Novel Role of vBcl2 in the Virion Assembly of Kaposi's Sarcoma-Associated Herpesvirus

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ABSTRACT The viral Bcl-2 homolog (vBcl2) of Kaposi's sarcoma-associated herpesvirus (KSHV) displays efficient antiapoptotic and antiautophagic activity through its central BH3 domain, which functions to prolong the life span of virus-infected cells and ultimately enhances virus replication and latency. Independent of its antiapoptotic and antiautophagic activity, vBcl2 also plays an essential role in KSHV lytic replication through its amino-terminal amino acids (aa) 11 to 20. Here, we report a novel molecular mechanism of vBcl2-mediated regulation of KSHV lytic replication. vBcl2 specifically bound the tegument protein open reading frame 55 (ORF55) through its amino-terminal aa 11 to 20, allowing their association with virions. Consequently, the vBcl2 peptide derived from vBcl2 aa 11 to 20 effectively disrupted the interaction between vBcl2 and ORF55, inhibiting the incorporation of the ORF55 tegument protein into virions. This study provides new insight into vBcl2's function in KSHV virion assembly that is separable from its inhibitory role in host apoptosis and autophagy.

IMPORTANCE KSHV, an important human pathogen accounting for a large percentage of virally caused cancers worldwide, has evolved a variety of stratagems for evading host immune responses to establish lifelong persistent infection. Upon viral infection, infected cells can go through programmed cell death, including apoptosis and autophagy, which plays an effective role in antiviral responses. To counter the host response, KSHV vBcl2 efficiently blocks apoptosis and autophagy to persist for the life span of virus-infected cells. Besides its anti-programmed-cell-death activity, vBcl2 also interacts with the ORF55 tegument protein for virion assembly in infected cells. Interestingly, the vBcl2 peptide disrupts the vBcl2-ORF55 interaction and effectively inhibits KSHV virion assembly. This study indicates that KSHV vBcl2 harbors at least three genetically separable functions to modulate both host cell death signaling and virion production and that the vBcl2 peptide can be developed as an anti-KSHV therapeutic application.

KEYWORDS KSHV, vBcl2, lytic replication, ORF55 tegument, virion assembly, Kaposi's sarcoma-associated herpesvirus, autophagy

Kaposi's sarcoma (KS)-associated herpesvirus (KSHV) (human herpesvirus 8 [HHV-8]) belongs to the gammaherpesvirus family, which also includes Epstein-Barr virus (EBV), herpesvirus saimiri (HSV), and murine gammaherpesvirus 68 (MHV-68) (1, 2). KSHV is etiologically linked to KS as well as two rare B-cell proliferative diseases, primary effusion lymphoma (PEL) and multicentric Castlemann's disease (3–5). It has two alternative life cycles, latency and lytic replication. Latency is a dormant state during which KSHV maintains its genome as an episome and expresses only a few viral genes, whereas the lytic cycle leads

Received 2 June 2017 Accepted 17 November 2017

Accepted manuscript posted online 22 November 2017

Citation Liang Q, Wei D, Chung B, Brulois KF, Guo C, Dong S, Gao S-J, Feng P, Liang C, Jung JU. 2018. Novel role of vBcl2 in the virion assembly of Kaposi's sarcoma-associated herpesvirus. *J Virol* 92:e00914-17. <https://doi.org/10.1128/JVI.00914-17>.

Editor Rozanne M. Sandri-Goldin, University of California, Irvine

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to the expression of a full panel of viral genes, the assembly and release of progeny viral particles, and *de novo* infection of other cells (6–8). Although latency is the predominant state in KSHV-induced tumors, lytic replication is detectable in some populations of cells in and around tumor tissues and plays a critical role for KSHV tumorigenesis. Lytic replication of infected cells provides a suitable microenvironment for the proliferation and tumor progression of latently infected cells by producing cytokines and virokines (6, 8, 9). Therefore, inhibiting lytic replication is as important as controlling latent infection for the treatment of KSHV-induced malignancies.

A typical herpesvirus virion consists of four morphologically distinct components: a linear double-stranded DNA (dsDNA) viral genome, an icosahedral capsid that encloses the viral genome, a tegument layer between the capsid and envelope, and an outer envelope with viral glycoproteins on the surface (2, 10). By mass spectrometry analysis of mature KSHV virions, several tegument proteins, such as open reading frame 33 (ORF33), ORF38, ORF45, ORF52, ORF55, ORF63, ORF64, and ORF75, have been identified (11). Among these tegument proteins, some are important for innate immune modulation to facilitate the establishment of persistent infection (12–17), and some are involved in virion tegumentation, assembly, and trafficking (18–23). However, how these tegument proteins are packaged into virions is still largely unknown. Interestingly, some viral proteins, which were previously classified as nonstructural proteins, have been found to be located in mature virions (24), providing new insight into the molecular mechanism of herpesvirus assembly.

Autophagy is an important homeostatic mechanism involving the formation of double-membrane vesicles, called autophagosomes, which sequester cytoplasmic damaged organelles, protein aggregates, or invading intracellular pathogens for degradation. Conserved from yeast to humans, autophagy takes place through a series of steps that include the initiation, elongation, and formation of autophagosomes, followed by fusion with lysosomes for cargo degradation (25, 26). Since autophagy functions in diverse cellular processes, it undergoes delicate regulations at each step (27–30). Recent studies have broadened our understanding of the mechanisms by which herpesviruses modulate autophagy machinery and cellular innate immune responses (1, 31). For instance, vBcl2 (ORF16) interacts with the Beclin-1 complex to downregulate autophagy induction (32), vFLIP (ORF71 or K13) suppresses autophagy at the vesicle elongation step by preventing the Atg3 E2 enzyme from binding and processing LC3 (27), and K7 interacts with Rubicon to impair autophagosome maturation (33). By investigating the roles of these antiapoptotic genes in the context of KSHV, we surprisingly found that vBcl2 is essential for KSHV lytic replication, whereas vFLIP and K7 are dispensable (34). More interestingly, this essential role of vBcl2 for lytic replication does not depend on its antiapoptotic and antiapoptotic activities but depends on its amino-terminal BH4 domain (34). In this report, we identified the tegument protein ORF55 as a novel binding partner of vBcl2 by yeast two-hybrid screening. vBcl2 bound ORF55 through its amino-terminal amino acids (aa) 11 to 20, and disruption of the vBcl2-ORF55 interaction by the vBcl2 peptide (vBcl2p) derived from vBcl2 aa 11 to 20 dramatically suppressed virion production. This study not only demonstrates the molecular mechanism by which vBcl2 controls KSHV virion production but also describes the potential therapeutic application of a vBcl2-derived peptide.

RESULTS

Identification of a vBcl2-derived peptide. Previously, 29 alanine scanning mutants (named A1 to A29) of KSHV vBcl2 carrying a replacement of 4 to 5 aa with alanines were reconstituted in iSLK cells carrying replication-incompetent KSHVΔvBcl2 (BAC16-ΔvBcl2), reconstituted iSLK-BAC16-ΔvBcl2 cells were treated with doxycycline (1 μg/ml) and sodium butyrate (1 mM) for 3 days to induce lytic replication, the progeny virus-containing supernatants were harvested and used to infect naive SLK cells, and the infectious units were finally quantified by flow cytometry analysis for green fluorescent protein (GFP)-positive SLK cells. That previous study showed that mutations of the four regions (A3-A4, A7-A8, A14-A15, and A23-A25) of vBcl2 considerably

FIG 1 vBcl2-derived peptide (vBcl2p) blocks KSHV progeny production. (A) Identification of the functional regions of vBcl2 for KSHV lytic replication. iSLK-BAC16- Δ vBcl2 cells were complemented with lentivirus carrying vBcl2 and its mutants, as indicated, and treated with doxycycline (1 μ g/ml) and sodium butyrate (1 mM) to induce lytic replication. After a 3-day induction, the supernatants containing progeny viruses were used to infect SLK cells, and the infectious units were quantified by fluorescence-activated cell sorter (FACS) analysis with GFP-positive cells. (B and C) iSLK-BAC16 cells were pretreated with increasing amounts of the indicated peptides, and lytic replication was subsequently induced. The progeny viruses were quantified by FACS analysis as described above for panel A.

reduced KSHV progeny production (Fig. 1A). This suggested that vBcl2 might interact with important host or viral factors through these regions and that these interactions with vBcl2 could be critical for effective lytic replication and/or progeny virus production. To test this hypothesis, four peptides (A3-A4, A7-A8, A14-A15, and A23-A25) containing these critical regions of vBcl2 were engineered by an N-terminal fusion with the HIV-Tat membrane penetration sequence (RRRQRRKKRGY). iSLK-BAC16 cells were pretreated with increasing amounts of the Tat control or Tat-vBcl2 peptide and stimulated with doxycycline-sodium butyrate for 3 days. The progeny virus-containing supernatants were then used to infect naive SLK cells to measure GFP-positive infectious titers by flow cytometry analysis. These peptides did not affect the overall morphology of SLK cells. Compared to treatment with the control HIV-Tat peptide, treatment of iSLK-BAC16 cells with the A3-A4 peptide drastically blocked KSHV progeny production. In contrast, treatment of iSLK-BAC16 cells with the A7-A8 peptide, the A14-A15 peptide, or the A23-A25 peptide showed little or no effect on KSHV progeny production (Fig. 1B). A serial truncation of this A3-A4 peptide, designated the vBcl2-derived peptide (vBcl2p), from either the N or C terminus resulted in a significant abrogation of its function to block KSHV progeny production (Fig. 1C). These results suggest that the 10-amino-acid sequence of the vBcl2 peptide is necessary for blocking KSHV progeny production.

vBcl2-derived peptide blocks virion production. To determine whether vBcl2 peptide treatment had an effect on viral gene expression, we took advantage of NanoString technology, a hybridization-based method sensitive enough to detect RNA without an amplification step. It relies on two probes, a capture probe, which allows the immobilization of the target mRNA and imaging on a solid surface, and a reporter probe, which is linked to a color-coded identifier for each gene of interest. We designed a customized panel of probes to quantify viral transcript levels across the entire KSHV genome. iSLK-BAC16 cells were pretreated with the Tat or vBcl2 peptide for 2 h, and the lytic cycle was induced by treatment with doxycycline and sodium butyrate. Total RNA was isolated from peptide-treated iSLK-BAC16 cells at 48 h postinduction (p.i.) and

FIG 2 vBcl2 peptide blocks KSHV assembly. (A) Genome-wide analysis of KSHV transcripts using NanoString technology with treatment with the Tat control peptide or vBcl2p. iSLK-BAC16 cells were pretreated with the Tat or vBcl2 peptide and then lytically induced for 48 h. RNA was isolated, and viral mRNA expression was analyzed with an nCounter analysis system using nSolver analysis software 2.0 (NanoString Technologies). The graph shows relative levels of KSHV transcripts upon treatment with the Tat or vBcl2 peptide. (B) vBcl2 peptide does not affect KSHV protein expression. iSLK-BAC16 cells were pretreated with the Tat or vBcl2 peptide and then lytically induced for 24, 48, and 72 h as described above. Whole-cell lysates were analyzed by immunoblotting (IB) using the indicated antibodies. (C) Relative amounts of viral DNA were quantified by using ORF11-specific primers as described previously (34). (D) iSLK-BAC16 cells were pretreated with the Tat or vBcl2 peptide and then lytically induced for 48 h. Cells were harvested and processed for transmission electron microscopy analysis. The numbers of viral particles in each cell were quantified ($P < 0.05$).

subjected to NanoString analysis. This showed no significant changes in most KSHV transcript levels upon either Tat peptide or vBcl2 peptide treatment (Fig. 2A). Moreover, KSHV proteins and DNA copy numbers were also similar under Tat peptide treatment conditions and vBcl2 peptide treatment conditions (Fig. 2B and C). These data indicate that treatment with the vBcl2 peptide does not affect KSHV gene expression and viral DNA replication. Finally, transmission electron microscopy (TEM) was used to determine whether treatment with the vBcl2 peptide affected virion production. As shown in Fig. 2D, Tat peptide-treated iSLK-BAC16 cells showed numerous premature virion particles in nuclei at 48 h p.i. (hpi), whereas vBcl2 peptide-treated iSLK-BAC16 cells showed few or no virion particles (Fig. 2D). These data indicate that treatment with the vBcl2 peptide drastically reduces KSHV progeny virus production.

vBcl2-derived peptide does not affect the antiapoptotic and antiautophagic functions of vBcl2. Since vBcl2 inhibits cellular apoptosis and autophagy by interacting with Bak and Beclin-1, respectively (32), we tested whether treatment with the vBcl2 peptide affected apoptosis and autophagy. During the autophagy process, microtubule-associated protein light chain 3 (LC3) is converted from the soluble form (LC3-I) to the lipidated form (LC3-II), and lipidated LC3-II is associated with the autophagosome membrane. Therefore, the ratio of LC3-II/LC3-I and the distribution of LC3 punctae indicate the level of intracellular autophagy (25). To examine the effect of the vBcl2 peptide on the inhibition of vBcl2-mediated autophagy, we pretreated SLK-vector and SLK-vBcl2 cells with increasing doses of the vBcl2 peptide, followed by rapamycin ($2 \mu\text{M}$) stimulation for 4 h. This showed that while vBcl2 expression apparently inhibited LC3-I→LC3-II conversion and GFP-LC3 puncta formation in SLK

FIG 3 vBcl2 peptide does not affect autophagy and apoptosis. (A) SLK-vector and SLK-vBcl2 cells were pretreated with the indicated peptides for 0.5 h, followed by rapamycin (Rapa) (2 μ M) stimulation for 4 h. Cells were harvested, and whole-cell lysates were subjected to immunoblotting with anti-LC3 and antiactin antibodies. DMSO, dimethyl sulfoxide. (B) SLK-GFP-LC3-vector and SLK-GFP-LC3-vBcl2 stable cells were pretreated with the indicated peptides and then stimulated with rapamycin (2 μ M) for 4 h. Cells were fixed, stained with DAPI (4',6-diamidino-2-phenylindole), and subjected to confocal microscopy. The numbers of GFP-LC3 punctae per cell and the percentages of GFP-LC3 puncta-positive cells were quantified. (C) SLK-vector and SLK-vBcl2 stable cells were pretreated with the indicated peptides and then treated with TNF- α (5 ng/ml) and CHX (5 μ g/ml) for 12 h. After treatment, cells were collected and subjected to TUNEL staining, and the numbers of apoptotic cells were quantified. Whole-cell lysates were subjected to an ELISA to measure caspase-3 cleavage. (D and E) vBcl2 peptide is not toxic to cells. SLK cells were treated with H₂O, the Tat peptide, or the vBcl2 peptide. Cell viability and growth were monitored as indicated ($P < 0.05$).

cells, vBcl2 peptide treatment did not affect either LC3-I→LC3-II conversion or GFP-LC3 puncta formation regardless of vBcl2 expression (Fig. 3A and B). SLK-vector cells and SLK-vBcl2 cells were also subjected to tumor necrosis factor alpha (TNF- α) and cycloheximide (CHX) treatments to induce apoptosis, followed by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining and an active caspase-3 enzyme-linked immunosorbent assay (ELISA) to measure apoptosis levels. This showed that vBcl2 expression effectively inhibited TNF- α -induced apoptosis, whereas vBcl2 peptide treatment had almost no effect on TNF- α -induced apoptosis as well as vBcl2-mediated inhibition of apoptosis (Fig. 3C). Finally, cell viability and growth rates also showed no significant change upon vBcl2 peptide treatment (Fig. 3D and E). These results indicate that treatment with the vBcl2 peptide does not affect the vBcl2-mediated inhibition of autophagy and apoptosis.

vBcl2 binds ORF55 tegument protein. To determine the molecular target of vBcl2, we used the vBcl2 functional region (aa 1 to 143) as bait to screen the KSHV yeast-two hybrid library and identified the tegument protein ORF55 as a potential binding partner for vBcl2. We also found that vBcl2 efficiently interacted with the ORF55 tegument protein during KSHV lytic replication in iSLK-BAC16-vBcl2-HA cells (Fig. 4A) and under overexpression conditions in HEK293T cells (Fig. 4B). In contrast, vBcl2 did not detectably interact with other viral proteins, including ORF30, -31, and -68 (Fig. 4B). Furthermore, protein purification showed that both vBcl2 and ORF55 were partially coeluted in fractions 30 to 34 by gel filtration chromatography (Fig. 4C). Since the A3-A4 region (aa 11 to 20) of vBcl2 was critical for KSHV lytic replication and the vBcl2 peptide effectively blocked KSHV virion production, we examined whether the vBcl2 A3-A4

FIG 4 vBcl2 binds to ORF55 through its aa 11 to 20. (A) vBcl2 binds to ORF55 during KSHV lytic replication. iSLK-BAC16-vBcl2-HA cells were lytically induced for 72 h as described in the legend of Fig. 1A. Whole-cell lysates (WCL) were subjected to immunoprecipitation (IP) and immunoblotting (IB) or fractionated and analyzed with the indicated antibodies (C). (B) vBcl2 specifically binds to ORF55. HEK293T cells were transfected with the indicated plasmids, and whole-cell lysates were harvested at 48 h posttransfection and subjected to immunoprecipitation and immunoblotting with the indicated antibodies. (C) iSLK-BAC16-vBcl2-HA cells were lytically induced for 72 h, and whole-cell lysates were fractionated and analyzed with the indicated antibodies. (D) vBcl2 binds to ORF55 through its A3-A4 region (aa 11 to 20). Forty-eight hours after transfection with the indicated plasmids, HEK293T cells were harvested, and whole-cell lysates were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. FL, full length. (E) The vBcl2 E14A mutant cannot bind to ORF55. Forty-eight hours after transfection with the indicated plasmids, HEK293T cells were harvested, and whole-cell lysates were subjected to immunoprecipitation and immunoblotting with the indicated antibodies. (F) vBcl2 binds to ORF55 through its A3-A4 region (aa 11 to 20) in yeast-two hybrid assays. The bait vector containing the indicated vBcl2 WT or mutant constructs and the prey vector harboring ORF55 were cotransformed into yeast and screened in 2-dropout (2DO) plates to check transformation and in 4-dropout (4DO) plates with X- α gal to check the interaction. (G) The vBcl2 peptide impairs the interaction between vBcl2 and ORF55 during KSHV lytic replication. iSLK-BAC16-vBcl2-HA cells were pretreated with the Tat or vBcl2 peptide and then lytically induced for 72 h. Whole-cell lysates were subjected to immunoprecipitation and immunoblotting with the indicated antibodies.

region was critical for the vBcl2-ORF55 interaction. Deletion or mutations of the vBcl2 A3-A4 region dramatically reduced the interaction between vBcl2 and ORF55 in HEK293 cells (Fig. 4D) or in yeast (Fig. 4F). In contrast, mutations of the vBcl2 A7 or A14 region showed no effect on this interaction (Fig. 4E). The vBcl2 E14A mutant could not interact with ORF55 as efficiently as wild-type (WT) vBcl2 (Fig. 4E). Furthermore, when iSLK-BAC16-vBcl2-HA cells were treated with the Tat control peptide or the vBcl2 peptide during KSHV lytic replication, the vBcl2-ORF55 interaction was detectably reduced by vBcl2 peptide treatment (Fig. 4G). These results suggest that vBcl2 binds the ORF55 tegument protein via its aa 11 to 20 and that treatment of the vBcl2 peptide markedly blocks their interaction.

vBcl2 is a virion-associated protein that is required for KSHV assembly. To test whether vBcl2 was packaged into virion particles, wild-type KSHV or recombinant KSHV-HA-vBcl2 carrying hemagglutinin (HA)-tagged vBcl2 was harvested, concentrated by ultracentrifugation, and subjected to immunoblot analysis with anti-HA or other antibodies. This showed that like other viral structural proteins, including gB, ORF45, ORF55, and ORF65, vBcl2 was readily detected in KSHV virion lysates (Fig. 5A). To exclude possible contamination or nonspecific virion associations, mature KSHV virions were treated with Triton X-100 to dissolve the outer layer of virions, as described previously (35). This showed that while the outer layer envelope proteins, including K8.1 and gB, were released from Triton X-100-treated virions, the inner layer tegument proteins (ORF45 and ORF55) and capsid protein (ORF65) were still tightly associated with Triton X-100-treated virions (Fig. 5B). Interestingly, vBcl2 was

FIG 5 vBcl2 is required for tegument integrity during KSHV lytic replication. (A) vBcl2 is detected in mature KSHV virions. iSLK-BAC16-vBcl2-HA cells were lytically induced for 96 h. Virions were harvested from supernatants, concentrated by ultracentrifugation, and then subjected to immunoblotting (IB) with the indicated antibodies. (B) KSHV virions were treated with Triton X-100 as described previously (35) and subjected to immunoblotting with the indicated antibodies. (C) KSHV virions were concentrated from iSLK-BAC16 cells (2 10-cm dishes), iSLK-BAC16- Δ vBcl2 cells (50 10-cm dishes), iSLK-BAC16-E14A cells (50 10-cm dishes), iSLK-BAC16 cells with vBcl2 peptide treatment (50 10-cm dishes), or iSLK-BAC16- Δ vBcl2 cells with lentivirus-mediated V5-vBcl2 expression (2 10-cm dishes). Purified virions were normalized by the viral DNA copy number, and equal amounts of virions were subjected to immunoblotting with the indicated antibodies. (D) iSLK-BAC16-vBcl2-HA cells were pretreated with the Tat or vBcl2 peptide and then lytically induced. Nuclear and cytosolic fractions were separated and subjected to immunoblotting with the indicated antibodies.

apparently detected in Triton X-100-treated virions, suggesting that vBcl2 is located in the inner layers of virions, as seen with tegument and capsid proteins (Fig. 5B). To further test the role of vBcl2 in virion assembly, we compared the amounts of the ORF45 and ORF55 tegument proteins and the ORF65 capsid protein between wild-type KSHV virions and KSHV Δ vBcl2 virions. This analysis showed that the ORF55 tegument protein was almost undetectable, and the level of the ORF45 tegument protein was evidently reduced in KSHV Δ vBcl2 virions compared with those in wild-type KSHV virions (Fig. 5C). However, the ORF65 capsid protein showed no change between KSHV Δ vBcl2 virions and wild-type KSHV virions (Fig. 5C). Consistently, compared to those with Tat peptide treatment, vBcl2 peptide treatment drastically reduced the amounts of virion-associated ORF55 and ORF45 tegument proteins and showed no effect on the amount of virion-associated ORF65 capsid (Fig. 5C). The lentivirus-mediated expression of V5-tagged vBcl2 in iSLK-BAC16 Δ vBcl2 cells restored the expression of ORF55 and ORF45 in mature virions (Fig. 5C). As a recent report described that KSHV vBcl2 localizes to the mitochondria and nuclei of infected cells and that the deletion of its amino-terminal 17 amino acids abrogates nuclear localization (36), we examined whether the vBcl2-ORF55 interaction was required for the nuclear localization of vBcl2 and ORF55. iSLK-BAC16 cells were pretreated with the Tat peptide or the vBcl2 peptide and subsequently subjected to lytic induction for 96 h, followed by an immunoblot assay. Upon Tat peptide treatment, the majority of vBcl2 and ORF55 localized in the cytoplasmic fraction, whereas a portion of vBcl2 and ORF55 was also present in the nuclear fraction of lytic replicating cells (Fig. 5D). Upon vBcl2 peptide treatment, however, vBcl2 and ORF55 were not detected in the nuclear fraction (Fig. 5D). These results demonstrate that the vBcl2-ORF55 interaction is critical for their nuclear localization and KSHV virion assembly, which is effectively suppressed by vBcl2 peptide treatment.

DISCUSSION

As a continuation of the study of our last report (34), here, we analyzed the functional regions of vBcl2 for KSHV lytic replication by comprehensive mutagenesis analysis and identified ORF55 as a molecular target of vBcl2 in controlling KSHV lytic replication. Moreover, vBcl2 bound ORF55 via its amino-terminal aa 11 to 20, and this

interaction played an important role in KSHV virion assembly. We also found that the vBcl2 peptide derived from its amino-terminal aa 11 to 20 considerably suppressed KSHV virion production by disrupting the interaction between vBcl2 and ORF55. Thus, our study identifies the molecular mechanism of how vBcl2 controls KSHV lytic replication and progeny virus production and the vBcl2 peptide that efficiently blocks KSHV lytic replication.

Controlling the host programmed cell death pathway, including autophagy and apoptosis, is critical for virus persistence and pathogenesis (37). Specifically, autophagy is an important host defense mechanism, which sequesters invading intracellular pathogens for degradation. Many gammaherpesviruses, including KSHV and EBV, delegate their genes to modulate each step of autophagy to establish persistent infection (25, 30, 35, 38). We have shown that KSHV vBcl2 displays efficient antiautophagic activity that functions to prolong the life span of virus-infected cells, ultimately enhancing virus replication and persistence. However, we and others surprisingly discovered that vBcl2 also plays an essential role in KSHV lytic replication (34, 36, 39). More importantly, this novel activity of vBcl2 does not depend on its central BH3 domain-mediated antiapoptotic and antiautophagic activities but is associated with its amino-terminal BH4 domain (34). Specifically, either the $\Delta 11-20$ deletion or the E14A mutation of vBcl2 led to both the inhibition of viral gene expression and the lack of virion production, suggesting that the N-terminal region of vBcl-2 may target multiple host/viral factors for viral gene expression and virion production. However, we found that the vBcl2 peptide specifically impaired the interaction between vBcl2 and ORF55, inhibiting virion assembly without affecting viral gene expression. This suggests that the linear epitope at the N-terminal region of vBcl-2 may be involved in the ORF55 interaction for virion assembly, which is suppressed by the vBcl2 peptide. However, the high-affinity interaction of vBcl2 with other factors for gene expression may require multiple contacts, which is not suppressed by the vBcl2 peptide alone. It is even more surprising that vBcl2 of the Old World primate virus rhesus rhadinovirus (RRV) is also essential for viral lytic replication, similarly to KSHV vBcl2, whereas vBcl2 proteins of the New World primate viruses herpesvirus saimiri (HVS) and MHV-68 are not required for lytic replication (34, 36). A recent report (36) also showed that the KSHV and RRV vBcl2 homologs localize to the mitochondria and nuclei of infected cells and that a deletion of the amino-terminal 17 amino acids of KSHV vBcl2 abrogates its nuclear localization and thus no longer supports lytic replication, suggesting that vBcl2 might execute its essential function in the nuclei of infected cells. This indicates that the vBcl2 proteins of KSHV and RRV differ from those of HVS and MHV-68, in which they are essential for viral replication. This also suggests that vBcl2 of Old World primate gamma-2-herpesviruses, including KSHV and RRV, may have further evolved to gain an additional nuclear function to regulate viral lytic replication.

Herpesvirus virions contain four morphologically distinct structures: a dsDNA genome, a capsid, a tegument, and an envelope. Although capsid proteins are well conserved among all herpesviruses (40–42), the arrangement and functions of tegument proteins are still largely unknown. Previous studies have revealed the roles of several KSHV tegument proteins in the assembly of mature virions, innate immune modulation, and efficient lytic replication (13, 14, 17–22, 42, 43). KSHV ORF55 is a tegument protein. RRV ORF55, MHV-68 ORF55, EBV BSRF1, and herpes simplex virus 1 (HSV-1) UL51 are homologs of KSHV ORF55. Interestingly, HSV-1 UL51 has been shown to play an important role in HSV-1 virion maturation and egress (44). However, no gammaherpesvirus ORF55 protein has been studied for its role in the viral life cycle. We found for the first time that KSHV vBcl2 binds ORF55 during lytic replication and that this interaction appears to be required for their nuclear localization and virion incorporation. In fact, disruption of the vBcl2-ORF55 interaction by the vBcl2 peptide reduced KSHV virion assembly in the nucleus. As KSHV vBcl2 localizes to the mitochondria and nuclei of infected cells (35), this vBcl2-ORF55 interaction appeared to be necessary for their nuclear translocation. In fact, several herpesviral tegument proteins are also localized in both the nucleus and cytoplasm and play important roles for

herpesvirus virion assembly and maturation (17, 42, 43). For instance, MHV-68 ORF45 localizes in both nucleus and cytoplasm and is required for nucleocapsid egress (18), and MHV-68 ORF33 is associated with both nuclear and cytoplasmic capsids and is required for herpesvirus tegumentation (45). It is possible that as tegument proteins are associated with capsid proteins during virion assembly and nucleocapsid egress, the vBcl2 interaction may enhance the association of the ORF55 tegument protein with a capsid protein(s) for the stability of the nucleocapsid. Further study is needed to investigate the detailed role of KSHV vBcl2 in virion assembly that is independent of its antiapoptosis and autophagy activities.

Unlike its cellular Bcl2 homologs that localize only in the cytoplasmic region, KSHV vBcl2 localizes in both the nucleus and cytoplasm, suggesting that it carries out unique activities at distinctive locations (36). In the cytoplasm, vBcl2 binds Beclin-1 and Bak to block autophagy and apoptosis, respectively (32). In the nucleus, vBcl2 binds ORF55 for their nuclear localization and virion assembly. Since vBcl2 does not contain a recognizable nuclear localization signal, it is also possible that some unknown cellular or viral partners of vBcl2 mediate this nuclear translocation, which could serve as a potential therapeutic target to inhibit KSHV replication. We also identified that vBcl2 peptide derived from vBcl2 aa 11 to 20 efficiently disrupted the interaction of vBcl2 and ORF55, reduced their nuclear localization, and ultimately suppressed KSHV virion assembly. Thus, as KSHV vBcl2 carries out multiple functions in viral lytic replication and persistence, it is a key target for the future development of anti-KSHV therapeutic intervention.

MATERIALS AND METHODS

Cell culture and viruses. HEK293T and SLK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin-streptomycin (Gibco-BRL). iSLK cells were cultured in the presence of 1 μ g/ml puromycin and 250 μ g/ml G418. Bacterial artificial chromosome 16 (BAC16) and its derivatives were introduced into iSLK cells via Eugene HD (Roche) transfection, and transfected iSLK cells were selected with 200 μ g/ml hygromycin (Invitrogen). WT KSHV and its mutant derivatives were all produced from iSLK-BAC16 or iSLK-BAC16 mutant cell lines upon doxycycline (1 μ g/ml) and sodium butyrate (1 mM) induction for 4 days. SLK stable cell lines were established by using lentivirus vector infection with 200 μ g/ml hygromycin selection.

Plasmid construction. vBcl2/ORF16, ORF30, ORF31, ORF55, and ORF68 coding sequences were amplified from KSHV BAC16 genomic DNA by PCR. vBcl2 was cloned into the pCDH-CMV-MS-e1-Hygromycin vector encoding an N-terminal HA tag (pCDH-HA-vBcl2). Deletions and mutations in the vBcl2 gene were generated by using a QuikChange site-directed mutagenesis kit (Stratagene). ORF55 was cloned into the pCDH-CMV-MS-e1-puromycin vector (System Biosciences). ORF30, ORF31, ORF55, and ORF68 were cloned into the pEGFP-C1 vector (Clontech Laboratories).

Immunoblotting. Cell lysates were collected in 1% NP-40 buffer and quantified by a Bradford protein assay (Thermo Scientific). Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by semidry transfer at 25 V for 30 min. All membranes were blocked in 5% milk in phosphate-buffered saline (PBS)-Tween (PBST) and probed overnight with the indicated antibodies in 5% bovine serum albumin (BSA) at 4°C. Primary antibodies included mouse Flag (Sigma), mouse GFP (Santa Cruz), mouse HA (Covance), rat KSHV LANA (Advanced Biotechnologies), rabbit KSHV ORF8/gB (GeneTex), rabbit KSHV viral interferon regulatory factor 3 (vIRF3) (Novus Biologicals), mouse LC3 (Cosmo Bio), mouse tubulin (Santa Cruz), mouse lamin A (Santa Cruz), and mouse actin (Santa Cruz) antibodies. Antibodies for mouse KSHV ORF45, mouse KSHV ORF65, rabbit KSHV K8.1, rabbit KSHV K3, rabbit KSHV K8, and rabbit KSHV ORF50/RTA were described previously (13, 30, 31). Rabbit KSHV ORF55 polyclonal antibody was generated by Covance using a recombinant 6 $\times$ His-ORF55 protein. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated on membranes in 5% milk, and bands were developed with ECL reagent (Thermo Scientific) and imaged on a Fuji LAS-4000 imager.

Production of progeny KSHV. iSLK-BAC16 cells were treated with doxycycline (1 μ g/ml) and sodium butyrate (1 mM) to induce lytic replication. After 96 h of induction, the culture supernatant was collected, filtered (0.45 μ m), and concentrated by ultracentrifugation (24,000 rpm for 4 h). The virion pellet was suspended with plain DMEM, and the virion particles were quantified by quantitative PCR (qPCR) and stored at -80°C .

Quantification of KSHV progeny. iSLK-BAC16 cells were treated with doxycycline (1 μ g/ml) and sodium butyrate (1 mM) to induce lytic replication. After 96 h of induction, the virus-containing media were cleared of cell debris by centrifugation at 2,000 rpm for 5 min and passed through a 0.45- μ m filter. A total of 50 μ l of filtered virus-containing medium was used to infect the SLK cells via spin infection (2,000 rpm for 45 min at 30°C). Fresh media were changed at 2 hpi, and the number of infected cells (based on the GFP signal) was determined by flow cytometry (FACSCanto II; BD Bioscience, San Jose, CA) at 24 hpi.

Transmission electron microscopy. Cells were fixed at 48 h postinduction (doxycycline and butyrate treatment), and the samples were prepared as previously described (34). The prepared samples were subjected to TEM at the USC/Norris Cell & Tissue Imaging Core within the Vision Research Center.

RNA extraction and quantitative reverse transcriptase PCR (RT-PCR). Total RNA was isolated from cells with the RNeasy minikit (Qiagen) and treated with RNase-free DNase according to the manufacturer's protocol. cDNA was reverse transcribed from 2 μ g of total RNA by using the iScript cDNA synthesis kit (Bio-Rad), and qPCR was performed with iQ SYBR green Supermix (Bio-Rad). qPCR primer sequences were described previously (34).

Autophagy analyses. Stable SLK cells expressing the WT or mutant forms of vBcl2 were treated with 2 μ M rapamycin (Sigma) in DMEM containing 1% FBS for 2 to 4 h. LC3 mobility shift was detected by immunoblotting. Quantitation of GFP-LC3 punctae was performed by confocal microscopy as previously described (33, 34, 46, 47).

Apoptosis analyses. SLK cells stably expressing the WT or mutant forms of vBcl2 were seeded at 10^6 cells per well of 6-well plates and grown for 24 h. The cells were then treated with fresh medium containing 5 ng/ml TNF- α plus 5 μ g/ml CHX for 12 h. For the analysis of apoptotic cells, the samples were prepared by using a Deadend fluorometric TUNEL system kit (Promega) according to the manufacturer's protocol. For the caspase-3 activation assay, cleaved caspase-3 from cell lysates was measured by using a Cleaved Caspase-3 (Asp175) Sandwich ELISA kit (Cell Signaling) according to the manufacturer's protocols.

Gel filtration chromatography. iSLK-BAC16-vBcl2-3HA cells were harvested 72 h after treatment with doxycycline and butyrate, washed with cold PBS, resuspended in buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1.5 mM MgCl₂, 20 mM β -glycerophosphate, 1 mM sodium orthovanadate, 10% glycerol, 0.5 mM EGTA, 0.5% Triton X-100 or 0.2% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 10 μ g/ml leupeptin), and lysed by three rounds of freezing and thawing followed by 10 s of sonication in a microultrasonic cell disrupter. Cell lysates were clarified by centrifugation at 13,000 rpm for 30 min at 4°C, followed by passage through a 0.22- μ m filter. The supernatants were fractionated on a Superose 6 gel column with a high-performance liquid chromatography (HPLC) system (Bio-Rad). Fractions were analyzed by Western blotting.

Yeast two-hybrid screen. The vBcl2 functional region (aa 1 to 143) was cloned into the bait vector pGBKT7. The reporter AH109 yeast strain (*Saccharomyces cerevisiae*) expressing vBcl2 (aa 1 to 143) was transformed with the KSHV yeast-two hybrid library in accordance with instructions for Matchmaker GAL4 Two-Hybrid System 3 (Clontech). For the yeast cotransformation assay, the bait vector containing the indicated vBcl2 constructs and the prey vector harboring ORF55 were cotransformed and screened in 4-dropout plates with X- α gal (5-bromo-4-chloro-3-indolyl- α -D-galactopyranoside).

Statistical analysis. All data were analyzed by using 2-tailed Student's *t* test with a minimum of 3 experiments. *P* values of less than 0.05 were considered significant.

ACKNOWLEDGMENTS

This work was partly supported by the National Natural Science Foundation of China (31770176); the Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning; the Shanghai Rising-Star Program (17QA1403200); Leukemia & Lymphoma Society grant 3367-16 (Q.L.); grants CA200422, CA180779, DE023926, AI073099, and AI116585; the Hastings Foundation; the Fletcher Jones Foundation; and the GRL Program (K2081500001) from the National Research Foundation of Korea (J.U.J.).

Q.L. and J.U.J. conceived and designed the experiments. Q.L., D.W., S.D., and C.G. performed the experiments. Q.L., D.W., B.C., K.F.B., C.G., S.D., S.-J.G., P.F., C.L., and J.U.J. analyzed the data. Q.L. and J.U.J. wrote the paper.

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