Caspase-7 Cleavage of Kaposi Sarcoma-associated Herpesvirus ORF57 Confers a Cellular Function against Viral Lytic Gene Expression*§

Received for publication, September 22, 2009, and in revised form, February 8, 2010. Published, JBC Papers in Press, February 16, 2010, DOI 10.1074/jbc.M109.068221

Vladimir Majercíak¹, Michael Kruhlak¹, Pradeep K. Dagar³, J. Philip McCoy, Jr.⁴, and Zhi-Ming Zheng⁵‡

From the ¹HIV and AIDS Malignancy Branch and the ⁵Experimental Immunology Branch, NCI, and the ⁴Flow Cytometry Core, NHLBI, National Institutes of Health, Bethesda, Maryland 20892

Kaposi sarcoma-associated herpesvirus (KSHV) ORF57 is a viral early protein essential for KSHV multiplication. We found that B cells derived from cavity-based B cell lymphoma with lytic KSHV infection display activation of caspase-8 and cleavage of ORF57 in the cytoplasm by caspase-7 at the aspartate residue at position 33 from the N terminus. Caspase-7 cleavage of ORF57 is prevented by pan-caspase inhibitor z-VAD, caspase-3 and position 33 from the N terminus. Caspase-7 cleavage of ORF57 in the cytoplasm by caspase-7 at the aspartate residue at position 33 from the N terminus. Caspase-7 cleavage of ORF57 may represent a cellular function against lytic KSHV infection.

Caspases (cysteine-dependent aspartate specific protease) (1) are the central mediators of apoptosis, a fundamental and tightly regulated cellular process of programmed cell death. Caspases are divided into initiator caspases with long prodomains (caspase-2, -8, -9, and -10) and effector caspases with short prodomains (caspase-3, -6, and -7) (2). Caspases are present within cells as inactive procaspases or proenzymes (zymogens) and after apoptotic stimulation become activated by proteolytic cleavage and oligomerization (3).

Apoptosis plays an important role in regulating homeostasis in multicellular organisms and is characterized by a series of morphological and biochemical changes resulting in cell self-destruction (4). Apoptosis can be initiated through two major signaling pathways. The extrinsic pathway is activated by ligand binding to cell death receptors on the cell surface. The intrinsic or mitochondrion-mediated pathway depends on signals within the cell, such as DNA damage, growth factor withdrawal, oxidative stress, or viral infection. Both pathways lead to a cascade of caspase activation: initiator caspases activate effector caspases, which in turn cleave a broad spectrum of intracellular substrates, leading to cell death.

KSHV (also called human herpesvirus 8 or HHV8), like other herpesviruses, establishes long term infection in the host, which is essential for development of all forms of Kaposi sarcoma (5, 6), primary effusion lymphoma, and multicentric Castleman disease (7, 8). KSHV exhibits two viral life cycles: latent and lytic infection. Only a few viral genes are expressed during latency, the role of which is to maintain the viral genome and ensure survival of infected cells (9). Lytic infection is associated with the expression of viral lytic genes and the production of infectious virus. KSHV ORF57 (also known as Mta or KS-SM) is a viral lytic gene that is essential for virus replication and the production of infectious virions in viral lytic infection (10, 11). It also promotes viral RNA splicing and the stability of a subset of viral lytic transcripts (12). During our previous investigations into the expression and function of KSHV ORF57 during infection, we consistently detected ORF57 as a doublet on Western blotting. We postulated that the lower band of ORF57 is a cleavage product of full-length ORF57, because the ORF57 gene contains only one ORF that does not encode a product with this size difference from the full-length protein in HEK-293 cells by transient transfection. Here we report that the KSHV ORF57 protein is cleaved by cellular caspase-7 in viral lytic infection and that inhibition of the caspase cleavage of ORF57 leads to increased expression of ORF57-dependent viral transcripts and, consequently, the increased production of cell-free virus particles.

EXPERIMENTAL PROCEDURES

Cells and KSHV Lytic Induction—KSHV latently infected primary effusion lymphoma cell lines BCBL-1 (EBV⁻) and JSC-1 (EBV⁺), and human cell lines HEK-293, HeLa, MCF-7, and Bac36-Δ57 (a HEK-293-derived stable cell line containing...

* This work was supported, in whole or in part, by the National Institutes of Health Intramural Research Program, National Cancer Institute, Center for Cancer Research.

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

§ To whom correspondence should be addressed: 10 Center Dr., Rm. 6N106, Bethesda, MD 20892-1868. Fax: 301-480-8250; E-mail: zhengt@mail.nih.gov.

1 The abbreviations used are: KSHV, Kaposi sarcoma-associated herpesvirus; ORF, open reading frame; Z, benzoyloxycarbonyl; fmk, fluoromethyl ketone; siRNA, small interfering RNA; VA, valproate; diox, doxycycline; shRNA, small hairpin RNA; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; NT, nontargeting; DMSO, dimethyl sulfoxide; PARP, poly(ADP-ribose) polymerase; wt, wild-type.
Caspase-7 Cleavage of KSHV ORF57

an ORF57 knock-out KSHV genome (11) were cultivated as described (13). To induce ORF57 expression and virus lytic replication, KSHV-infected cells were treated with 0.6 mM valproate (VA), 0.3 mM butyrate, or 20 ng/ml of 12-O-tetradecanoylphor-13-acetate. Doxycline (dox)-inducible TREX BCBL-1 RTA and vector control cell lines were cultured as described (14).

Plasmids—Plasmids pVM7 (KSHV ORF57 with a FLAG tag on its C terminus), pVM9 (KSHV ORF56 with FLAG tag on its C terminus), and pST3 (KSHV K8β cDNA) were described previously (15–17). Plasmid pVM52 expresses KSHV ORF57 fused to a FLAG tag on its N terminus. Plasmids pVM58 (D25A plus D29A), pVM59 (D30A plus D33A), pVM60 (D30A), and pVM61 (D33A) carrying double or single mutations of aspartic acid residues at the indicated positions in ORF57 were derived from pVM7. Plasmid pVM74 (Δ1–33 amino acids) expresses KSHV ORF57 from amino acids 34 to 455 with a C-terminal FLAG tag.

UV Irradiation and Induction of Apoptosis in HeLa Cells—Because UV irradiation induces activation of both caspase-8- and caspase-9-mediated cell apoptosis (18–20), HeLa cells at 24 h after transfection were washed with cold phosphate-buffered saline and exposed to UV light (254 nm, 200 mJ/cm²) in Stratalinker (Stratagene, La Jolla, CA) as described (21). After irradiation, the cells were covered with fresh medium and incubated for an additional 4 h before harvesting.

Caspase Inhibitors—All of the peptide-based caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, and z-VEID-fmk, and negative inhibitor control z-FA-fmk were obtained from EMD (Gibbstown, NJ). Each inhibitor or inhibitor control was used at a final concentration of 50 μM in culture medium as indicated in each study. Cells grown in the inhibitor-containing medium at a time point as scheduled were collected for protein and RNA detection.

Preparation of Cytoplasmic Extracts for Caspase Digestion—The cytoplasmic extracts from apoptotic or virus-infected cells were prepared as described (22) with some modifications. After washes with cold phosphate-buffered saline, the cell pellets were resuspended in 5 volumes of ice-cold hypotonic buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol) and incubated for 15 min on ice. The swollen cells were disrupted by three freeze and thaw cycles. The nuclei were pelleted by 10 min of centrifugation at 10,000 × g at 4 °C. The resulting cytoplasmic extracts were used in caspase cleavage assays.

Protein and RNA Detection—The protein samples were blotted with the following antibodies: rabbit polyclonal anti-ORF57 antibody prepared by immunization with a synthetic peptide representing amino acids 119–132 of ORF57 (11), mouse anti-FLAG M2 (Sigma), anti-β-tubulin (Sigma), anti-PARP1 (clone C-2–10; EMD), anti-caspase-7 (clone 4G2; MBL International, Woburn, MA), anti-caspase-8 (clone 1C12; Cell Signaling, Danvers, MA), anti-Myc (Sigma), anti-KSHV K8α (ProMab, Albany, CA), rabbit anti-caspase-3 (EMD), and anti-caspase-9 (Cell Signaling).

Total cell RNA (5 μg) was analyzed by Northern blotting as described (16). The following ³²P-labeled oligonucleotide probes were used to detect specific KSHV transcripts: oVM73 (5′-GTCCACCGTCACCGCATAGT-3′) for ORF59, oVM164 (5′-AGCCTAGGCACGTAAATTGTCA-3′) for PAN RNA, and oST30 (5′-TAGTTCGTTAATGTGGTGACG-3′) for both RTA and K8. Glyceraldehyde-3-phosphate dehydrogenase mRNA was detected for sample loading with oZM270 (5′-TGAGCTCCTTCAGATACACAA-3′).

RNA Interference—The expression of individual endogenous caspases was knocked down by RNA interference with siGenome SMARTpool siRNAs (Dharmacon, Lafayette, CO). ON-TARGETplus siCONTROL (nontargeting siRNA 1; Dharmacon) served as a negative control. Mission lentiviral transduction particles (Sigma) containing a viral vector expressing small hairpin RNA (shRNA) were used to knock down the expression of caspase-7 in BCBL-1 cells. Briefly, BCBL-1 cells were simultaneously transduced with three different stocks of viral particles containing three different shRNAs against human caspase-7 (TRCN03521, TRCN03522, and TRCN03523) at a multiplicity of infection of 1 by using ExpressMag Super magnetic kit (Sigma) or transduced with MISSION nontargeting (NT) shRNA control transduction particles at the same multiplicity of infection. Puromycin (1 μg/ml) was added for selection 24 h after transduction. The surviving cells after 2 weeks of selection were induced with VA for 20 h for KSHV lytic infection and were analyzed by Western blotting.

In Vitro Caspase Cleavage Assays—All human active recombinant caspases were purchased from EMD and BIOMOL (Plymouth Meeting, PA). A 20-μl cleavage reaction containing 200 ng of substrate protein and 2.5 units of active caspase dissolved in caspase buffer (100 mM NaCl, 50 mM HEPES, pH 7.4, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS) was incubated for 4 h at 37 °C. The reaction was stopped with an equal amount of 2× SDS sample buffer and was immunoblotted. When cytoplasmic extracts were used as a source of active caspases, 10 μl of cytoplasmic extracts was mixed with an equal volume of 2× SDS sample buffer containing substrate protein.

Immunodepletion—Before immunodepletion of individual endogenous caspases, the cytoplasmic extracts (100 μg) were supplemented with NaCl to a final concentration of 100 mM. The specific antibodies (5 μg) and 50 μl of prewashed protein G-agarose beads (Upstate, Billerica, MA) were added to the cytoplasmic extracts and incubated overnight at 4 °C. The immunocomplexes were removed by centrifugation, and the supernatants were used in caspase cleavage assays. The immunodepletion efficiency for caspase-3 or caspase-7 was measured by Western blotting. The extracts incubated with the beads only or with beads covered with nonspecific mouse IgG served as negative controls for the depletion.

Multicolor Immunostaining—Cell multicolor immunostaining was performed as described (11). Briefly, the cells on coverslips were fixed, permeabilized, blocked with bovine serum albumin, and incubated with one of the following primary antibodies: mouse monoclonal anti-FLAG M2 (Sigma), anti-KSHV ORF59 (Advanced Biotechnologies, Columbia, MD), anti-RTA (ORF50) (gift from Dr. K. Yamanishi), rabbit polyclonal anti-KSHV ORF57, and anti-active (cleaved) caspase-3, -6, -7, -8, or -9 (Cell Signaling). After extensive washes, the cells on the slides were stained with secondary antibodies conjugated with
Caspase-7 Cleavage of KSHV ORF57

AlexaFlour467, AlexaFlour546 or AlexaFlour488 (Molecular Probes, Carlsbad, CA). RTA-Myc fusion in TREx BCBL-1 RTA stable cells was detected with monoclonal anti-Myc antibody conjugated with fluorescein isothiocyanate (Sigma). The cell nuclei were stained with 4′,6′-diamino-2-phenylindole. The specific signal was imaged by epifluorescence or confocal microscopy and quantified by using an Image J software.

Flow Cytometry—After 15 h of induction with VA, BCBL-1 cells with stable expression of caspase-7 shRNA or NT shRNA were washed and fixed with 1.6% paraformaldehyde for 15 min. Intracellular staining for ORF57 and ORF59 was performed using eBioscence staining kit (catalog number 00-5523-00) based on Foxp3 staining protocol from eBioscience (San Diego, CA). Rabbit anti-ORF57 polyclonal and mouse anti-ORF59 monoclonal antibodies were labeled, respectively, with AlexaFlour488 and AlexaFlour546 fluorescent dyes using appropriate Zenon labeling kits (Invitrogen). The obtained fluorescence-activated cell sorter data were analyzed with Flowjo v8.8.6 software (Tree Star Inc., Ashland, OR).

Virus Production—Viral replication in BCBL-1 cells (10⁶/ml x 20 ml) was induced by VA in the presence of DMSO (vehicle), 25 μM pan-caspase inhibitor z-VAD-fmk, caspase-3- and caspase-7-specific inhibitor z-DEVD, inhibitor negative control z-FA-fmk, or 1 mM phosphonoacetic acid. The culture medium was collected at 96 h after induction and cleared of cell debris by low speed centrifugation (5,000 x g) for 10 min at 4°C, followed by filtration through 0.45-μm filters. The cell-free virus particles were then pelleted by high speed centrifugation (30,000 x g) for 1 h at 4°C and washed once with cold phosphate-buffered saline. The virus pellets were resuspended in an equal amount of SDS sample buffer. The amount of virus particles in each sample was determined by the level of KSHV tegument protein ORF45 as assessed by Western blot with anti-KSHV ORF45 (a gift from Yan Yuan).

RESULTS

Induction of KSHV Lytic Infection in KSHV+ B Lymphocytes Activates Initiator Caspase-8 and Production of a Caspase-Cleaved KSHV ORF57—In cell lysates of KSHV+ BCBL-1 and JSC-1 cell lines after reactivation of the virus with a low dose of chemical inducers (Fig. 1A), we noticed KSHV ORF57 expressed as a doublet on Western blotting. The lower band was assumed to be a cleavage product of full-length ORF57, whereas VA was a better inducer than butyrate for the
Caspase-7 Cleavage of KSHV ORF57

**FIGURE 2. Identification of a caspase cleavage site in KSHV ORF57.** A, diagrams of ORF57 fusions with an N- or C-terminal FLAG tag and the anti-ORF57 (α-ORF57) binding site. The dotted vertical line represents the putative caspase cleavage sites on the N terminus of ORF57. Shown below are the N-terminal 40 amino acid residues of ORF57 with putative caspase cleavage sites indicated by arrows. B, cleavage of FLAG-tagged KSHV ORF57 in apoptotic HeLa cells. Cells transfected with expression vectors pVM7 (ORF57-FLAG) and pVM52 (FLAG-ORF57) were UV-irradiated to induce apoptosis and were then blotted for full-length (FL) and cleaved ORF57 fusions with anti-ORF57 and anti-FLAG antibodies. C, identification of the aspartic acid residue at position 33 from the N terminus of ORF57 as a caspase cleavage site by point mutational analysis. Four putative caspase cleavage sites at the N terminus of ORF57 were mutated (Asp → Ala) individually or in combination in ORF57-FLAG expression vectors. The mutants were expressed in HeLa cells exposed to UV and blotted with anti-FLAG. PARP cleavage served as an internal control for apoptotic induction and sample loading. D, cleavage of wt or mutant D33A ORF57 by VA-induced BCBL-1 cell extracts containing active caspases. Each form of ORF57-FLAG fusion was expressed from HEK-293 cells, affinity-purified with anti-FLAG beads, and digested with BCBL-1 extracts prepared 48 h after VA treatment. Western blot was performed with an anti-FLAG antibody. E, ORF57 cleavage activity of the cytoplasmic extracts isolated from BCBL-1 cells with or without VA induction for 48 h. The cytoplasmic extracts were mixed with purified ORF57-FLAG proteins, and the cleavage of ORF57 was assessed by Western blot with an anti-FLAG antibody.

induction of ORF57 in both cell types. Because VA is not toxic to a noninfected Burkitt lymphoma B cell line (23), we used VA for further analyses.

When BCBL-1 cells were induced with VA in the presence or absence of pan-caspase inhibitor z-VAD-fmk, production of the lower band of ORF57 was strongly reduced in the presence of z-VAD-fmk, which inhibits the caspase cleavage of poly(ADP-ribose) polymerase (PARP; Fig. 1B). To exclude the possible nonspecific effects of chemical inducers, this observation was further verified in TREx BCBL-1 RTA cells (14), which inducibly express RTA (ORF50), a potent transactivator for inducibly expressing RTA (ORF50), a potent transactivator for KSHV lytic induction (24). RTA induction by dox in these cells led to the production of both full-length and cleaved ORF57, along with the production of cleaved PARP (Fig. 1C). Caspase inhibitor z-VAD-fmk inhibited the cleavage, indicating that VA- or RTA-induced cleavage of ORF57 is mediated by active caspases during viral lytic induction (Fig. 1, B and C). To confirm the connection between caspase activation and ORF57 cleavage, we transfected HeLa cells with a vector expressing full-length ORF57 with a FLAG tag on its N terminus. We observed that ORF57, like PARP, was actively cleaved in HeLa cells when activation of initiator caspase-8 (supplemental Fig. S1) and apoptosis was induced by UV irradiation as reported (18, 19, 25), and the cleavage was reduced by z-VAD-fmk (Fig. 1D). These data suggest that caspase activity is needed for ORF57 cleavage.

Because activation of initiator caspase-8 triggers the extrinsic apoptosis pathway, which differs from the intrinsic apoptosis pathway initiated by activation of initiator caspase-9 (2), we examined whether lytic KSHV induction activates a particular apoptosis pathway. We found that only initiator caspase-8 was triggered in BCBL-1 cells with lytic KSHV induction by VA, along with cleavage of expressed ORF57 (Fig. 1E). Consistent with this, RTA expression in TREx BCBL-1 RTA cells led to the production of active caspase-8, but not active caspase-9, from its inactive full-length pro-caspase, along with the induced production of cleaved PARP and ORF57 (Fig. 1, C and F).

**Mapping the Aspartate Residue at Position 33 from the N Terminus of ORF57 as a Caspase Cleavage Site**—To understand where the cleavage takes place on ORF57 protein, HeLa cells were transfected with a vector expressing ORF57 tagged with either an N- or C-terminal FLAG (Fig. 2A) and were UV-irradiated to induce cell apoptosis. The cleavage products of the ORF57 fusions in the cells were then analyzed by anti-ORF57 or anti-FLAG Western blotting. Because the anti-ORF57 antibody was derived from immunization with a N-terminal peptide spanning amino acid residues 119–132 of ORF57, any cleavage product containing this region should be recognized by this anti-ORF57 antibody. Using this strategy by anti-FLAG Western blotting, we showed that the cleavage takes place in the N-terminal region of ORF57, upstream of the antibody recognition site (Fig. 2B, compare the anti-ORF57 and anti-FLAG Western blotting of FLAG-ORF57).

Next, we analyzed the cleavage efficiency of ORF57 in UV-irradiated HeLa cells by introducing D-to-A point mutations into each of the four putative caspase cleavage sites in the N-terminal region of ORF57 that were predicted by using a software program, CASVM (26). We identified the aspartic acid residue at position 33 from the N terminus of ORF57 responsible for the cleavage (Fig. 2, A and C). A cleavage product of ORF57 was obtained only from wild-type (wt) ORF57, and not from mutant D33A ORF57 (Fig. 2D), when wt and mutant D33A ORF57 expressed from HEK-293 cells were separately incubated with BCBL-1 extracts bearing VA-induced active caspases (Fig. 2E). Together, these data identify the caspase cleavage site of ORF57 as 30DETD33 followed by an alanine at the cleavage site P33 position (27). Thus, the cleaved ORF57 lacks the first 33 residues from the N terminus of ORF57.

**Identification of Cellular Caspase-7 as an Essential Caspase in the Cleavage of KSHV ORF57**—Because the mapped caspase cleavage site on ORF57 resembles a cleavage site for caspase-2, -3, and -7 (27), we performed a series of caspase digestions with...
10 commercially available caspases on a recombinant ORF57-FLAG fusion expressed from baculovirus. Caspase-7 was identified as a caspase primarily responsible for the cleavage of ORF57 (Fig. 3A). Even though both caspase-3 and caspase-7 utilize a cleavage site composed of the amino acid residues DEXD (27), found in the classic caspase-3 substrate PARP, and the caspase-3 used in the cleavage assay was active (supplemental Fig. S2), caspase-7 was the primary candidate that cleaved ORF57. Although ORF57 could be cleaved to a much lower extent by caspase-10 and caspase-5 (Fig. 3A), titration assays demonstrated that significantly more caspase-10 compared with caspase-7 was needed to cleave ORF57 (Fig. 3B). All other caspases tested showed no cleavage activity for ORF57. ORF57 digestion by caspase-7 and less efficiently by caspase-10, but not by caspase-3, was also verified with FLAG-tagged ORF57 expressed from mammalian cells HEK-293 (28, 29), indicating that protein modification of ORF57 in mammalian cells does not affect caspase-7 cleavage. When purified wt ORF57-FLAG and mutant D33A ORF57-FLAG fusions expressed from HEK-293 cells were compared in caspase-7 cleavage assays, only the wt ORF57 was cleaved (Fig. 3D), confirming the mapped caspase cleavage site (Fig. 2C) as the site for caspase-7 cleavage of ORF57.

Cellular Caspase-7 Plays a Key Role in ORF57 Cleavage in BCBL-1 Cells during KSHV Lytic Infection—We next examined the expression kinetics of KSHV ORF57 and caspase-7 in VA-treated BCBL-1 cells. Both the cleavage product of ORF57 and the production of active caspase-7 from its catalytically inactive procaspase appeared 24 h after VA induction, similar to the time course of cleaved PARP appearance (Fig. 3E, left panels). We verified the production of active caspase-7 and the cleavage of ORF57 and PARP during KSHV lytic infection in dox-inducible TREx BCBL-1 RTA cells (Fig. 3E, right panels). Both the cleavage of ORF57 and the production of active caspase-7 appeared only in the cytoplasm (Fig. 3F), in contrast to PARP, which is cleaved in the nucleus by caspase-3 (28).

We further verified the role of caspase-7 in ORF57 cleavage in BCBL-1 cells by using four additional approaches. First, we used z-DEVD-fmk, a caspase-3/7 inhibitor (29, 30), in addition to the pan-caspase inhibitor z-VAD-fmk and found that ORF57 cleavage was reduced in VA-treated BCBL-1 cells when production of active caspase-7 decreased (Fig. 4A). Because z-VEID-fmk, a caspase-3/6 inhibitor (31), had no effect on ORF57 cleavage (Fig. 4A), we ascribed the reduced ORF57 cleavage in the presence of z-DEVD-fmk to the inhibition of active caspase-7. Second, depletion of caspase-7 from the cytoplasmic extracts of VA-induced BCBL-1 cells reduced the cleavage of ORF57, but depletion of caspase-3 did not (Fig. 4B). Third, knocking down caspase-7 expression in UV-irradiated HeLa cells (Fig. 4C, left panels) or in BCBL-1 cells (Fig. 4C, right panels) by RNA interference prevented the cleavage of ORF57, whereas knocking down caspase-3 expression in HeLa cells (Fig. 4C, left panels) had no effect on cleavage. Fourth, actinomycin D (32) induced the cleavage of ORF57 in caspase-3-deficient MCF-7 cells (Fig. 4D). Collectively, the data indicate that caspase-7 does play a major role in ORF57 cleavage in these cells.

Inverse Correlation between ORF57 Expression and Active Caspase-7 Production in KSHV Lytic Infection—We wished to determine whether caspase-7 activation occurred in direct correlation with ORF57 expression. Despite the existence of a few spontaneous apoptotic cells with active caspase-7, which have latent KSHV infection, virus reactivation in BCBL-1 cells dramatically increased the number of active caspase-7-positive cells, consistent with the results from Western blotting. We also observed an inverse correlation between active caspase-7...
production and ORF57 (Fig. 5A and supplemental Fig. S3) or RTA (supplemental Fig. S4) expression in BCBL-1 cells with lytic KSHV infection. This relationship is more remarkable in dox-induced TREx BCBL-1 RTA cells in which the majority of dox-induced cells expressing ORF57 did not display active caspase-7 or vice versa (Fig. 5B). This was also observed for active caspase-3 and caspase-8 (supplemental Fig. S3). Further analysis showed that among the ORF57-positive BCBL-1 cells, only 19% of them at 24 h of induction were positive for active caspase-7. The number of double positive cells increased to 43% at 12 h (Fig. 6A) or 24 h (Fig. 6B) of induction, showed a prominent reduction of ORF57. The cells with active caspase-7 staining and apoptotic nuclear bodies exhibited much more reduced expression of ORF57 (Fig. 6B), when compared with the cells without apoptotic nuclear bodies (Fig. 6). In the apoptotic cells, a reduced expression of ORF57 could be further diminished by active caspase-7, and the resulting cells appeared to have no detectable ORF57 (compare cell 1 with cell 2 in Fig. 6B, single color versus overlay).

Given the fact that ORF57 expression and active caspase-7 production is inversely correlated, we investigated whether the cells expressing active caspase-7 ever underwent KSHV reactivation. We cotained BCBL-1 TREx RTA cells induced with 1 μg of dox for coexpression of RTA, ORF57, and active caspase-7 and analyzed by confocal microscopy. As shown in Fig. 7, more than 99% of ORF57+ cells counted coexpressed RTA, but the majority of the ORF57+ or RTA+ cells expressed no active caspase-7 or vice versa. There were only 24% of active caspase-7+ cells coexpressing both RTA and ORF57, with 10% coexpressing RTA only and none for coexpression of ORF57 alone. Because RTA expresses earlier than ORF57, RTA coexpression with active caspase-7, but not with ORF57, during KSHV lytic induction suggests that the cells are either at the early stage of induction or at a stage in preventing further reactivation by active caspase-7. Nevertheless, these data confirmed that the cells positive only for active caspase-7 did not undergo full KSHV reactivation, or their viral reactivation was completely diminished by active caspase-7 at an early stage of the reactivation.

Caspase Cleavage of ORF57 Attenuates Its Function in Promoting Viral Lytic Gene Expression and Virus Production—ORF57 promotes the expression of KSHV ORF56 (16), a gene encoding a viral DNA primase, and K8 (11, 13), a gene encoding
a K-bZIP protein. We analyzed the effect of caspase cleavage of ORF57 on the expression of these two genes in HEK-293 cells by cotransfecting assays with increasing amounts of full-length ORF57, a truncated (Δ1–33 amino acids) mutant that mimics caspase-cleaved ORF57, or a D33A mutant (Fig. 2). Although all three versions of ORF57 appeared as nuclear proteins (data not shown), the truncated (Δ1–33 amino acids) mutant was deficient in promotion of ORF56 expression (Fig. 8A) or K8α (K-bZIP) production (Fig. 8B) from K8β transcripts (17). The reduction of K8α production from K8β transcripts indicates that the truncated mutant is deficient in promoting viral RNA splicing (13). When coexpressed with full-length ORF57, the truncated mutant in a small amount was suppressive for the full-length ORF57 activity in promotion of K8β RNA splicing (supplemental Fig. S5).

We also assessed the functional deficiency of caspase-cleaved ORF57 in a Bac36-Δ57 stable cell line, which contains an ORF57-null KSHV genome and expresses no ORF57 and thus has reduced expression of a subset of viral lytic genes (ORF56, ORF59, K8, etc.) upon lytic induction (11). Three versions of ORF57 (full-length, truncated Δ1–33, and D33A) were all efficiently expressed in Bac36-Δ57 stable cells after transfection (Fig. 8C). When KSHV ORF59 expression was used as a readout, both full-length and D33A, and not the truncated Δ1–33, were able to efficiently induce the expression of ORF59, a viral DNA polymerase processivity factor. As predicted, the D33A, which is resistant to caspase-7 cleavage, worked even better (≈2.7-fold) than the full-length ORF57 in the complement assay to restore ORF59 expression from Bac36-Δ57 stable cells (Fig. 8C).

Whether caspase cleavage of ORF57 affects virus production was examined in BCBL-1 cells with lytic induction. As shown in Fig. 9A, BCBL-1 cells induced with VA in the presence of the pan-caspase inhibitor z-VAD-fmk showed a great increase in the expression of ORF59 and PAN RNA (polyadenylated nuclear RNA with unknown function) and an intermediate increase in the expression of K8 and RTA. There were also more ORF59 cells in the presence of z-VAD-fmk but not in the presence of the inhibitor negative control z-FA-fmk or DMSO, despite a similar level of ORF57 expression among the three groups (supplemental Fig. S6). Together, these data suggest that caspase cleavage of ORF57 during viral lytic infection permits the host cells to restrain the expression of viral lytic genes and to control virus production.

To quantify cell-free virus particles in the culture medium of BCBL-1 cells induced with VA in the presence or absence of z-VAD-fmk, we enriched the cell-free virus particle in the cul-
expression and virus production. In both studies, we observed far define the role of caspase-7 activation in KSHV gene

RTA with single (box 2), double (box 1), or triple (box 3) staining are shown on the top left panel. Below are three selected cells numbered as 1, 2, and 3 on the top left being further enlarged and displayed for individual color stain, with RTA-Myc in green, ORF57 in red, and active caspase-7 in pink. Cell 4 was taken from a separate image to show coexpression of RTA and active caspase-7.

that inhibition of caspase-7 activity promoted the expression of ORF59, as monitored by Northern blot (Fig. 9C) or by flow cytometry (Fig. 9, D and F) and the production of cell-free virus particles as determined by ORF45 immunoblot (Fig. 9C). In particular, we observed by fluorescence-activated cell sorting a remarkable reduction of the cells with cleaved ORF57 (cells with ORF57+ staining only) along with a nearly 2-fold increase of the cells with ORF57+/ORF59+ (double positive) staining when the VA-induced BCBL-1 cells with caspase-7 knockdown were compared with the cells with NT siRNA treatment (Fig. 9,
with VA for 96 h in the presence of DMSO, 25 were isolated by centrifugation from the culture medium of BCBL-1 cells treated increases the production of cell-free virus particles. The cell-free virus particles

each treatment.

lated for the expression of individual genes according to the normalized value in

was used for Northern blot. A relative ratio (fold) of z-VAD to DMSO was calcu-

negative control z-FA-fmk. Total RNA isolated from the cells 48 h after induction

phosphonoacetic acid and were quantified by Western blot for virus particle-

FIGURE 9. Blockade of caspase cleavage of ORF57 in BCBL-1 cells promotes

expression of a subset of viral lytic genes and the production of cell-free

virus particles. A, blockade of caspase cleavage of ORF57 promotes the expres-
sion of KSHV lytic genes. Lytic infection was induced in BCBL-1 cells by VA in the

presence of DMSO (vehicle), 50 μM pan-caspase inhibitor z-VAD-fmk, or inhibitor

negative control z-FA-fmk. Total RNA isolated from the cells 48 h after induction

was used for Northern blot. A relative ratio (fold) of z-VAD to DMSO was calcu-

lated for the expression of individual genes according to the normalized value in
each treatment. B, inhibition of caspase cleavage of ORF57 in BCBL-1 cells

increases the production of cell-free virus particles. The cell-free virus particles

were isolated by centrifugation from the culture medium of BCBL-1 cells treated

with VA for 96 h in the presence of DMSO, 25 μM z-VAD-fmk, z-VAD-fmk, or 1 mM

phosphonoacetic acid and were quantified by Western blot for virus particle-

associated ORF45. SE, short time exposure; LE, long time exposure. C, blockade

of ORF57 cleavage by caspase-3/7 inhibitor z-DEVD-fmk enhances ORF59 expres-
sion at 48 h and virus production at 96 h after lytic induction. See other details in
A and B. D and E, knocking down caspase-7 expression in BCBL-1 cells by RNA

interference increases ORF59 expression. BCBL-1 cells with stable expression of

NT or caspase-7 shRNA were treated with VA for 15 h and analyzed by flow

cytometry for cells with single or double staining of ORF57 and/or ORF59. The

arrows in D indicate gated cells with ORF57 and/or ORF59 and, with ORF57

and ORF59 double positive cells in the top right quadrant. The bar graph in

E shows the numbers of ORF57+/59- only cells and of both ORF57 and ORF59 (double

positive) cells. See Fig. 4C (right panel) for caspase-7 knockdown efficiency.

D and E). These data clearly indicate an important role of caspase-7 cleavage of ORF57 in regulation of the expression of ORF57 targets.

DISCUSSION

KSHV ORF57 encodes a viral nuclear protein essential for viral gene expression and virus multiplication during viral lytic infection. In this report, we have demonstrated that KSHV ORF57 contains a classical caspase cleavage site, 30DETD 32, in its N terminus and can be cleaved by cellular caspase-7 during virus lytic induction (Fig. 1). Although the cleaved ORF57 retains three intact NLSs (15), it remains mainly in the cytoplasm of infected cells. The consequence of caspase-7 cleavage of ORF57 in lytic KSHV infection is reducing a subset of viral lytic gene expression and virus production. However, when Δ1–33 ORF57 with an added N-terminal methionine was over-expressed as a FLAG-tagged protein, with the FLAG on its C terminus, it can be translocated into the nucleus, but its ability to promote the expression of viral lytic genes (ORF56, ORF59, and K8) remains attenuated in cotransfection assays (Fig. 8). It is unclear how the N-terminal truncation contributes to the attenuated function of ORF57. Computer predictions using NetPhos (34) and ScanSite 2.0 (35) suggest that the first 33 N-terminal amino acid residues also contain several putative phosphorylation sites that could potentially be important to various ORF57 functions, as has been reported for its homologue Epstein-Barr virus EB2 (36). Mutation of these putative sites leads to disruption of ORF57 functions (data not shown).

Nevertheless, our data clearly indicate that caspase-7 cleavage of ORF57 appears to be a cellular function against virus infection in viral lytic infection.

Viral lytic infection leads to cell apoptosis (23) and production of active caspases. As reported in this study, KSHV lytic infection activates caspase-8-mediated apoptosis pathway, resulting in the activation of caspase-3 and -7 in the infected B cells. However, it remains to be understood how this apoptosis pathway can be induced during viral lytic infection, which is beyond our focus in the present study. Caspase-3 and -7 have been found to share 53% sequence identity, high structural similarity, and nearly identical substrate preference, with both enzymes targeting the same DEXD motif (27). Gene knock-out experiments have shown that caspase-3 and -7 have similar phenotypes (37). However, given the overlap between caspase-3 and -7 in their substrate specificity, it is remarkable that caspase-7, but not caspase-3, has a specific role in the cleavage of KSHV ORF57 at a classical DETD ↓ A cleavage site. Recent studies have suggested that substrates can have a substantial preference for caspase-7 over caspase-3 or vice versa. The reported substrates with specificity for caspase-7, but not for the closely related caspase-3, are Nogo-B (SSTD ↓ S) (38), claspin (DEYD ↓ G) (39), and Ataxin-7 (PKMD ↓ G and FDPD ↓ I) (40). A substrate preference for caspase-3 over caspase-7 is found in protein phosphatase-1 inhibitor-3 (DTVD ↓ G) (41) and in excitatory amino acid transporter EAAT2 (DTID ↓ S) (42). These data suggest that caspase-7 and caspase-3 have distinct functions. Moreover, caspase-3 and -7 preferentially function in different compartments of apoptotic (28) and virus-infected cells, with caspase-3 acting in the nucleus and caspase-7 in the cytoplasm.
Caspase-7 Cleavage of KSHV ORF57

The preferential cleavage of ORF57 by caspase-7 in the cytoplasm of infected cells provides a great advantage for the cells to attenuate ORF57 immediately after its translation. Because ORF57 functions mainly in the nucleus, caspase-7 cleavage of ORF57 in the cytoplasm enables the infected cells to take action even before ORF57 gets into the nucleus, an attractive strategy to prevent KSHV replication and virus production.

The insensitivity of the characterized caspase-7 cleavage site \(^{39}\text{DETD}^{33}\) to caspase-3 might be evolutionarily conserved. After the expression of ORF57 in lytic infection, KSHV expresses K7, a caspase-3-specific inhibitor (43), and vIRF3, which preferentially suppresses caspase-3 and only slightly suppresses caspase-7 (44). Therefore, having an ORF57 that is resistant to caspase-3 cleavage, a key mediator of apoptosis in mammalian cells with promiscuous substrates, could allow the virus to have enough ORF57 at early infection to promote the expression of the ORF57 targets.

Caspase-10 showed some activity for ORF57 cleavage in vitro. However, we were unable to determine its role in vivo because of the lack of a specific antibody (45). We are assuming that caspase-10, as well as caspase-5, which displayed a little activity in vitro, plays only minimal roles in ORF57 cleavage in vivo, because both immunodepletion of caspase-7 from cell extracts and siRNA-mediated knockdown of caspase-7 expression from cells dramatically reduced ORF57 cleavage.

KSHV also expresses protein inhibitors of caspase-8 and caspase-9 during viral latent infection. Notably, all of the latent proteins are expressed during viral lytic infection, when they presumably execute a similar function to prevent the lytically infected cells from apoptosis. However, activation of initiator caspase-8 and induction of caspase-8-mediated apoptosis pathway during lytic KSHV infection suggests that these viral caspase inhibitors do not function efficiently in lytic infection. KSHV latent vFLIP (ORF71/K13) is a caspase-8 inhibitor but also inhibits caspase-9 activity (46) to prevent death receptor-mediated apoptosis. Thus, vFLIP is essential for the survival of infected lymphoma cells (47). KSHV latent K15 is a HAX-1 (HS-1-associated protein-1)-binding protein that acts as a potent inhibitor of Bax-induced apoptosis (48) by inhibiting caspase-9 (49). By doing so, KSHV equips itself to develop persistent and latent infection for cell proliferation and transformation by protecting latently infected cells from apoptosis.

In KSHV lytic infection, these proteins, together with lytic K7, vIRF3, and vBcl-2, presumably prevent viral lytic infection-induced apoptosis of the infected cells to ensure a complete viral life cycle and virus production. These could be the cells represented by predominant expression of ORF57 and/or RTA but no active caspase-7 (Figs. 5–7 and supplemental Figs. S3 and S4). However, KSHV lytic infection does induce caspase-8-mediated apoptosis pathway, which activates caspase-7 as feedback in fighting against virus infection. This leads to caspase-7 cleavage of ORF57 in the infected cells very early in the lytic infection. The consequence of this battle is to limit the expression of this essential viral protein as shown in the cells mainly with active caspase-7 staining (Figs. 5–7 and supplemental Figs. S3 and S4) and to delay virus productivity even before the virus to complete its life cycle. We have the following two observations to support this scenario: (a) the majority of cells with active caspase-7 expression alone underwent apoptosis, with a fragmented nucleus and without, or at least suppressed, KSHV reactivation, whereas other cells with ORF57 and/or RTA coexpression did not exhibit apoptotic nuclear morphology and greatly diminished the presence of active caspase-7 (Figs. 6 and 7); and (b) specific reduction of caspase-7 by RNA interference or a peptide-based inhibitor z-DEVD could prevent ORF57 from cleavage and enhance the expression of ORF57 targets in more cells with KSHV lytic infection (Fig. 9, C–E).

Acknowledgments—We thank Jae Jung (University of Southern California) for providing doxycycline-inducible TREx BCBL-1 RTA and vector cell lines and Chu-Xia Deng (NIDDK, National Institutes of Health) for providing MCF-7 cells.

REFERENCES
1. Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996) Cell 87, 171
2. Riedl, S. J., and Shi, Y. (2004) Nat. Rev. Mol. Cell. Biol. 5, 897–907
3. Creagh, E. M., Conroy, H., and Martin, S. J. (2003) Immunol. Rev. 193, 10–21
4. Galluzzi, L., Mairui, M. C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L., and Kroemer, G. (2007) Cell Death Differ. 14, 1237–1243
5. Chang, Y., Cesarman, E., Pessin, M. S., Lee, F., Culppepper, J., Knowles, D. M., and Moore, P. S. (1994) Science 266, 1865–1869
6. Schulz, T. F. (1998) J. Gen. Virol. 79, 1573–1591
7. Cesarman, E., Chang, Y., Moore, P. S., Said, J. W., and Knowles, D. M. (1995) N. Engl. J. Med. 332, 1186–1191
8. Soulier, J., Grollet, L., Oksenhendler, E., Cacoub, P., Cazals-Hatem, D., Babinet, P., d’Agay, M. F., Clavelu, J. P., Raphael, M., Degos, L., and Sigaud, F. (1995) Blood 86, 1276–1280
9. Zhong, W., Wang, H., Herndier, B., and Ganem, D. (1996) Proc. Natl. Acad. Sci. U.S.A. 93, 6641–6646
10. Han, Z., and Swaminathan, S. (2006) J. Virol. 80, 5251–5260
11. Majerciak, V., Pripuzova, N., McCoy, J. P., Gao, S. J., and Zheng, Z. M. (2007) J. Virol. 81, 1062–1071
12. Majerciak, V., and Zheng, Z. M. (2009) Front. Biosci. 14, 1516–1528
13. Majerciak, V., Yamaneci, K., Allemand, E., Kruhlak, M., Krainer, A. R., and Zheng, Z. M. (2008) J. Virol. 82, 2792–2801
14. Nakamura, H., Lu, M., Gwack, Y., Souvlis, J., Zeichner, S. L., and Jung, J. U. (2003) J. Virol. 77, 4205–4220
15. Majerciak, V., Yamaneci, K., Nie, S. H., and Zheng, Z. M. (2006) J. Biol. Chem. 281, 28365–28378
16. Majerciak, V., Yamaneci, K., and Zheng, Z. M. (2006) J. Virol. 80, 11968–11981
17. Yamaneci, K., Tang, S., and Zheng, Z. M. (2005) J. Virol. 79, 14207–14221
18. Aragane, Y., Kulms, D., Metze, D., Wilkes, G., Poppelmuller, B., Luger, T. A., and Schwarz, T. (1998) J. Cell Biol. 140, 171–182
19. Wang, Y., and Li, G. (2008) J. Biol. Chem. 283, 11887–11893
20. Kuribayashi, K., Mayes, P. A., and El-Deiry, W. S. (2006) Cancer Biol. Ther. 5, 763–765
21. Nijhawan, D., Fang, M., Traer, E., Zhong, Q., Gao, W., Du, F., and Wang, X. (2003) Genes Dev. 17, 1475–1486
22. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
23. Klass, C. M., Krug, L. T., Pozharskaya, V. P., and Offermann, M. K. (2005) Blood 105, 4028–4034
24. Sun, R., Lin, S. F., Gradoville, L., Yuan, Y., Zhu, F., and Miller, G. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 10866–10871
25. Kulms, D., and Schwarz, T. (2002) Biochem. Pharmacol. 64, 837–841
26. Watanabe, T., Tan, T. W., and Ranganathan, S. (2006) BMC Bioinformatics 7 (Suppl. 5), S14
27. Timmer, J. C., and Salvesen, G. S. (2007) Cell Death Differ. 14, 66–72
28. Kamada, S., Kikkawa, U., Tsujimoto, Y., and Hunter, T. (2005) J. Biol. Chem. 280, 857–860
29. Gausdal, G., Gjertsen, B. T., Fladmark, K. E., Demol, H., Vandekerckhove, J., and DSkeland, S. O. (2004) Leukemia 18, 1989–1996
30. Hardy, J. A., Lam, J., Nguyen, J. T., O’Brien, T., and Wells, J. A. (2004) Proc. Natl. Acad. Sci. U.S.A. 101, 12461–12466
31. McStay, G. P., Salvesen, G. S., and Green, D. R. (2008) Cell Death Differ. 15, 322–331
32. Jänicke, R. U., Sprengart, M. L., Wati, M. R., and Porter, A. G. (1998) J. Biol. Chem. 273, 9357–9360
33. Zhu, F. X., and Yuan, Y. (2003) J. Virol. 77, 4221–4230
34. Blom, N., Gammeltoft, S., and Brunak, S. (1999) J. Mol. Biol. 294, 1351–1362
35. Obenauer, J. C., Cantley, L. C., and Yaffe, M. B. (2003) Nucleic Acids Res. 31, 3635–3641
36. Medina-Palazon, C., Gruffat, H., Mure, F., Filhol, O., Vingtdeux-Didier, V., Drobecq, H., Cochet, C., Sergeant, N., Sergeant, A., and Manet, E. (2007) J. Virol. 81, 11850–11860
37. Lakhani, S. A., Masud, A., Kuida, K., Porter, G. A., Jr., Booth, C. J., Mehal, W. Z., Inayat, I., and Flavell, R. A. (2006) Science 311, 847–851
38. Schweigreiter, R., Stasyk, T., Contarini, I., Frauscher, S., Oertle, T., Klimaschewski, L., Huber, L. A., and Bandtlow, C. E. (2007) Proteomics. 7, 4457–4467
39. Clarke, C. A., Bennett, L. N., and Clarke, P. R. (2005) J. Biol. Chem. 280, 35337–35345
40. Young, J. E., Goush, L., Propp, S., Sopher, B. L., Taylor, J., Lin, A., Hermel, E., Logvinova, A., Chen, S. F., Chen, S., Brezden, D. E., Truant, R., Ptacek, L. J., La Spada, A. R., and Ellerby, L. M. (2007) J. Biol. Chem. 282, 30150–30160
41. Huang, H. S., and Lee, E. Y. (2008) J. Biol. Chem. 283, 18135–18146
42. Boston-Howes, W., Gibb, S. L., Williams, E. O., Pasinelli, P., Brown, R. H., Jr., and Trott, D. (2006) J. Biol. Chem. 281, 14076–14084
43. Wang, H. W., Sharp, T. V., Koomi, A., Koentges, G., and Boshoff, C. (2002) EMBO J. 21, 2602–2615
44. Wies, E., Mori, Y., Hahn, A., Kremmer, E., Stürzl, M., Fleckenstein, B., and Neipel, F. (2008) Blood 111, 320–327
45. Jänicke, R. U., Sohn, D., Totzke, G., and Schulze-Osthoff, K. (2006) Science 312, 1874
46. Djerbi, M., Screpanti, V., Catrina, A. I., Bogen, B., Biberfeld, P., and Gran- dien, A. (1999) J. Exp. Med. 190, 1025–1032
47. Guasparri, I., Keller, S. A., and Cesaran, E. (2004) J. Exp. Med. 199, 993–1003
48. Sharp, T. V., Wang, H. W., Koomi, A., Hollyman, D., Endo, Y., Ye, H., Du, M. Q., and Boshoff, C. (2002) J. Virol. 76, 802–816
49. Han, Y., Chen, Y. S., Liu, Z., Bodyak, N., Rigo, D., Bisping, E., Pu, W. T., and Kang, P. M. (2006) Circ. Res. 99, 415–423
50. Olson, N. E., Graves, J. D., Shu, G. L., Ryan, E. J., and Clark, E. A. (2003) J. Immunol. 170, 6065–6072