



Specific Akt Family Members Impair Stress-Mediated Transactivation of Viral Promoters and Enhance Neuronal Differentiation: Important Functions for Maintaining Latency

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ABSTRACT Neurotropic *Alphaherpesvirinae* subfamily members such as bovine herpesvirus 1 (BoHV-1) and herpes simplex virus 1 (HSV-1) establish and maintain life-long latent infections in neurons. Following infection of ocular, oral, or nasal cavities, sensory neurons within trigeminal ganglia (TG) are an important site for latency. Certain external stressors can trigger reactivation from latency, in part because activation of the glucocorticoid receptor (GR) stimulates productive infection and promoters that drive expression of key viral transcriptional regulators. The Akt serine/threonine protein kinase family is linked to maintaining latency. For example, Akt3 is detected in more TG neurons during BoHV-1 latency than in reactivation and uninfected calves. Furthermore, Akt signaling correlates with maintaining HSV-1 latency in certain neuronal models of latency. Finally, an active Akt protein kinase is crucial for the ability of the HSV-1 latency-associated transcript (LAT) to inhibit apoptosis in neuronal cell lines. Consequently, we hypothesized that viral and/or cellular factors impair stress-induced transcription and reduce the incidence of reactivation triggered by low levels of stress. New studies demonstrate that Akt1 and Akt2, but not Akt3, significantly reduced GR-mediated transactivation of the BoHV-1 immediate early transcription unit 1 (IEt1) promoter, the HSV-1 infected cell protein 0 (ICP0) promoter, and the mouse mammary tumor virus long terminal repeat (MMTV-LTR). Akt3, but not Akt1 or Akt2, significantly enhanced neurite formation in mouse neuroblastoma cells, which correlates with repairing damaged neurons. These studies suggest that unique biological properties of the three Akt family members promote the maintenance of latency in differentiated neurons.

IMPORTANCE External stressful stimuli are known to increase the incidence of reactivation of *Alphaherpesvirinae* subfamily members. Activation of the glucocorticoid receptor (GR) by the synthetic corticosteroid dexamethasone (DEX) stimulates bovine herpesvirus 1 (BoHV-1) and herpes simplex virus 1 (HSV-1) reactivation. Furthermore, GR and dexamethasone stimulate productive infection and promoters that drive expression of viral transcriptional regulators. These observations lead us to predict that stress-induced transcription is impaired by factors abundantly expressed during latency. Interestingly, activation of the Akt family of serine/threonine protein kinases is linked to maintenance of latency. New studies reveal that Akt1 and Akt2, but not Akt3, impaired GR- and dexamethasone-mediated transactivation of the BoHV-1 immediate early transcription unit 1 and HSV-1 ICP0 promoters. Strikingly, Akt3, but not Akt1 or Akt2, stimulated neurite formation in mouse neuroblastoma cells, a requirement for neurogenesis. These studies provide insight into how Akt family members may promote the maintenance of lifelong latency.

KEYWORDS AKT signaling, HSV-1, bovine herpesvirus 1, latency, neurogenesis, stress-induced transcription

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Herpes simplex virus 1 (HSV-1) and bovine herpesvirus 1 (BoHV-1) infections cause important diseases in their respective hosts (1, 2). Their ability to latently infect neurons and periodically reactivate from latency is crucial for virus transmission and, in the case of HSV-1, for recurrent disease. Sensory neurons in trigeminal ganglia (TG) are a primary site for latency when acute infection is initiated in the ocular, oral, or nasal cavity (1–3). Establishment and maintenance of latency requires that infected neurons survive, neuronal damage is repaired, lytic cycle viral gene expression is silenced, and little or no virus is produced (4–6). The BoHV-1-encoded latency-related (LR) RNA and the HSV-1 latency-associated transcript (LAT) are readily detected in latently infected neurons. LAT (7) and the LR gene (8) encode multiple products that can promote survival of infected neurons by inhibiting apoptosis (9–11) and expression of crucial viral regulatory proteins (12, 13). A cellular microRNA reduces expression of an HSV-1 regulatory protein (ICP0), demonstrating that cellular factors play a role in promoting latency (14). Thus, viral and cellular functions actively promote establishment and maintenance of lifelong latency.

“Stress” increases the incidence of BoHV-1 (1, 2, 15, 16), HSV-1 (17–19), and canine herpesvirus 1 reactivation from latency (20, 21). Strikingly, the genomes of BoHV-1 (22) and HSV-1 (C Jones and F. Meyer, unpublished results) contain many putative glucocorticoid receptor (GR) response elements (GREs). The immediate early transcription unit 1 (IEt1) promoter of BoHV-1 contains two functional GREs that are essential for transactivation by GR and the synthetic corticosteroid dexamethasone (DEX) (22, 23). This viral promoter drives expression of two important viral transcriptional regulators (bICP0 and bICP4). Recent studies demonstrated that GR and Krüppel-like transcription factor 15 (KLF15) cooperatively transactivate the HSV-1 ICP0 promoter (24). While the ICP0 promoter does not contain a whole GRE, it contains five half-GREs that are important but not required for stress-induced promoter activation.

Recent studies suggested that the three Akt family members play a significant role in the latency-reactivation cycle of BoHV-1 and HSV-1. For instance, Akt3 RNA is significantly higher in TG neurons of calves latently infected with BoHV-1; conversely, 30 min after DEX is administered to initiate reactivation, Akt3 expression is reduced more than 50-fold and the number of Akt3-positive TG neurons is significantly reduced (25). Furthermore, the number of TG neurons that express Akt3 is significantly higher during latency relative to the number of TG neurons from uninfected calves or from those undergoing DEX-induced reactivation from latency. With respect to the HSV-1 latency-reactivation cycle, several studies indicate that Akt signaling is important. For example, inhibiting the phosphatidylinositol 3-kinase (PI3K)/Akt signaling axis induces reactivation from latency in two neuronal models of HSV-1 latency, primary rat sympathetic neurons (26, 27) and Lund human mesencephalic (LUHMES) neuronal cells (28). Furthermore, interfering with Akt kinase functions impairs the ability of the HSV-1 latency-associated transcript (LAT) to interfere with apoptosis and neurite formation (29, 30). The Wnt/ β -catenin pathway is induced during BoHV-1 latency but repressed during DEX-induced reactivation (25, 31, 32). Mice latently infected with wild-type HSV-1 contain significantly more TG neurons that express β -catenin than those in an LAT-null mutant (33). Akt family members and the Wnt signaling pathway form a positive regulatory loop (34–39), suggesting that these signaling pathways promote the maintenance of latency.

While the three Akt family members are similar and are generally associated with cell survival, regulating metabolism, and promotion of tumor development, they also have nonredundant functions, reviewed in Manning and Toker (40). For example, Akt1^{-/-} mice exhibit growth defects and increased perinatal lethality (41). In contrast, Akt2^{-/-} mice develop diabetes-like symptoms (42). Furthermore, Akt1 interferes with metastatic spread of breast cancer, whereas Akt2 promotes metastatic spread (43–45). Akt3^{-/-} mice have decreased brain volume and exhibit neurodevelopmental problems (46, 47). Akt3 prevents stroke-induced neuronal injury (48), inhibits apoptosis in neurons, and stimulates axonal development more efficiently than Akt1 and Akt2 (49).

Currently, proteins phosphorylated by specific Akt isoforms that mediate the unique biological properties of these kinases have not been identified.

In this study, we tested whether Akt family members regulate stress-induced transactivation of two key alphaherpesvirus promoters, BoHV-1 IETu1 and HSV-1 ICP0. We also compared these results to the mouse mammary tumor virus long terminal repeat (MMTV-LTR) because it is widely known to be activated by GR and DEX (50). Akt1 and Akt2, but not Akt3, interfered with GR-mediated transactivation of these three promoters. Interestingly, a protein kinase dead Akt1 mutant did not inhibit stress-induced transactivation of the IETu1 and MMTV-LTR. Conversely, this Akt1 protein kinase mutant inhibited stress-induced transactivation of the ICP0 promoter. Additional studies revealed that Akt3, but not Akt1 or Akt2, enhanced neurite formation during differentiation of Neuro-2A cells. In summary, these studies identified novel Akt functions that are predicted to be important for maintaining a lifelong latent infection.

RESULTS

Akt1 reduced GR- and DEX-mediated activation of BoHV-1 IETu1 collapsed promoter in a dose-dependent manner. While the antiapoptotic functions of Akt family members (40, 51) are likely to play an important role in maintaining latency, we predicted that additional Akt functions are important for this crucial phase of the latency-reevaluation cycle. For example, mammals face stressful stimuli every day, but reevaluation from latency is not a daily occurrence. Support for this statement comes from the finding that calves latently infected with BoHV-1 do not frequently shed virus prior to DEX treatment (C. Jones, personal communication). Interestingly, Akt1 is frequently activated in acute lymphoblastic leukemia, which correlates with development of glucocorticoid resistance (52, 53). Based on these observations, we predicted that cellular or viral factors actively restrain stress-induced stimulation of viral gene expression during latency. Consequently, we tested whether Akt1, Akt2, or Akt3 influenced GR-mediated transactivation of viral promoters in Neuro-2A cells. Neuro-2A cells were used for these studies because they are a mouse neuroblastoma cell line that can be readily transfected and differentiated into dopamine-like neurons (54)—thus, they have certain neuron-like properties.

Initially we examined the IETu1 collapsed promoter because it is a minimal IETu1 promoter construct efficiently transactivated by GR plus DEX or by GR plus KLF15 plus DEX (22, 23, 55). The IETu1 promoter contains two consensus GREs required for activation by GR and DEX. Akt1 or Akt2, but not Akt3, significantly reduced GR-mediated transactivation of the BoHV-1 IETu1 collapsed promoter when cultures were treated with the synthetic corticosteroid DEX (Fig. 1A). Increasing Akt1 concentrations reduced GR-mediated activation in a dose-dependent manner (Fig. 1B). Dose-dependent effects on GR-mediated transactivation of IETu1 collapsed promoter activity were also observed with Akt2 but not with Akt3 (data not shown). Conversely, none of the Akt family members significantly reduced basal promoter activity. Akt1 is a serine/threonine protein kinase, and the Akt1 kinase dead mutant (1014 pcDNA3 T7, which contains 3 point mutations, namely K179M, T308A, and S473A; Addgene) (56) was used to test whether kinase activity was important for inhibiting GR-mediated transcription. The Akt1 kinase mutant (Akt1m) did not significantly reduce GR-mediated transactivation of the IETu1 collapsed promoter (Fig. 1B). These studies demonstrated that Akt1 and Akt2 impaired GR-mediated activation of the IETu1 collapsed promoter and that Akt1 kinase activity was required for the inhibitory effect.

Akt1 and Akt 2 reduced GR-mediated activation of the MMTV-LTR promoter. The effect of Akt family members on stress-induced activation of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) was also examined because this retroviral promoter is strongly stimulated by GR plus DEX, as well as by estrogen, due to multiple GREs in the LTR (50, 57). Consequently, the MMTV-LTR is an excellent model promoter to explore the mechanism by which stress-induced transcription occurs. Hence, we examined the effect Akt family members had on MMTV LTR promoter activity as a comparison to the BoHV-1 IETu1 promoter. Akt1 and Akt2, but not Akt3,

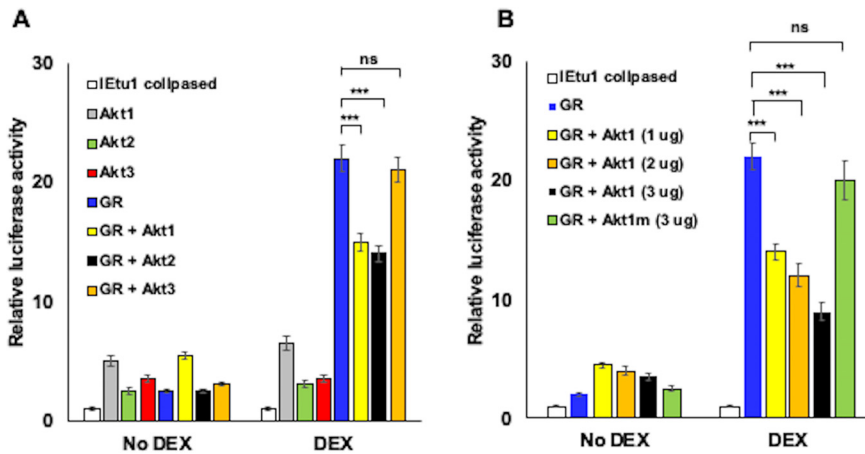


FIG 1 Akt family members influence GR- and DEX-mediated transactivation of the IETu1 collapsed promoter. (A) Neuro-2A cells were transfected with the IETu1 collapsed promoter construct containing the firefly luciferase reporter gene (0.5 μ g) and, where indicated, plasmids that expressed GR (1.0 μ g), Akt1, Akt2, or Akt3 (1.0 μ g). (B) Neuro-2A cells were transfected with the IETu1 collapsed promoter (0.5 μ g) and, where indicated, plasmids that expressed GR (1.0 μ g), Akt1 (1.0 μ g, 2.0 μ g, or 3.0 μ g), or Akt1 kinase mutant construct (3.0 μ g). All transfections contained a plasmid that expresses Renilla luciferase (0.05 μ g) to normalize firefly luciferase values. To maintain the same amount of DNA in each sample, empty vector was included in certain samples. Cells were incubated with 2% stripped fetal bovine serum (FBS) at approximately 24 h after transfection, and then certain cultures were treated with DEX (10 μ M). At 48 h after transfection, cells were harvested and protein lysate subjected to a dual-luciferase assay. The results are the average of 3 independent experiments, and error bars denote the standard error. Student's *t* test was used for statistical analysis. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

significantly reduced GR-mediated transactivation of the MMTV-LTR promoter in a DEX-dependent manner (Fig. 2A). However, Akt1, Akt2, and Akt3 each had little effect on basal promoter activity (no DEX addition). These studies also revealed that Akt1-dependent inhibition was dose dependent (Fig. 2B). The Akt1 kinase mutant (Akt1m)

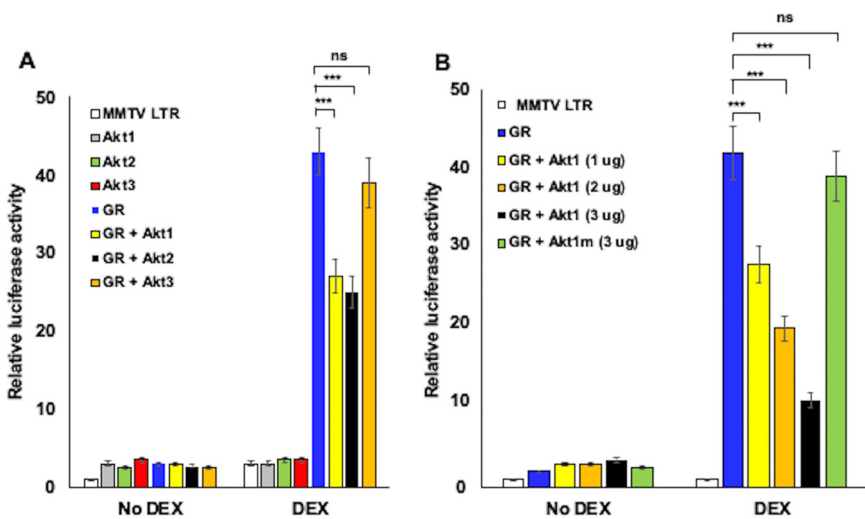


FIG 2 Akt1 and Akt2 impair GR-mediated activation of the MMTV-LTR promoter. (A) Neuro-2A cells were transfected with the MMTV-LTR promoter construct (0.5 μ g) and, where indicated, plasmids that express GR (1.0 μ g), Akt1, Akt2, or Akt3 (1.0 μ g). (B) Neuro-2A cells were transfected with the MMTV-LTR promoter construct (0.5 μ g) and, where indicated, plasmids that expressed GR (1.0 μ g), Akt1 (1.0 μ g, 2.0 μ g, or 3.0 μ g), or Akt1 kinase mutant construct (3.0 μ g). All transfections contained a plasmid that expresses Renilla luciferase (0.05 μ g) to normalize firefly luciferase values. To maintain the same amount of DNA in each sample, empty vector was included in certain samples. Cells were incubated with 2% stripped FBS at approximately 24 h after transfection and then certain cultures were treated with DEX (10 μ M). At 48 h after transfection, cells were harvested and protein lysate subjected to a dual-luciferase assay. The results are the average of 3 independent experiments and error bars denote the standard error. Student's *t* test was used for statistical analysis. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$.

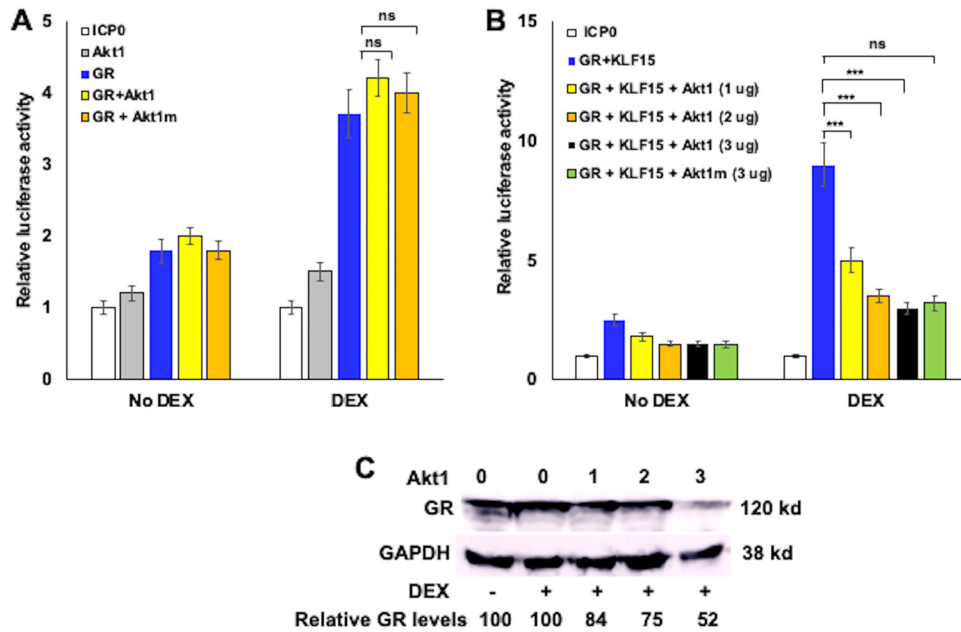


FIG 3 Akt1 significantly reduces GR- and KLF15-mediated transactivation of the HSV-1 ICP0 promoter. (A) Neuro-2A cells were transfected with the HSV-1 ICP0 promoter construct (0.5 μ g), plasmids that express Akt1 or Akt1m (1.0 μ g), and a plasmid that expresses GR (1.0 μ g). (B) Neuro-2A cells were transfected with the ICP0 promoter construct (0.5 μ g) and, where indicated, plasmids that expressed GR (1.0 μ g), KLF15 (1.0 μ g), Akt1 (1.0 μ g, 2.0 μ g, or 3.0 μ g), or Akt1 kinase mutant construct (3.0 μ g). All transfections contained a plasmid that expresses Renilla luciferase (0.05 μ g) to normalize firefly luciferase values. To maintain the same amount of DNA in each sample, empty vector was included in certain samples. Cells were incubated with 2% stripped FBS 24 h after transfection and then certain cultures were treated with DEX (10 μ M). At 48 h after transfection, cells were harvested and protein lysate subjected to a dual-luciferase assay. The results are the average of 3 independent experiments, and error bars denote the standard error. Student's *t* test was used for statistical analysis. ns, not significant; **, $P < 0.01$; ***, $P < 0.001$. (C) Neuro-2A cells were transfected with a plasmid that expresses Akt1 (1.0, 2.0 or 3.0 μ g of the expression vector as denoted), and a plasmid that expresses GR (1.0 μ g). Cells were incubated with 2% stripped FBS 24 h after transfection and then certain cultures were treated with DEX (10 μ M). At 48 h after transfection, cells were harvested, cell lysate prepared, and Western blot analysis performed to detect GR and the loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Fifty μ g of protein was loaded in each lane. The results are the average of 3 independent experiments. kd, kilodalton.

did not significantly reduce GR-mediated transactivation (Fig. 2B), indicating that Akt1 kinase activity was essential for reducing GR-mediated transactivation of the MMTV-LTR, which was similar to that of the I Etu1 promoter.

Akt1 reduced GR+KLF15 mediated transactivation of the HSV-1 ICP0 promoter. The HSV-1 ICP0 promoter has numerous transcription factor binding sites and is stimulated by heat stress-induced factors, (58). Expression of the multifunctional ICP0 protein is believed to be important for successful reactivation from latency (reviewed in reference 15). Unlike the I Etu1 collapsed promoter and MMTV-LTR, there are no consensus GREs in the ICP0 promoter (24). Akt1 had little effect on ICP0 promoter activity when cultures were not treated with DEX, in part because the basal activity of the promoter was low in Neuro-2A cells (Fig. 3A). Since DEX only stimulated promoter activity by approximately 3-fold, the effects of Akt family members were nominal. KLF15, GR, and DEX treatment cooperatively transactivate ICP0 promoter activity (24). When KLF15 was cotransfected with GR (no DEX treatment), ICP0 promoter activity was not influenced dramatically by Akt1 (Fig. 3B). However, Akt1 significantly reduced the activity of GR plus KLF15 plus DEX mediated transactivation of the ICP0 promoter in a dose-dependent manner (Fig. 3B). In contrast to the I Etu1 promoter and MMTV-LTR, the Akt1 kinase mutant (Akt1m) also significantly reduced ICP0 promoter activity (Fig. 3B). As with the MMTV-LTR and I Etu1 promoter, Akt2, but not Akt3, reduced ICP0 promoter activity stimulated by GR, KLF15, and DEX treatment (data not shown).

To address whether Akt1 influenced GR steady-state proteins, increasing amounts of Akt1 were cotransfected with the GR expression construct, and Western blot analysis

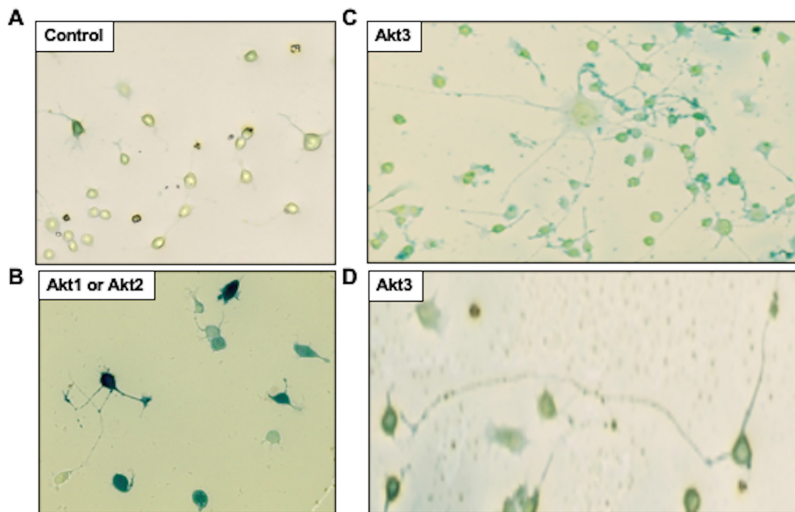


FIG 4 Akt3 efficiently promotes neurite formation in Neuro-2A cells. Neuro-2A cells were cotransfected with an empty vector (pcDNA3.1) (A), a plasmid expressing Akt1 or Akt2 (Panel B), or Akt3 (C and D) ($1 \mu\text{g}$ plasmid DNA) and a plasmid expressing the *lacZ* gene ($0.1 \mu\text{g}$ plasmid) to mark transfected cells. (B) A typical result from cells transfected with Akt1 or Akt2. To induce neurite sprouting, 24 h after transfection, cells were seeded into new plates at a low density ($2,000 \text{ cells}/\text{cm}^2$) and then incubated with minimal essential medium (MEM) that contained 0.5% serum for 3 days. Cells were fixed, and $\beta\text{-Gal}^+$ cells were detected by staining.

were performed. The GR construct we used for these studies expresses a 120-kilodalton protein, as previously demonstrated (23). In the presence of DEX, we consistently observed that $3 \mu\text{g}$ Akt1 reduced GR protein levels by approximately 50% relative to the loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 3C).

Akt3 enhances Neuro-2A cell differentiation. When Neuro-2A cells are transfected with a LacZ expression vector and growth factors are removed, $\beta\text{-galactosidase}$ -positive ($\beta\text{-Gal}^+$) neurites were detected when cells were seeded at a low density and then serum starved for 3 days (Fig. 4A, “control”), which is consistent with previous studies (54). Numerous cells detach from the plate because they do not survive growth factor withdrawal and consequently undergo apoptosis (27). When Akt1 or Akt2 was transfected with the LacZ expression vector and neurite formation assessed, Akt1 and Akt2 appeared to slightly increase the length of neurites (Fig. 4B). However, Akt1 and Akt2 did not significantly increase the frequency of neurite formation (Fig. 5). In stark contrast to Akt1 or Akt2, Akt3 dramatically increased the efficiency of neurite formation (Fig. 4C and D and Fig. 5). For example, clusters of neurite-positive cells that contained $\beta\text{-Gal}^+$ cells were readily detected (Fig. 4C). Furthermore, neurites were generally longer relative to controls or Neuro-2A cells transfected with Akt1 or Akt2. In fact, we observed $\beta\text{-Gal}^+$ Neuro-2A cells that had differentiated and contained neurites more than 20 times longer than the cell body (Fig. 4D). Since neurite sprouting is synonymous with regeneration of damaged axons and dendrites (59), the ability of Akt3 to enhance neurite formation and length of neurites may be important for repairing and restoring normal neuronal functions following infection.

DISCUSSION

UV light, heat stress (fever), trauma, and increased corticosteroids as a result of stress increase the incidence of reactivation from latency in humans (1–3, 17, 19, 60, 61). While these reactivation stimuli appear to be dissimilar, heat stress and UV light activate the GR. For example, cyanoketone, a glucocorticoid synthesis inhibitor, reduces corticosterone synthesis and the efficiency of HSV-1 reactivation in latently infected mice (62). Furthermore, heat stress or DEX increased the incidence of reactivation from latency in cultured TG cells (63). UV light-induced GR phosphorylation occurs via a ligand-independent mechanism that correlates with GR-mediated transcriptional activation

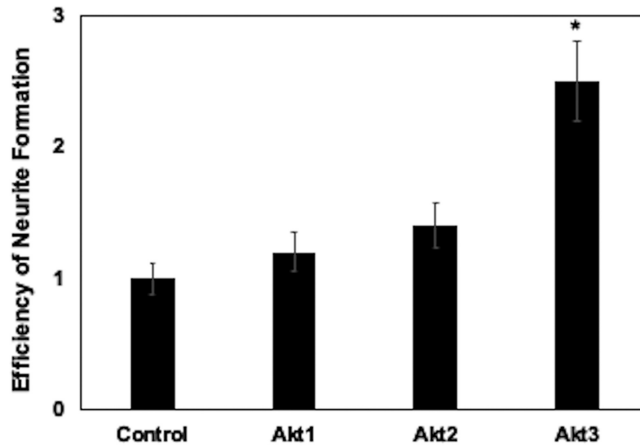


FIG 5 Akt3, but not Akt1 or Akt2, significantly increased neurite formation in Neuro-2A cells. The relative efficiency of β -Gal⁺ cells containing neurites was calculated by dividing the number of β -Gal⁺ cells with a neurite length at least twice the diameter of the cell by the total number of β -Gal⁺ cells. The % of β -Gal⁺ cells with neurites in the control was set at 1. The other samples were compared to the control to obtain the relative efficiency of neurite formation. The average of three independent experiments is shown with the respective standard deviation. An asterisk denotes significant differences ($P < 0.05$) in β -Gal⁺ Neuro-2A cells containing neurites following transfection with the Akt family member relative to the number of β -Gal⁺ Neuro-2A cells with neurites following transfection with an empty vector, as determined by the one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) multiple means comparison tests.

(64, 65). UV light also induces expression of certain enzymes regulated by GR activation (66) and activates a serine/threonine protein kinase, c-Jun N-terminal kinase (JNK) (65). JNK is crucial for remodeling HSV-1 chromatin during reactivation from latency in an *in vitro* neuronal model for latency (67). These studies suggest that several known reactivation stimuli activate GR; consequently, GR-mediated transactivation of viral and cellular promoters is predicted to trigger early events during reactivation from latency.

Several independent studies concluded that the phosphatidylinositol-3-kinase (PI3K)/Akt/mTOR signaling axis promotes HSV-1 latent or quiescent infections in certain neuronal cell culture models of latency. For example, inhibiting nerve growth factor, PI3K, or Akt signaling induces reactivation from latency in two *in vitro* models for latency, primary rat sympathetic neurons (26, 27, 68) and human LUHMES cells (a human mesencephalic neuronal cell line) (28). Numerous growth factor signaling pathways activate PI3K/Akt signaling pathways (38–40, 69). PI3K activation increases phosphatidylinositol(3,4,5)-trisphosphate (PIP3); consequently, 3-phosphoinositide-dependent protein kinase-1 (PDK1) and Akt signaling are activated (reviewed in references 40 and 70). Akt can directly phosphorylate β -catenin, which leads to increased β -catenin-dependent transcription (71). N-cadherin is also activated by Wnt/ β -catenin and N-cadherin activates Akt, which promotes neuronal differentiation during cortical development (38).

We propose that Akt signaling has multiple effects on the ability of neurotropic *Alphaherpesvirinae* subfamily members to maintain latency (summarized in Fig. 6). This model is based on published studies using the following different latency models: (i) Akt3 is expressed in more TG neurons during BoHV-1 latency (25) and (ii) the PI3K/Akt/mTOR pathway maintain an HSV-1 latent/quiescent infection in neuronal models of latency (26–28, 68). Strikingly, Akt3 RNA levels in TG are reduced more than 50-fold, and the number of Akt3⁺ TG neurons is significantly reduced during DEX-induced reactivation of BoHV-1. In contrast, Akt1 and Akt2 RNA levels are similar in TG of uninfected calves and in those with latency or DEX-induced reactivation. Studies presented in Fig. 1 and 3 revealed that Akt1 and Akt2 can potentially support maintenance of latency by impairing stress-induced activation of viral promoters that drive expression of transcriptional regulators. The finding that Akt2 inhibited GR-mediated transactivation is supported by a published study demonstrating that Akt2 kinase activity interferes with

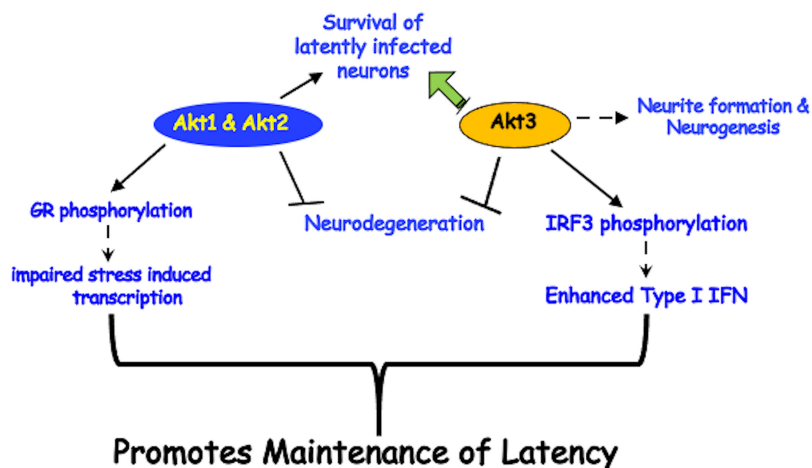


FIG 6 Schematic of Akt functions that can influence maintenance of latency. Summary of how Akt family members may contribute to maintenance of latency. For specific details, see the text.

glucocorticoid resistance in certain lymphoid malignancies (72). Furthermore, Akt1 directly phosphorylates GR at position serine-134, which interferes with GR nuclear localization and transactivation (53). Multiple GR isoforms are generated by alternative splicing and alternative translation initiation at the amino terminus of the GR (73, 74), suggesting that certain GR isoforms may not be phosphorylated by Akt1 and Akt2. While our studies indicated that overexpression of Akt1 in Neuro-2A cells slightly reduced GR steady protein levels (Fig. 3C), we suggest that the ability of Akt1 to directly phosphorylate GR at serine-134 was more important.

Surprisingly, Akt1 kinase activity was not important for interfering with the HSV-1 ICP0 promoter. In contrast to I Etu1 and MMTV-LTR, the ICP0 promoter lacks “whole” GREs but contains half-GREs that are important but not required for stress-induced transcription (24). GR and KLF15 stably interact with each other to form a feed-forward transcription loop that cooperatively transactivates the ICP0 promoter and the BoHV-1 I Etu1 promoter (23, 24, 75). While GR monomers can bind and transactivate certain half-GREs (76), we do not fully understand how GR and KLF15 transactivate promoters that lack “whole” GREs. Of note, Akt1 was reported to stably interact with transcriptional activators or repressors (77, 78), including an H₃ methyltransferase that coordinates gene silencing (79). Based on these observations, we suggest that interactions between the Akt1 kinase mutant and transcriptional repressors serve as a molecular scaffold that impair GR- and KLF15-mediated transactivation of the HSV-1 ICP0 promoter. Studies designed to determine whether specific Akt family members regulate the BoHV-1 or HSV-1 latency-reactivation cycle *in vivo* need to be performed. However, these studies will be complicated and are not within the scope of this study.

While Akt3 had no obvious effect on interfering with GR-mediated transactivation, novel functions of Akt3 are predicted to mediate certain aspects of maintaining latency. For example, Akt3 enhanced neurite formation in Neuro-2A cells significantly better than Akt1 or Akt2. In general, Akt signaling pathways stimulate neurite outgrowth (80), impair neurodegeneration (81), promote neuronal survival following stressful stimuli (82), and coregulate neuronal differentiation (83). However, Akt3, but not the other two Akt family members, is required for nerve growth factor (NGF)-mediated antiapoptotic signaling in PC12 neuron-like cells (84) and apoptosis in motor neurons (85). As discussed above, Akt3 is more important than Akt1 and Akt2 for preventing stroke-induced neuronal injury (48) and for promoting the growth of axons (49). Interestingly, a recent study concluded that Akt3 directly binds and phosphorylates interferon response factor 3 (IRF3), thus stimulating expression of antiviral type I interferons (86), which may interfere with reactivation via the antiviral activity of IRF3 and type 1 interferons. In summary, novel Akt3 functions are predicted to contribute to the maintenance of latency (summarized in Fig. 6).

Several independent studies concluded that the PI3k/Akt signaling pathways is coopted by several different viruses, including HSV-1 (87), to enhance virus entry, cell survival, transcription, protein synthesis, and virus transmission (88, 89). HSV-1-encoded protein kinases (US3 and UL13) regulate PI3K/Akt kinase activity to enhance virion packaging, which increases virus yield (90). During BoHV-1 productive infection, Akt phosphorylation is also increased dramatically (91) suggesting that these virally encoded kinases have similar effects on virus yield. While these findings appear to be discordant with the findings presented in this study and the model proposed in Fig. 6, tissue-specific effects of the three Akt family members in terminally differentiated sensory neurons are predicted to be responsible for their putative roles during lifelong latent infections.

MATERIALS AND METHODS

Cells, plasmids, and antibodies. Murine neuroblastoma cells (Neuro-2A; CCL-131) were obtained from ATCC (Manassas, VA) and grown in minimal essential medium (MEM; Life Technology) supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 μ g/ml).

A plasmid that expresses Akt1 was a gift from Jie Chen (pCDNA3-HA-Akt1, plasmid 73408; Addgene). A plasmid that expresses Akt3 was a gift from William Sellers (1236 pcDNA3 Myr HA Akt3, plasmid 9017; Addgene). A plasmid that expresses Akt2 was a gift from William Sellers (1227 pcDNA3 Myr HA Akt2, plasmid 9016; Addgene). The Akt1 kinase dead mutant (1014 pcDNA3 T7; Addgene) includes 3 points mutations, K179M, T308A, and S473A. The HSV-1 ICPO luciferase construct (−800 to +150) was obtained from Priscilla Schaffer and was described in previous studies (58, 92). The BoHV-1 IEtu1 collapsed promoter construct was previously described (93). The MMTV LTR luciferase reporter construct (pGL3-MMLV-LTR-Luc) was obtained from Stephen Goff (catalog no. 67831; Addgene). All plasmids were transfected into Neuro-2A cells in 60-mm dishes or 12-well plates using Lipofectamine 3000 transfection reagent (L3000075; Invitrogen) according to the manufacturer's instructions.

Dual-luciferase assay. To test the effect of Akt1, Akt2 and Akt3 on GR-mediated activation, Neuro-2A cells were seeded into 60-mm dishes containing MEM with 10% FBS at 24 h prior to transfection. At the time of transfection, approximately 6×10^5 Neuro-2A cells were present in each 60-mm dish. Two h before transfection, cells were cultured with antibiotic-free medium containing 2% stripped FBS. Cells were cotransfected with the designated plasmids and a plasmid carrying Renilla luciferase under the control of a minimal herpesvirus thymidine kinase (TK) promoter (50 ng). To maintain equal plasmid amounts in the transfection mixtures, an empty expression vector was added as needed. At approximately 24 h after transfection, water soluble DEX (Sigma; catalog no. D2915) number was added to the designated cultures. At 48 h after transfection, cells were harvested, and protein lysate subjected to a dual-luciferase assay by using a commercially available kit (catalog number E1910; Promega) according to the manufacturer's instructions. Luminescence was measured with a GloMax 20/20 luminometer (catalog number E5331; Promega).

Neurite formation assay. Neuro-2A cells grown in 24-well plates were cotransfected with plasmids that express Akt1, Akt2, or Akt3 (1 μ g) and a pCMV- β -Gal plasmid (1 μ g). At the time of transfection, approximately 6×10^5 Neuro-2A cells were present in each 60-mm dish. To induce neurite sprouting, 24 h after transfection cells were seeded onto 60-mm dishes at a low density (2,000 cells/cm²) and cells were starved in medium with 0.5% serum for 3 days. Cells were then fixed, stained, and a β -Gal assay was performed as previously described (94). The percentage of cells with β -Gal⁺ neurites was calculated by dividing the number of β -Gal⁺ cells with neurite length at least twice the diameter of the cell by the total number of β -Gal⁺ cells. The results are averages of three independent experiments.

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