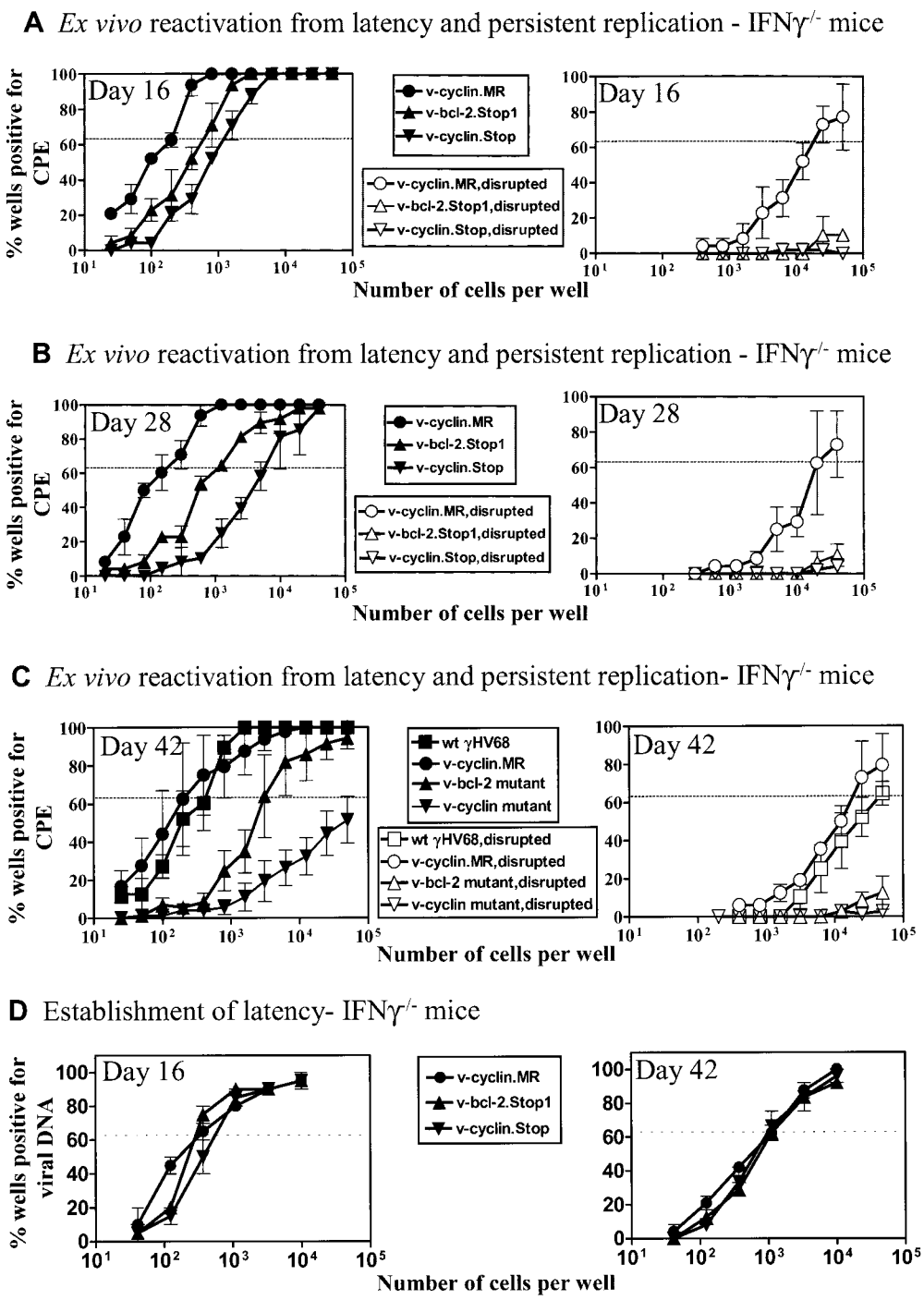

Figure 4. *v-bcl-2* and *v-cyclin* are critical for persistent replication of γHV68 in IFN-$\gamma^{-/-}$ mice. IFN-$\gamma^{-/-}$ mice were infected with 10^6 PFU of virus and peritoneal cells were harvested at 16, 28, or 42 d after infection for quantitation of frequency of cells reactivating virus (A–C, left panels), amount of persistent virus (A–C, right panels), and frequency of latently infected cells (D). Data represent results of two to three experiments (mean ± SEM), with each experiment containing cells pooled from five mice. Since at day 42 time point, no significant difference between v-cyclin.MR and wt γHV68 was observed, only v-cyclin.MR was checked for day 16 and day 28 time points along with two independent isolates of v-bcl-2.Stop1 and v-cyclin.Stop. For day 42 experiments, *v-bcl-2* mutant infection included two experiments with v-bcl-2.Stop1 and one experiment with v-bcl-2.Stop2. Similarly, *v-cyclin* mutant infection included two experiments with v-cyclin.Stop and one experiment with v-cyclin.LacZ at day 42 time point. The decreased frequencies of ex vivo reactivation and persistent productive replication of both *v-bcl-2* mutant and *v-cyclin* mutant compared with wt γHV68 and v-cyclin.MR were statistically significant as follows: day 16 ex vivo reactivation, v-bcl-2.Stop1 versus v-cyclin.MR, $P = 0.007$; v-cyclin.Stop versus v-cyclin.MR, $P = 0.003$; for day 16 persistent productive replication, v-bcl-2.Stop1 versus v-cyclin.MR, $P = 0.014$; v-cyclin.Stop versus v-cyclin.MR, $P = 0.011$, for day 28 ex vivo reactivation, v-bcl-2.Stop1 versus v-cyclin.MR, $P = 0.0009$, v-cyclin.Stop versus v-cyclin.MR, $P = 0.0001$; for day 28 persistent productive replication, v-bcl-2.Stop1 versus v-cyclin.MR, $P = 0.0326$, v-cyclin.Stop versus v-cyclin.MR, $P = 0.028$, and for day 42 ex vivo reactivation, *v-bcl-2* mutant versus wt γHV68, $P = 0.0008$, *v-bcl-2* mutant versus v-cyclin.MR, $P = 0.0003$, *v-cyclin* mutant versus wt γHV68, $P = 0.0001$, *v-cyclin* mutant versus v-cyclin.MR, $P = 0.0001$; for day 42 persistent productive replication, *v-bcl-2* mutant versus wt γHV68, $P = 0.027$, *v-bcl-2* mutant versus v-cyclin.MR, $P = 0.008$, *v-cyclin* mutant versus wt γHV68, $P = 0.032$, *v-cyclin* mutant versus v-cyclin.MR, $P = 0.011$. The difference in frequency of genome positive cells at day 16 and day 42 were not statistically significant between the v-cyclin.MR versus v-bcl-2.Stop1 and v-cyclin.MR versus v-cyclin.Stop.

the v-cyclin.MR, *v-bcl-2* mutant, and the *v-cyclin* mutant virus infected mice (data not shown).

We noted that the phenotype of v-bcl-2 and v-cyclin deficient viruses during chronic infection of IFN-$\gamma^{-/-}$ was in contrast to the lack of a phenotype for these viruses in growth during acute infection of normal and IFN-$\gamma^{-/-}$ mice (Fig. 2 C). We considered the hypothesis that data on acute infection using IFN-$\gamma^{-/-}$ is not comparable to data on chronic infection from IFNγR$^{-/-}$ mice. However, the phenotypes of IFN-$\gamma^{-/-}$ mice and IFNγR$^{-/-}$ are similar using a number of different routes of infection and doses of wt γHV68 (32, 38). To determine if decreased virulence of

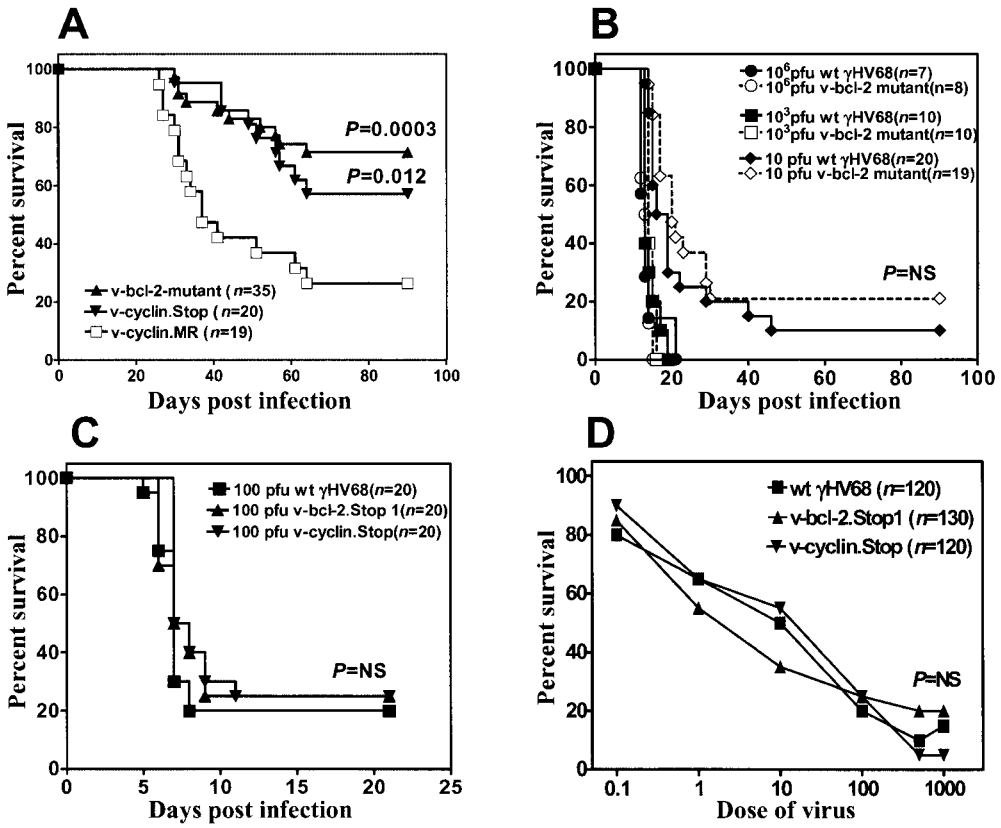

Figure 5. *v-bcl-2* and *v-cyclin* are necessary for lethal chronic disease but not for lethal acute disease. (A) IFNγR$^{-/-}$ mice were infected intraperitoneally with v-cyclin.MR, v-bcl-2.Stop, or v-cyclin.Stop mutant virus and observed for lethality over 90 d after infection. The increase in percent survival of mice infected with *v-bcl-2* mutant virus or v-cyclin.Stop mutant virus compared with v-cyclin.MR virus was statistically significant as follows: v-bcl-2.Stop versus v-cyclin.MR, $P = 0.0003$; v-cyclin.Stop versus v-cylin.MR, $P = 0.012$. (B) Survival of RAG-1$^{-/-}$ mice infected intraperitoneally with 10^6, 10^3, or 10 PFU of virus. Data are pooled from two experiments (n = number of mice). (C and D) Survival of CD1 mice (3–4 wk old) infected intracerebrally with v-cyclin.MR, v-bcl-2.Stop 1, or v-cyclin.Stop virus and observed for lethality over 21 d after infection. Data represent 20–30 mice per point pooled from 2–3 independent experiments. P = NS indicates that there is no statistical difference between the indicated groups.

v-bcl-2 and *v-cyclin* mutant viruses was specific to chronic disease, we tested mutant viruses in two additional models of virulence during acute infection. The virulence of wt γHV68 and v-bcl-2.Stop1 was comparable in RAG-1$^{-/-}$ mice infected with 10, 10^3, or 10^6 PFU (Fig. 5 B). We previously showed that *v-cyclin* mutant γHV68 is not attenuated in this same model (18). γHV68 also causes acute lethal meningitis when administered intracerebrally into weanling mice (39). The virulence of *v-cyclin* and *v-bcl-2* mutants in this lethal meningitis model was indistinguishable from that of wt γHV68 (Fig. 5, C and D). Thus, the lack of virulence of *v-bcl-2* and *v-cyclin* mutant viruses during chronic disease was not seen in two different models of acute disease, further supporting our conclusion that *v-bcl-2* and *v-cyclin* are viral genes with a physiologically important and specific role during chronic infection.

Discussion

We show here for the first time that a γ-herpesvirus *v-bcl-2*, as previously shown for a *v-cyclin*, is necessary for efficient ex vivo reactivation from latency. The importance of both anti-apoptotic and cell cycle regulatory proteins in ex vivo reactivation from latency suggests that the reactivation process requires cell cycle progression and triggers host or cellular apoptotic pathways that serve to prevent reactivation unless the virus counters with anti-apoptotic molecules.

Of special importance for understanding how herpesviruses cause disease, we demonstrate for the first time that herpesviruses have genes (*v-bcl-2* and *v-cyclin*) that are important for persistent replication and chronic disease in an immunocompromised host, but not acute replication and acute disease. This identifies persistent replication as a process that is genetically distinct from replication during acute infection, and shows that genes important for persistent replication are key virulence determinants.

v-bcl-2 Is Required for Efficient Ex Vivo Reactivation from Latency. γHV68 establishes latency in a number of cells types including macrophages, dendritic cells, and B cells (28, 31, 40, 41). We found that establishment of latency does not require v-bcl-2. In this respect, *v-bcl-2* is similar to other γHV68 genes we have analyzed including the *v-cyclin* and *M1* (18, 29). However, the *v-bcl-2* mutation resulted in inefficient ex vivo reactivation of virus from latently infected cells. In this respect the *v-bcl-2* gene is similar to *v-cyclin*, but distinct from the *M1* locus since mutations in the *M1* locus enhance the efficiency of ex vivo reactivation (18, 29). Thus, γHV68 contains genes that both enhance and inhibit ex vivo reactivation, suggesting that the balance between latency and ex vivo reactivation is under tight regulation by multiple viral genes. The importance of *v-bcl-2* for ex vivo reactivation from latency and persistent replication is consistent with our prior demonstration that the *v-bcl-2* gene is actively transcribed in latently infected tissues (42). It is possible that additional

phenotypes of v-bcl-2 mutants may be identified using different route of infection.

Why are both *v-bcl-2* and *v-cyclin* important for efficient ex vivo reactivation from latency? We speculate that latently infected cells are in a resting G_0 state, and that the process of ex vivo reactivation requires the *v-cyclin* for induction of cell cycle progression. In this scenario, the *v-bcl-2* would prevent apoptosis induced either by expression of viral genes critical for ex vivo reactivation or by proapoptotic host genes that come into play during ex vivo reactivation. It is interesting that the KSHV v-cyclin can induce apoptosis in cells when overexpressed, and that this is blocked by coexpression of the KSHV v-bcl-2 (43). Similarly, the γHV68 v-cyclin induces cell cycle progression in transgenic thymocytes and is oncogenic, despite causing increased apoptosis (26). Thus, the v-bcl-2 may be needed to prevent an apoptotic response caused by v-cyclin expression.

It is also possible that the v-bcl-2 prevents apoptosis induced by host proteins. For example, the γHV68 and HVS v-bcl-2 can block both Fas and TNF-α mediated apoptosis when over-expressed (6, 44), and the EBV v-bcl-2 can inhibit granzyme-mediated apoptosis (45). This latter is particularly significant since deficiency of perforin (which is key for induction of apoptosis by host granzymes) results in increased number of latently infected cells (unpublished data). It will be important to determine the precise host and viral pathways for apoptosis induction that are inhibited by v-bcl-2 expression from the viral genome, as opposed to overexpression in cultured cells. It is possible that the v-bcl-2 is critical for blocking apoptosis induced by both host and viral proteins. Identification of apoptotic pathways inhibited by v-bcl-2 may lead to therapies for γ-herpesvirus associated diseases, as enhancement of such pathways, for example by ablating *v-bcl-2* function, may prevent viral reactivation or inhibit persistent replication.

Different Genetic Requirements for Acute and Persistent Replication In Vivo. The demonstration that both the *v-bcl-2* and the *v-cyclin* are critical for persistent replication in an immunocompromised host, but not for acute infection, identifies persistent infection as a genetically distinct phase of herpesvirus infection in vivo. Thus, γ-herpesvirus infection in vivo has the following experimentally distinguishable components: acute replication, establishment of latency, maintenance of latency, persistent replication, and reactivation from latency. The presence of conserved genes with a specialized function during persistent replication rather than replication during acute infection suggests that persistent replication is a physiologically important component of herpesvirus infection.

It is likely that persistent replication contributes to spread of the virus within the population. In addition, it has been suggested that persistent replication may contribute to tumorigenesis by γ-herpesviruses (46). It is therefore possible that genes of the virus that are necessary for persistent replication will also contribute to tumorigenesis even if they are not independently oncogenic. This hypothesis predicts that induction of tumors by γ-herpesviruses could be prevented by targeting the function of genes essential for persistent replication, even when the genes have no role in replication in tissue culture, replication during acute infection, or detectable transforming activity. It is important to note that there is no direct evidence to date that persistent replication occurs in normal hosts. However, intermittent reactivation does occur in normal hosts, and considerable evidence shows that T cells recognizing lytic γHV68 antigens are continuously stimulated during latency, suggesting that lytic viral gene expression, and perhaps persistent replication at a level undetectable by current assays, does occur in normal hosts (47–53).

It is interesting that two genes that are important for efficient reactivation ex vivo (*v-cyclin* and *v-bcl-2*) are also important for persistent replication in an immunocompromised host. This strongly supports the hypothesis that persistent virus is derived from reactivation events rather than continued passage of infectious virus from one lytically infected cell to the next. In this model, persistent virus would derive from reactivation events with expansion of the reactivation-derived virus limited by components of the immune system. One such host factor is IFN-γ, which controls latency, persistence, and chronic disease due to infection with both the γ-herpesvirus γHV68 (54; unpublished data) and the γ-herpesvirus murine cytomegalovirus (32, 38, 55). It is possible that effective control of chronic γ-herpesvirus infection would best be accomplished by simultaneous blockade of the function of genes critical for reactivation and persistent replication and enhancement of immune functions such as IFN-γ that are critical for controlling reactivation from latency and persistence.

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