The Latency-Related Gene of Bovine Herpesvirus 1 Inhibits Programmed Cell Death

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Although viral gene expression occurs in the peripheral nervous system during acute infection, bovine herpesvirus 1 (BHV-1) gene expression is extinguished, many neurons survive, and latency ensues. The only abundant viral transcript detected during latency is the latency-related (LR) RNA, which is alternatively spliced in trigeminal ganglia during acute infection (L. Devireddy and C. Jones, J. Virol. 72:7294–7301, 1998). A subset of neurons express a protein encoded by the LR gene and the LR protein (LRP) is associated with cyclin-dependent kinase 2 (Cdk2)/cyclin complexes during productive infection (Y. Jiang, A. Hossain, M. T. Winkler, T. Holt, A. Doster, and C. Jones, J. Virol. 72:8133–8142, 1998). LR gene products inhibit cell cycle progression, perhaps as a result of LRP interacting with Cdk2/cyclin complexes. During acute infection, expression of cyclin A occurs in trigeminal ganglionic neurons (L. M. Schang, A. Hossain, and C. Jones, J. Virol. 70:3807–3814, 1996). Inappropriate expression of G1- and S-phase cyclins can initiate programmed cell death (PCD), apoptosis, in neurons, suggesting that LR gene products inhibit PCD. To test this hypothesis, we modified an assay to measure PCD frequency in transiently transfected cells. β-ceramide, fumonisin B1 (FB1), or etoposide was used to initiate PCD following transfection of cells with plasmids expressing LR gene products and the β-galactosidase gene. Transfected cells that survived were quantified by counting β-galactosidase-positive cells. Plasmids that expressed LR gene products promoted survival of monkey kidney (CV-1), human lung (IMR-90), or mouse neuroblastoma (neuro-2A) cells after induction of PCD. Plasmids with termination codons at the beginning of LR open reading frames or deletion of sequences that mediate splicing of LR RNA did not promote cell survival following PCD induction. We hypothesize that LR gene products play a role in promoting survival of postmitotic neurons during acute infection or reactivation.

Bovine herpesvirus 1 (BHV-1) is a significant bovine pathogen, which causes respiratory disease, abortion, genital disease, or occasionally encephalitis (reviewed in reference 29). Like other members of the Alphaherpesvirinae subfamily, BHV-1 establishes latent infection in sensory ganglionic neurons (reviewed in reference 29). Viral DNA persist in these neurons for the lifetime of infected cattle but can periodically reactivate and spread. In contrast to the 70 to 80 viral genes expressed during productive infection, latency-related (LR) RNA is the only abundant viral transcript detected in latently infected neurons. A small fraction of LR RNA is polyadenylated and alternatively spliced in trigeminal ganglia (TG), suggesting that this RNA is translated into an LR protein (LRP) (8, 21, 27). LR gene products inhibit S-phase entry, and LRP is associated with cyclin-dependent kinase 2 (Cdk2)/cyclin complexes (27, 46). Cdk2/cyclin complexes regulate the transition from G1 to S to G2 (reviewed in reference 19) and are required for DNA replication (30). Cdk2 activity is stimulated after herpes simplex virus type 2 (HSV-2) infection (22) and is important for HSV-1 infection (47, 48). It is reasonable to hypothesize that members of the Alphaherpesvirinae subfamily utilize Cdk2 and perhaps other Cdns to stimulate viral DNA replication and transcription. Although the functional significance of the interactions between LRP and Cdk2/cyclin is not known, these interactions are likely to be important.

Herpesviruses can induce programmed cell death (PCD), or apoptosis, when cultured cells are infected (reviewed in references 18 and 51). BHV-1 also induces PCD after infection of cultured cells (9, 14–17) or calves (53). Neuronal PCD occurs during neurodegenerative disorders, trauma, or imbalances of growth factors and cytokines (reviewed in references 10, 25, and 54). Expression of cell cycle regulatory proteins is frequently observed in neurons undergoing PCD, suggesting that altered Cdk activity initiates PCD (reviewed in reference 49). The HSV-1 US3 kinase plays a role in preventing PCD (1, 33), suggesting that regulation of PCD is crucial for pathogenesis. Inhibiting neuronal damage and/or PCD may also be important because the primary site of latency for BHV-1 is sensory neurons.

This study demonstrated that LR gene products inhibit or delay PCD following transient transfection of CV-1 cells, low-passage human fibroblasts, or mouse neuroblastoma cells. Although there was a correlation between LR protein expression and cell survival, we cannot exclude the possibility that LR RNA by itself is important. We hypothesize that LR gene products promote neuronal survival by inhibiting PCD.

MATERIALS AND METHODS

Cells. Cells were plated at a density of 5 × 10^4/100-mm-diameter plastic dish in Earle’s modified Eagle’s medium supplemented with 5% fetal bovine serum (FBS). CV-1 cells were split at a 1:3 ratio every 4 or 5 days. Human primary lung fibroblasts (IMR-90 cells) were obtained from the American Type Culture Collection (ATCC, Rockville, Md. and split in a 1:3 ratio every 5 days. IMR-90 cells were split five to seven times and then discarded. Mouse neuroblastoma (neuro-2A; ATCC CCL131) cells were grown in Earle’s minimal essential medium supplemented with 5% FBS. All media contained penicillin (10 U/ml) and streptomycin (100 μg/ml).

β-Gal cotransfection and analysis of cell death. To quantitatively measure cell death, we modified a previously described assay (23, 24, 31, 35) that entails cotransflecting a β-galactosidase (β-Gal) expression plasmid (pCMV-β-gal) and...
a gene of interest by calcium phosphate precipitation (5, 13). If a gene induces PCD or is toxic to cells, the number of β-Gal-positive cells decrease. Conversely, a gene that inhibits PCD maintains the number of β-Gal-positive cells after inducing PCD. Cells were plated at a density of $2 \times 10^5/\text{well}$ in six-well plastic plates ($35 \text{ mm/well}$) 12 to 16 h prior to transfection. After transfection, a glycerol shock ($20\% \text{ glycerol–phosphate-buffered saline [PBS]}$) was performed for 4 min, followed by two PBS washes. Fresh medium containing 5% FCS and 5 mM sodium butyrate was added to the cells to facilitate transfection efficiency. Cells were then treated (37°C for 48 h) with 25 μM fumonisin B1 (FB1) (5, 52) or 15 μM C₆-ceramide (2, 40) to initiate PCD.

Neuro-2A cells were transfected as described above except that the glycerol shock was not performed and cultures were treated with 2.5 mM sodium butyrate. Cultures were subsequently treated with 15 μM etoposide (catalogue no. E1383; Sigma) to induce PCD. Etoposide is an anticancer agent that inhibits topoisomerase II. Cells treated with etoposide have higher levels of DNA damage (double- and single-stranded DNA), especially cells that are in late S and G₂ (reviewed in references 13a and 39). At 48 h after etoposide treatment, β-Gal-positive cells were observed microscopically. The number of stained blue cells was counted by identifying the same area of each plate. At least five fields per plate were counted (>500 cells), and the average number of cells per field was calculated.

**Plasmids.** The various constructs (see Fig. 3) were generated by standard recombinant techniques and as previously described (21, 46). To construct LRTΔSmaI, plasmid LRTWT was digested with SmaI, and the large fragment was
RESULTS

Analysis of PCD in cells transfected with the LR gene. Previous studies concluded LR gene products inhibit G1-to-S transition (46) and LRP is associated with Cdk2/cyclin complexes (27). Several independent studies have concluded that cell cycle factors, in addition to regulating cell cycle progression, play a role during PCD (11, 38, 41–43, 49). To test whether the LR gene influences cell survival, we modified a β-Gal cotransfection assay (24, 27, 31, 35) to measure the effects of various genes on PCD. Two sphingoid bases, FBa or Cε-ceramide, can induce PCD in mammalian cells (2, 5, 40, 52). Cells treated with FBa or Cε-ceramide exhibit the hallmarks of PCD: DNA laddering, formation of apoptotic bodies, and condensation of chromatin. Both agents kill approximately 80% of treated cells (5, 52). Cε-ceramide is one of the central regulators of the sphingomyelin signal transduction pathway, a ubiquitous signaling system that links specific cell surface receptors and environmental stresses to the nucleus. The sphingomyelin pathway is crucial during PCD initiated by tumor necrosis factor alpha, FAS, and ionizing radiation (2, 26, 40). FBa inhibits ceramide synthase, the enzyme that synthesizes complex sphingolipids (including ceramide). We have characterized the differences between the mechanism of PCD initiated by these two sphingoid bases (5, 52) and thus are useful reagents for analyzing PCD. Monkey kidney (CV-1) and primary human lung (IMR-90) cells were used for these studies because these cell types are nontumorigenic and susceptible to PCD.

A plasmid that expresses LR gene products (LRwt) enhanced cell survival after treatment with Cε-ceramide or FBa, as judged by an increase in the number of surviving β-Gal-positive CV-1 cells (Fig. 1 and 2A). As expected, the frequency of β-Gal-positive cells was reduced dramatically when CV-1 cells were cotransfected with pCDNA/3.1 and pCMV-β-Gal followed by treatment with Cε-ceramide or FBa (5) (Fig. 1). A plasmid that expresses bICP0 was used as a control because it contains sequences that overlap the LR gene. Overexpression of bICP0 in CV-1 cells reduced the number of β-Gal-positive CV-1 cells (Fig. 1) independent of treatment with Cε-ceramide or FBa, suggesting that it was toxic. After transfection with bICP0, the β-Gal-positive cells were smaller and rounded compared to those transfected with LRwt or pCDNA/3.1 (Fig. 1). Further studies will be necessary to determine whether bICP0 induces PCD or was merely toxic. The adenosivirus EIA gene, which induces PCD (7, 45), reduced the number of β-Gal-positive cells independent of treatment with FBa or Cε-ceramide (Fig. 2A). As expected, pCIAP enhanced the survival of cells after treatment with Cε-ceramide or FBa (Fig. 2A).

To ensure these findings were not a peculiarity of CV-1 cells, this study was repeated with IMR-90 cells. After transfection with LRwt or pCIAP, a higher frequency of cells survived Cε-ceramide or FBa treatment relative to cultures transfected with the blank expression vector (Fig. 2B). LRwt and pCIAP were statistically different from the vector alone. However, the difference between LRwt and pCIAP was not statistically significant. EIA reduced the number of blue cells purified and religated. LRStop contains a stop codon linker in the Spel sites located at positions 781 and 812 of the LR gene. This was accomplished by insertion of the 3′ flanking fragment containing the first 981 nucleotides (nt) of the LR gene into the pBlueBacHis vector and digestion with Spel. Large fragment was purified, and an Spel linker containing stop codons in all three open reading frames (ORFs) (5′-CAGAATTCATGGTAGTACGATG-3′) was ligated into the amino terminus of LR ORF2. This linker also contains an EcoRI site to facilitate screening.

pCMVbCIAP (hereafter referred to as pCIAP), a plasmid that contains the baculovirus antipapopoptotic gene iap (6), was obtained from Lois Miller (University of Georgia, Athens). The adenovirus EIA gene was obtained from E. White (Rutgers University, Piscataway, N.J.). pCMV-β-gal was purchased from Clontech (Palo Alto, Calif.). Plasmid E2.6 contains the BHV-1 ICP0 gene (bICP0) and was obtained from M. Schwyzer (Zurich, Switzerland). Two rounds of cesium chloride centrifugation were used to purify plasmids after bacteria were lysed with alkali and sodium dodecyl sulfate (SDS).

Preparation of RNA and RT-PCR. RNA from transfected CV-1 cells was prepared as described previously (8, 21). RNA was quantified spectrophotometrically (optical density at 260 nm) and stored at -80°C in 3 volumes of ethanol. Reverse transcriptase PCR (RT-PCR) and LRT primers were described previously (21). The Actin primer is (5′-TTCTCTGGGCTC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′). The L3B upstream sense primer spans nt 1755 to 1775 (5′-CGAATTCATGGTAGTACGATG-3′). The L3A downstream antisense primer is (5′-TTCTCTGGGCTC-3′). The L3A upstream sense primer spans nt 1672 to 1693 (5′-CGAATTCATGGTAGTACGATG-3′). The Actin primer is (5′-CTTCTTTAATGTGACGCCAGGATCTACATC-3′).

Western blot analysis. Preparation of extracts and Western blot analysis were performed as described previously (4, 21, 22, 46). The P2 antibody is directed against an 18-kDa protein in infected or transiently transfected cells (21, 27).

Statistical analysis. Statistical analysis was performed with the SSPS program, student version. Values shown in Fig. 2 and 6 are normalized to those for the vector-alone transfected controls (defined as 0). Data are average mean differences from vector-alone control (n = 7). P values represent the probability that the result occurred by chance, using 95% confidence; P < 0.05 is statistically significant. Error bars represent the standard error of the mean differences.
and L3B (Fig. 3C). As expected, LRTwt synthesized RNA that was amplified in every sample (Fig. 4D). Since primers L3A and L3B are downstream of the LR gene constructs synthesized RNA that was amplified by primers L3A and L3B. Only LRTstop (lane D) or LRTΔSmaI (lane E) or in mock-transfected cells (lane A). Since COS-7, U2-OS, and 293 cells are highly transformed and readily form tumors in immunodeficient mice, they are more resistant to apoptotic agents such as FB1 and Cε- ceramide. Consequently, these cell lines are not good models for studies of apoptosis. In summary, all four plasmids containing the LR gene constructs synthesized RNA in CV-1 cells that was amplified by primers L3A and L3B. Only LRTΔHX expressed a 40-kDa protein in transiently transfected cells (CV-1, neuro-2A, and primary human fibroblasts) is due to lower transfection efficiency. However, it cannot be ruled out that factors in these cell lines inhibit stable expression of LRP or the protein is not expressed at detectable levels (50). 293 cells transfected with LRTwt and LRTΔHX expressed a 40-kDa protein that was recognized by the P2 antibody (Fig. 5, lanes B and C, respectively). The same antibody did not recognize a 40-kDa protein in cells transfected with LRTstop (lane D) or LRTΔSmaI (lane E) or in mock-transfected cells (lane A). Since COS-7, U2-OS, and 293 cells are transformed, RNA was prepared 48 h after transfection, and RT-PCR was performed with LR-specific primers L3A and L3B (Fig. 3C). As expected, LRTΔHX synthesized RNA that was amplified with primers L3B (Fig. 4A, lane 5) and L3A (Fig. 4B, lane 5). L3A (Fig. 4B) and L3B (Fig. 4A) primers also amplified a similar-sized cDNA fragment, using RNA prepared from CV-1 cells transfected with LRTΔHX (lane 2), LRTstop (lane 3), or LRTΔSmaI (lane 4). These bands were amplified cDNA because no bands were observed when RT was omitted from the reaction (Fig. 4C). As expected, β-actin RNA was amplified in every sample (Fig. 4D). Since primers L3A and L3B are downstream of the SmaI restriction site (Fig. 3C), the SmaI deletion was not expected to interfere with amplification of the 3' terminus of LR RNA.

Transient transfection of COS-7 (21, 46), U2-OS (27), and 293 cells with the LR gene leads to expression of a 40-kDa protein that is recognized by the P2 antibody. We believe that the difficulty in detecting LRP in other transiently transfected cells (CV-1, neuro-2A, and primary human fibroblasts) is due to lower transfection efficiency. However, it cannot be ruled out that factors in these cell lines inhibit stable expression of LRP or the protein is not expressed at detectable levels (50). 293 cells transfected with LRTwt and LRTΔHX expressed a 40-kDa protein that was recognized by the P2 antibody (Fig. 5, lanes B and C, respectively). The same antibody did not recognize a 40-kDa protein in cells transfected with LRTstop (lane D) or LRTΔSmaI (lane E) or in mock-transfected cells (lane A). Since COS-7, U2-OS, and 293 cells are transformed, RNA was prepared 48 h after transfection, and RT-PCR was performed with LR-specific primers L3A and L3B (Fig. 3C). As expected, LRTΔHX synthesized RNA that was amplified with primers L3B (Fig. 4A, lane 5) and L3A (Fig. 4B, lane 5). L3A (Fig. 4B) and L3B (Fig. 4A) primers also amplified a similar-sized cDNA fragment, using RNA prepared from CV-1 cells transfected with LRTΔHX (lane 2), LRTstop (lane 3), or LRTΔSmaI (lane 4). These bands were amplified cDNA because no bands were observed when RT was omitted from the reaction (Fig. 4C). As expected, β-actin RNA was amplified in every sample (Fig. 4D). Since primers L3A and L3B are downstream of the SmaI restriction site (Fig. 3C), the SmaI deletion was not expected to interfere with amplification of the 3' terminus of LR RNA.
There was also an increase in CV-1 cell survival after transfection with LRTΔHX compared to LRTwt. This was not observed in neuro-2A cells treated with etoposide and may reflect differences in cell types or the mechanism by which etoposide kills cells. In summary, this study demonstrated that LRTstop and LRTΔSmaI did not protect CV-1 and neuro-2A cells from PCD but LRTwt and LRTΔHX did.

DISCUSSION

This study provided evidence that LR gene products inhibited PCD induced by C6-ceramide, FB1, or etoposide. Three different cell types, including a cell line of neuronal origin, were used. LRTstop and LRTΔSmaI were unable to prevent PCD in any cell type tested and did not express a 40-kDa protein in 293 cells that was recognized by the P2 antibody. Although it is tempting to speculate that expression of LRP is required for preventing PCD, it is clear that LRTΔSmaI and LRTstop would express transcripts that are different from those expressed by LRTwt and LRTΔHX. Consequently, it cannot be ruled out that subtle quantitative or qualitative differences in the transcripts encoded by LRTΔSmaI and LRTstop play a role in cell survival. Further studies are necessary to prove that continual expression of LRP is necessary for inhibiting PCD.

The ability of LR gene products to inhibit cell cycle progression (46) and LRP to bind Cdk2/cyclins (27) may play a role in preventing PCD. A link between cell cycle regulatory proteins and PCD has been established. For example, cyclin A-dependent kinase activity is stimulated during PCD (37), and PCD is suppressed by dominant negative mutants of Cdk2 or Cde2 (38). Second, cell cycle inhibitors promote survival of postmitotic neurons (41–43), and the Cdk inhibitor p21 can protect cells from PCD (12, 36). Third, proteolytic enzymes that are activated during PCD (caspases) induce cdk/cyclin activity in the early stages of PCD (34, 55). Genes that regulate PCD, Bcl-2 and BAX, modulate cdk2 activation during thymocyte apoptosis (11). Finally, FB1 treatment of CV-1 cells induces a transient increase in Cdk2 and Cdk4 activity (5). At this time, the factors that direct cell cycle regulators to initiate PCD but not cell cycle progression have not been identified.
BHV-1 induces PCD in lymphocytes (14–17), bovine kidney cells (9), and acutely infected cattle (53), suggesting that cells in the peripheral nervous system undergo PCD. During pathological states, neurons undergo PCD (10, 49, 54), indicating they are usually resistant to PCD. LR RNA and LRP may promote neuronal survival during establishment and maintenance of latency (outlined in Fig. 7). The LR gene inhibits the transactivation potential of bICP0 in transient transfection assays presumably because LR RNA interferes with bICP0 expression (3) and thus may interfere with productive infection. The interaction between LRP and Cdk2 (28) may also repress productive infection because roscovitine, a chemical that inhibits Cdk2, Cdc2, and Cdk5 activity (47, 48), inhibits HSV transcription and DNA replication. Cdk2 activity is also required for initiation of cellular DNA replication (30). Based on these observations, we hypothesize that LR RNA and LRP act in concert to inhibit productive viral gene expression and neuronal PCD during establishment and maintenance of latency.

During reactivation, LR gene products may promote neuronal survival in the face of productive viral gene expression and DNA replication, thus maximizing virion production. Only 20% of neurons latently infected with BHV-1 actually reactivate following dexamethasone injection (44), suggesting that 80% of latently infected neurons resume latency. Thus, LR gene products may enhance neuronal survival in the event of incomplete reactivation. During reactivation, TG neurons may be more vulnerable to PCD because dexamethasone inhibits LR promoter activity (28), represses LR RNA expression in TG but initiates viral gene expression (44), and induces PCD (20). Considering LR RNA is alternatively spliced in neurons (8), it is possible that reactivation-specific factors facilitate reactivation. This hypothesis can be tested directly in cattle when a LR-negative mutant is constructed.

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REFERENCES


