**The serum and glucocorticoid-regulated protein kinases (SGK) stimulate bovine
herpesvirus 1 and herpes simplex virus 1 productive infection.**

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ABSTRACT

Serum and glucocorticoid-regulated protein kinases (SGK) are serine/threonine protein kinases that contain a catalytic domain resembling other protein kinases: AKT/protein kinase B, protein kinase A, and protein kinase C-Zeta for example. Unlike these constitutively expressed protein kinases, SGK1 RNA and protein levels are increased by growth factors and corticosteroids. Stress can directly stimulate SGK1 levels as well as stimulate bovine herpesvirus 1 (BoHV-1) and herpes simplex virus 1 (HSV-1) productive infection and reactivation from latency suggesting SGK1 can stimulate productive infection. For the first time, we provide evidence that a specific SGK inhibitor (GSK650394) significantly reduced BoHV-1 and HSV-1 replication in cultured cells. Proteins encoded by the three BoHV-1 immediate early genes (bICP0, bICP4, and bICP22) and two late proteins (VP16 and gE) were consistently reduced by GSK650394 during early stages of productive infection. In summary, these studies suggest SGK may stimulate viral replication following stressful stimuli.

KEYWORDS: bovine herpesvirus 1 (BoHV-1), herpes simplex virus 1 (HSV-1), serum and glucocorticoid-regulated protein kinases, productive infection

INTRODUCTION

Alphaherpesvirinae subfamily members, including herpes simplex virus 1 (HSV-1) and bovine herpesvirus 1 (BoHV-1), initiate infection on mucosal layers, reviewed by (Jones, 1998, 2003, 2006, 2009; Jones, 2007). Although immune responses clear infection, life-long latency is established in sensory neurons. Periodically, reactivation from latency occurs, which is crucial for virus transmission. In general, increased “stress” correlates with a higher incidence of reactivation from latency in humans (Cassidy et al., 1997; Glaser et al., 1985; Padgett et al., 1998). In keeping with these observations, the synthetic corticosteroid dexamethasone accelerates reactivation from latency in TG neuronal cultures or TG organ cultures prepared from mice latently infected with HSV-1 (Du et al., 2012; Halford et al., 1996). Stress, as a result of food and water deprivation during shipping of cattle, weaning, and/or dramatic weather changes increases corticosteroid levels and the incidence of BoHV-1 reactivation from latency, reviewed by (Jones, 2013, 2014). Furthermore, the synthetic corticosteroid dexamethasone initiates BoHV-1 reactivation from latency 100% of the time (Inman et al., 2002; Jones, 1998, 2003, 2006; Jones et al., 2000; Rock et al., 1992; Sheffy and Davies, 1972; Shimeld et al., 1990). Stressful stimuli generally lead to increased corticosteroids levels, which readily diffuse into a cell, and activate the glucocorticoid receptor (GR) or mineralocorticoid receptor (MR) by binding to these receptors, reviewed in (Oakley and Cidlowski, 2013). Activation of the GR and/or MR may stimulate reactivation from latency by several distinct mechanisms.

The family of serum and glucocorticoid-regulated protein kinases (SGK) contains three members (SGK1, SGK2, and SGK3), reviewed by (Pearce et al., 2010). SGKs are serine/threonine protein kinases that have a catalytic domain similar to the Akt/protein kinase B,

protein kinase A, protein kinase C-zeta, and rat p70S6K/p85S6K kinases, reviewed by (Pearce et al., 2010). SGK1, but not SGK2, mRNA and protein levels are rapidly stimulated by corticosteroids, mitogenic, and cellular stress signals in a cell type and stimulus-dependent manner (Bell et al., 2000; Brenan and Fuller, 2000; Buse et al., 1999; Imaizumi et al., 1994; Maiyar et al., 1996; Mizuno and Nishida, 2001; Webster et al., 1993). All SGK family members can transduce signals that are vital for cell survival and proliferative responses (Brunet et al., 2001; Mikosz et al., 2001).

In this study, we tested whether inhibiting SGK kinase activity influenced BoHV-1 and HSV-1 productive infection. Our results demonstrated that a specific SGK inhibitor (GSK650394) consistently and significantly reduced viral replication. Additional studies revealed GSK650394 interfered with BoHV-1 protein expression during early stages of productive infection. However, GSK650394 did not interfere with dexamethasone-mediated activation of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter or the BoHV-1 immediate early transcription unit 1 (IEt1) promoter. In summary, this study suggested SGK family members might stimulate viral replication following stressful stimuli.

MATERIALS AND METHODS:

Cells

Murine neuroblastoma cells (Neuro-2A), bovine kidney cells (CRIB) and African green monkey kidney cells (Vero) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% FCS, penicillin (10 U/ml), and streptomycin (100 µg/ml).

Viruses

The Cooper strain of BoHV-1 (wt virus) was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services, Ames, IA. BoHV-1 stocks were prepared in CRIB cells. The KOS strain of HSV-1 was propagated in Vero cells.

Cell viability assay

CRIB or Vero cells were seeded at 3.5×10^6 cells/ml in 24-well plates and then incubated overnight at 37° C. Cells were treated with GSK650394 (Catalogue #3572, Tocris Biosciences, Bristol, UK) at various concentrations (1-100 nM) for 24 hours. To assess cell viability, ATP levels in cultures were measured using CellTiter-Glo Luminescent Cell Viability Assay (Promega; G7572) according to the manufacturer's instruction.

Viral plaque assays

Confluent CRIB or Vero cells in 6 well plates were infected with BoHV-1 or HSV-1 respectively. An agarose mixture was prepared by combining equal volumes of 1.2% sea plaque agar and 10% FCS containing growth media. After infection, supernatants were removed and the agarose mixture applied to each well. Cells were incubated at 37°C until plaques were visible. After removal of overlays, cells were fixed with formaldehyde and stained with crystal violet. Infectivity was determined by counting plaques.

Plasmids

pIE1 (IEt_u1cat) was obtained from Vikram Misra (University of Saskatchewan) and was described previously (Misra V, 1994; Misra V, 1995 Sep). A mouse GR expression vector was obtained from Dr. Joseph Cidlowski, NIH. The mouse mammary tumor virus (MMTV) long terminal repeat (LTR) was obtained from Dr. Kevin Trotter (NIEHS, NIH). All plasmids were prepared from bacterial cultures by alkaline lysis and 2 rounds of cesium chloride centrifugation.

Measurement of CAT activity

Neuro-2A cells grown in 60 mm dishes were transfected with the designated plasmids as indicated in the respective figure legends using NeuroTransIt (MIR2145; Mirus). CAT assays were performed as previously described (Kook et al., 2015b).

Western blot analysis

Procedures for Western blot analysis were previously described (Kook et al., 2015b). The SGK1 antibody was purchased from Abcam (ab59337). A peptide-specific rabbit antibody directed against bICP0 (Affinity Bioreagents, Golden, CO) was affinity purified. This antibody specifically recognizes bICP0 in infected or transfected cells. A VP16-specific rabbit polyclonal antibody was obtained from Vikram Misra (University of Saskatchewan, Saskatoon, CA). Peptide specific bICP4 and bICP22 antiserum was generated in rabbits. A BoHV-1 gE specific antiserum was a gift from Lynn Enquist (Princeton).

RESULTS

GSK650394 inhibits viral replication in a dose-dependent manner

To test whether SGK kinases regulate productive infection, the effects of the only commercially available inhibitor of SGK enzymes (GSK650394) were examined. GSK650394 has 50% inhibitory concentration (IC₅₀) values of 62 nM and 103 nM for SGK1 and SGK2 respectively (Sherk, 2008) and (Selleckchem.com). Since SGK3 shares extensive similarity to SGK1 and SGK2, it is assumed GSK650394 inhibits SGK3 activity. Cells were pretreated with the designated levels of GSK650394 for 3 hours and then infected with BoHV-1 at an MOI of 0.01. Although DMSO (solvent control) had no effect on viral replication when cultures were grown in 10% FBS, it slightly reduced BoHV-1 titers when CRIB cells were incubated with

stripped FBS after infection (Figure 1A). BoHV-1 replication was reduced by GSK650394 in a dose dependent manner in cells containing normal FBS or stripped FBS. Viral replication in cells incubated with stripped FBS cell culture was affected more than normal FBS when treated with 10 or 25 nM GSK650394. Furthermore, plaque formation was not observed at 50 nM and 100 nM when cells were incubated with stripped FBS prior to GSK650394 and infection.

Additional studies assessed whether GSK650394 inhibited HSV-1 replication. As described for BoHV-1 studies, Vero cells (pretreated with GSK650394 for 3 hours) were infected with HSV-1 at an MOI of 0.01. HSV-1 replication was significantly reduced when 50 or 100 nM levels of GSK650394 were used (Figure 1B). As with BoHV-1, the effect of GSK650394 on viral replication was more profound when cells were incubated with stripped FBS after infection because no plaques were observed when 50 or 100 nM GSK650394 were used. At 1 nM GSK650394 concentration, viral replication was not significantly inhibited. If a moi of 1 was used for BoHV-1 and HSV-1, GSK650394 had a strong inhibitory effect on viral replication (data not shown).

The effects of GSK650394 on CRIB and Vero cell viability were examined by measuring ATP content in cells, which is a reliable measure of metabolically active cells. Cell viability was not significantly reduced by GSK650394 in CRIB or Vero cells using drug concentrations ranging from 1 to 100 nM for 24 hours (Figure 1C). In summary, these studies demonstrated that the SGK inhibitor GSK650394 reduced BoHV-1 and HSV-1 replication in CRIB or Vero cells respectively.

Analysis of SGK1 protein expression following infection

160 We examined the effects of BoHV-1 productive infection on SGK1 protein levels
because SGK1, but not SGK2 or SGK3, is stimulated by stressful stimuli (Webster et al., 1993).
162 Infection consistently led to a slight increase of SGK1 protein levels at 4 hours, but not 8 hours
after infection (Figure 2). SGK1 expression was reduced at 16 hours after infection, which may
164 reflect a function of the virus-host shutoff function or depletion of growth factors in media. At 8
and 16 hours after infection, a faint high molecular weight band was recognized by the antibody,
166 which was readily detected upon prolonged exposure (Figure 2, middle panel). Since SGK1
isoforms exist as a result of alternative splicing and the protein can be phosphorylated (Burgeon
168 et al., 2014; Prince et al., 2004; Webster et al., 1993), this band may represent a phosphorylated
band or a novel SGK1 isoform. In summary, these studies demonstrated that BoHV-1 infection
170 did not dramatically alter SGK1 protein levels during early stages of productive infection.

GSK650394 effects on viral protein synthesis

172 We next examined whether GSK650394 influenced steady state levels of certain BoHV-1
during productive infection. Since we were interested in comparing viral protein levels during
174 early stages of infection, we increased the MOI to 2. Steady state protein levels of the three
BoHV-1 immediate early genes (bICP0, bICP4, and bICP22) were consistently reduced by 25
176 nM GSK650394 at 4 and 8 hours after infection. In particular, bICP22 protein expression was
reduced 10 fold at 4 hours after infection in the presence of GSK650394. At 16 hours after
178 infection, similar levels of bICP0 and bICP22 proteins were detected in DMSO control treated

and GSK650394 treated cells whereas a slight reduction of bICP4 protein was still observed in the presence of GSK650493. GSK650394 inhibited steady state protein levels of a true late gene (VP16) by approximately 100 fold at 8 hours after infection. In contrast to VP16, steady state protein levels of a leaky late gene (gE) was detected at 4, 8, and 16 hours after infection: however gE protein levels were only reduced 3-fold by GSK650394. The gE protein is a glycosylated protein: however cultures treated with GSK650394 did not contain obvious differences in gE isoforms. In summary, these data demonstrated GSK650394 reduced steady state viral protein levels, which is consistent with its ability to interfere with productive infection.

Effects of GSK650394 on DEX mediated viral promoter activation

Previous studies demonstrated that the synthetic corticosteroid dexamethasone stimulated the immediate early transcription unit 1 (IEtu1) promoter via glucocorticoid receptor elements (GREs) located in the promoter (Kook et al., 2015). For these studies, we utilized mouse neuroblastoma cells (Neuro-2A) because they can be readily transfected and we know DEX stimulates IEtu1 promoter activity in these cell lines (Kook et al., 2015b). Conversely, CRIB cells are not readily transfected. The mouse mammary tumor virus long terminal repeat (MMTV LTR) was also tested because it contains GREs, which are activated by dexamethasone (Foy and Hertz, 2003; Funder, 1997; Oakley and Cidlowski, 2013). At 24 h after transfection, 10 nM dexamethasone was added to cultures in the presence of DMSO or different concentrations of GSK650394. As expected, IEtu1 (Figure 4A) and MMTV LTR promoter (Figure 4B) activation were induced by dexamethasone treatment and dexamethasone stimulated the MMTV LTR more

than the IETu1 promoter (Kook et al., 2015b). However, 25 or 100 nM GSK650394 did not significantly decrease MMTV LTR or IETu1 promoter activity.

DISCUSSION

In this study, new evidence is provided demonstrating that GSK650394 interferes with BoHV-1 and HSV-1 replication. Three SGK genes have been identified in humans, SGK1, SGK2, and SGK3, and each has at least 80% similarity in their catalytic domains (Kobayashi et al., 1999). SGK1 and SGK3 are expressed in every tissue, whereas SGK2 was reported to be expressed primarily in the liver, kidney, pancreas, and brain (Kobayashi et al., 1999). There are at least four alternatively spliced isoforms for each gene in human neutrophils (Burgeon et al., 2014; Prince et al., 2004). Since GSK650394 has an effect on each SGK family member, additional studies will be necessary to identify which SGK isoform(s) are important for inhibiting viral replication. In particular, it will be of interest to determine which isoforms are expressed in sensory neurons and whether SGK influences reactivation from latency. The increased antiviral effect GSK650394 had when cells were incubated with stripped serum implicated SGK1 as being more important than the other family members because its mRNA and protein levels are regulated by serum and corticosteroids (Webster et al., 1993). Nearly all cells contain nuclear GR when grown in the presence of FBS (Kook et al., 2015b), which is indicative of an activated GR, reviewed in (Oakley and Cidlowski, 2013; Schonevild et al., 2004). Conversely, the GR is localized to the cytoplasm in most cells following incubation with stripped FBS (Kook et al., 2015b).

The finding that GSK650394 had no effect on the ability of DEX and the GR to stimulate

the MMTV LTR and the BoHV-1 IETu1 promoter was somewhat expected because the ability of the GR to stimulate transcription does not require protein synthesis, reviewed in (Oakley and Cidlowski, 2013; Schonevild et al., 2004). However, SGK1 gene expression is induced by growth factors and glucocorticoids (Webster et al., 1993): consequently de novo SGK1 protein synthesis is necessary. Based on these observations, it appears that activation of the MMTV LTR and IETu1 promoter by dexamethasone is upstream of SGK kinase activity.

Although SGK protein kinase activity may regulate certain aspects of productive infection and conceivably reactivation from latency, corticosteroids are likely to influence reactivation by additional means. For example, HSV-1 productive infection is stimulated when cells are pre-treated with dexamethasone (Erlandsson et al., 2002). This stimulatory effect may occur because a functional GR binding site is present in a HSV-1 origin of replication (Hardwicke and Schaffer, 1997). BoHV-1 productive infection is also stimulated by dexamethasone, in part because an activated GR directly stimulates the BoHV-1 IETu1 promoter (Kook et al., 2015b). The IETu1 promoter drives expression of two viral regulatory proteins, bICP0 and bICP4 (Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991). Two BoHV-1 regulatory proteins (bICP0 and VP16) are induced during reactivation from latency and their expression is primarily detected in GR+ sensory neurons (Frizzo da Silva et al., 2013; Kook et al., 2015a) underscoring the impact that an activated GR has on stimulating viral gene expression during reactivation. In addition to the direct effects that the GR may have on viral transcription, dexamethasone rapidly stimulates expression of cellular transcription factors in sensory neurons that can activate the ICP0 promoters of BoHV-1 (Workman et al., 2012) and HSV-1 (Sinani et al., 2013).

Increased corticosteroid levels can also interfere with immune responses, reviewed by
248 (Barnes, 1998; Funder, 1997; Schonevild et al., 2004), which we suggest will indirectly promote
reactivation from latency. For example, T cells undergo apoptosis following corticosteroid
250 treatment (Barnes, 1998; Funder, 1997; Migliorati et al., 1994; Schonevild et al., 2004), and
soluble factors produced by T cells can interfere with reactivation from latency (Decman et al.,
252 2005; Knickelbein et al., 2008). Furthermore, the activated GR can interact with two cellular
transcription factors, activating protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer
254 of activated B cells (NF- κ B), reviewed in (Oakley and Cidlowski, 2013; Rhen and Cidlowski,
2005; Smoak and Cidlowski, 2004). Interactions between the GR and AP-1 and NF- κ B would
256 repress expression of tumor necrosis factor-alpha (TNF-alpha), interleukin 1-beta, as well as
other inflammatory cytokines because AP-1 and/or NF- κ B trans-activate the promoters of these
258 respective genes. AP-1 and NF- κ B are also required for activating the beta-interferon
promoter/enhancer following virus infection (Goodbourn et al., 1985), which is significant
260 because beta-interferon has anti-herpesvirus activity, including the ability to reactivate from
latency (Hendricks et al., 1991; Katze et al., 2002; Leib et al., 1999; Liu et al., 2001; Melroe et
262 al., 2004; Mikloska and Cunningham, 2001). In summary, the ability of corticosteroids to
interfere with immune responses would likely increase herpesvirus replication and virus
264 shedding during the course of reactivation from latency.

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REFERENCES

- 272 Barnes, P.J., 1998. Anti-inflammatory actions of glucocorticoids: molecular mechanisms.
Clinical Science 94, 557-572.
- 274 Bell, L.M., M.L. Leong, B. Kim, E. Wang, J. Park, B.A. Hemmings, and G.L. Firestone, 2000.
Hyperosmotic stress stimulates promoter activity and regulates cellular utilization of the serum-
276 and glucocorticoid-inducible protein kinase (Sgk) by a p38 MAPK-dependent pathway. J Biol
Chem 275, 2562-25272.
- 278 Brenan, F.E. and P.J. Fuller, 2000. Rapid upregulation of serum and glucocorticoid-regulated
kinase (sgk) gene expression by corticosteroids in vivo. Mol Cell Endocrinol 166, 129-136.
- 280 Brunet, A., J. Park, H. Tran, L.S. Hu, B.A. Hemmings, and M.E. Greenberg, 2001. Protein
kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor
282 FKHL (FOXO3a). Mol Biol Cell 21, 952-965.
- Burgeon, J., A.L. Roertson, P. Sadiku, X. Wang, E. Hooper-Greenhill, L.R. Prince, P. Walker,
284 E.E. Hoggett, J.R. Ward, S.N. Farrow, W.J. Zuercher, P. Jeffrey, C.O. Savage, P.W. Ingham,
A.F. Hurlstone, M.K.B. Whyte, and S.A. Renshaw, 2014. Serum and glucocorticoid-regulated
286 kinase 1 regulates neutrophil clearance during inflammation resolution. J of Immunology 192,
1796-1805.
- 288 Buse, P., S.H. Tran, E. Luther, P.T. Phu, G.W. Aponte, and G.L. Firestone, 1999. Cell cycle and
hormonal control of nuclear-cytoplasmic localization of the serum- and glucocorticoid-inducible
290 protein kinase, Sgk, in mammary tumor cells. A novel convergence point of anti-proliferative
and proliferative cell signaling pathways. J Biol Chem 274, 7253-7263.
- 292 Cassidy, L., J. Meadows, J. Catalan, and S. Barton, 1997. Are stress and coping style associated
with frequent recurrence of genital herpes? Genitourin Med 73, 263-266.
- 294 Decman, V., Freeman, M.L., Kinchington, P.R., Hendricks, R.L., 2005. Immune control of HSV-
1 latency. Viral Immunol 18, 466-473.
- 296 Du, T., G. Zhou, and B. Roizman, 2012. Induction of apoptosis accelerates reactivation from
latent HSV-1 in ganglionic organ cultures and replication in cell cultures. Proc Natl Acad Sci
298 USA 109, 14616-14621.
- Erlandsson, A.C., L.-C. Bladh, P. Stierna, T. Yucel-Lindberg, O. Hammersten, T. Modeer, J.
300 Harmenberg, and A.-C. Wikstrom, 2002. Herpes simplex virus type 1 infection and
glucocorticoid treatment regulate viral yield, glucocorticoid receptor and NF-kB levels. Journal
302 of Endocrinology 175, 165-176.
- Foy, R. and W.Hortz, 2003. Sequence-specific positioning of nucleosomes over the steroid-
304 inducible MMTV promoter. Embo J 6, 2321-2328.
- Frizzo da Silva, L., I. Kook, A. Doster, and C. Jones, 2013. Bovine herpesvirus 1 regulatory
306 proteins, bICP0 and VP16, are readily detected in trigeminal ganglionic neurons expressing the
glucocorticoid receptor during the early stages of reactivation from latency. J Virol 87, 11214-
308 11222.
- Funder, J.W., 1997. Glucocorticoids and mineralocorticoid receptors: biology and clinical
310 relevance. Annu Rev Med 48, 231-240.
- Glaser, R., J.K. Kiecolt-Glaser, C.E. Speicher, and J.E. Holliday., 1985. Stress, loneliness, and
312 changes in herpesvirus latency. Journal of Behavioral Medicine 8, 249-260.
- Goodbourn, S., Zinn, K. and T. Maniatis, 1985. Human beta-interferon gene expression is
314 regulated by an inducible enhancer element. Cell 41, 509-520.

Halford, W.P., B.M. Gebhardt, and D.J. Carr, 1996. Persistent cytokine expression in trigeminal ganglion latently infected with herpes simplex virus type 1. *J Immunol* 157, 3542-3549.

Hardwicke, M.A. and P.A. Schaffer, 1997. Differential effects of nerve growth factor and dexamethasone on herpes simplex virus type 1 oriL- and oriS-dependent DNA replication in PC12 cells. *J Virol* 71, 3580-3587.

Hendricks, R.L., P.C. Weber, J.L. Taylor, A. Koumbis, T.M. Tumpey, J.C. Glorioso, 1991. Endogenously produced interferon alpha protects mice from herpes simplex virus type 1 corneal disease. *J Gen Virol* 72 (Pt 7), 1601-1610.

Imaizumi, K., M. Tsuda, A. Wanaka, M. Tohyama, and T. Takagi, 1994. Differential expression of sgk mRNA, a member of the Ser/Thr protein kinase gene family, in rat brain after CNS injury. *Brain Res Mol Brain Res* 26, 189-196.

Inman, M., L. Lovato, A. Doster, and C. Jones, 2002. A mutation in the latency related gene of bovine herpesvirus 1 interferes with the latency-reactivation cycle of latency in calves. *J Virol* 76, 6771-6779.

Jones, C., 1998. Alphaherpesvirus latency: its role in disease and survival of the virus in nature. *Adv Virus Res* 51, 81-133.

Jones, C., 2003. Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Micro Rev* 16, 79-95.

Jones, C., 2009. Regulation of innate immune responses by bovine herpesvirus 1 and infected cell protein 0. *Viruses* 1, 255-275.

Jones, C., 2013. Bovine herpes virus 1 (BHV-1) and herpes simplex virus type 1 (HSV-1) promote survival of latently infected sensory neurons, in part by inhibiting apoptosis. *J of Cell Death* 6, 1-16.

Jones, C., 2014. Reactivation from latency by alpha-herpesvirinae subfamily members: a stressful stimulation. *Current Topics in Virology* 12, 99-118.

Jones, C., T.J. Newby, T. Holt, A. Doster, M. Stone, J. Ciacci-Zanella, C.J. Webster, M.W. Jackwood, 2000. Analysis of latency in cattle after inoculation with a temperature sensitive mutant of bovine herpesvirus 1 (RLB106). *Vaccine* 18, 3185-3195.

Jones, C., V. Geiser, G. Henderson, Y. Jiang, F. Meyer, S. Perez, and Y. Zhang., 2006. Functional analysis of bovine herpesvirus 1 (BHV-1) genes expressed during latency. *Vet Micro* 113, 199-210.

Jones, C. and S. Chowdhury, 2007. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex, and development of improved vaccines. *Adv in Anim Health* 8, 187-205.

Katze, M.G., Y. Heng, and M. Gale, 2002. Viruses and interferon: fight for supremacy. *Nat Rev Immunol* 2, 675-686.

Knickelbein, J.E., K.M. Khanna, M.B. Yee, C.J. Baty, P.R. Kinchington, and R.L. Hendricks, 2008. Noncytotoxic lytic granule-mediated CD8+ T cell inhibition of HSV-1 reactivation from neuronal latency. *Science* 322, 268-272.

Kobayashi, T., M. Deak, N. Morrice, and P. Cohen, 1999. Characterization of the structure and regulation of two novel isoforms of serum- and glucocorticoid induced protein kinase. *Biochem J* 344, 189-197.

Kook, I., A. Doster, and C. Jones, 2015a. Bovine herpesvirus 1 regulatory proteins are detected in trigeminal ganglionic neurons during the early stages of stress-induced escape from latency. *J Neurovirology* 21, 585-591.

360 Kook, I., C. Henley, F. Meyer, F.G. Hoffmann, and C. Jones, 2015. Bovine herpesvirus 1
 362 productive infection and immediate early transcription unit 1 promoter are stimulated by the
 synthetic corticosteroid dexamethasone. *Virology* 484, 377-385.
 Leib, D.A., T.E. Harrison, K.M. Laslo, M.A. Machalek, N.J. Moorman, and H.W. Virgin, 1999.
 364 Interferons regulate the phenotype of wild-type and mutant herpes simplex viruses in vivo. *J Exp*
Med 189, 663-672.
 366 Liu, T., K.M. Khanna, B.N. Carriere, and R.L. Hendricks, 2001. Gamma interferon can prevent
 herpes simplex virus type 1 reactivation from latency in sensory neurons. *J Virol* 75, 11178-
 368 11184.
 Maiyar, A.C., A.J. Huang, P.T. Phu, H.H. Cha, and G.L. Firestone, 1996. p53 stimulates
 370 promoter activity of the sgk serum/glucocorticoid-inducible serine/threonine protein kinase gene
 in rodent mammary epithelial cells. *J Biol Chem* 271, 12414-12422.
 372 Melroe, G.T., N.A. DeLuca, and D.M. Knipe, 2004. Herpes simplex virus 1 has multiple
 mechanisms for blocking virus-induced interferon production. *J Virol* 78, 8411-8420.
 374 Migliorati, G., I. Nicoletti, F. D'Adamio, A. Spreca, C. Pagliacci, and C. Riccardi, 1994.
 Dexamethasone induces apoptosis in mouse natural killer cells and cytotoxic T lymphocytes.
 376 *Immunology* 81, 21-26.
 Mikloska, Z., A.L. Cunningham, 2001. Alpha and gamma interferons inhibit herpes simplex
 378 virus type 1 infection and spread in epidermal cells after axonal transmission. *J Virol* 75, 11821-
 11826.
 380 Mikosz, C.A., D.R. Brickley, M.S. Sharkey, T.W. Moran, and S.D. Conzen, 2001.
 Glucocorticoid receptor-mediated protection from apoptosis is associated with induction of the
 382 serine/threonine survival kinase gene, sgk-1. *J Biol Chem* 276, 16649-16654.
 Misra V, B.A., D. Carpenter, and P. O'Hare, 1994. Protein and DNA elements involved in
 384 transactivation of the promoter of the bovine herpesvirus (BHV) 1 IE-1 transcription unit by the
 BHV alpha gene trans-inducing factor. *J Virol* 68, 4898-4909.
 386 Misra V, W. S Walker, S. Hayes, and P. O'Hare, 1995. The bovine herpesvirus alpha gene trans-
 inducing factor activates transcription by mechanisms different from those of its herpes simplex
 388 virus type 1 counterpart VP16. *J Virol* 69, 5209-5216.
 Mizuno, H. and E. Nishida, 2001. The ERK MAP kinase pathway mediates induction of SGK
 390 (serum- and glucocorticoid-inducible kinase) by growth factors. *Genes and Cells* 6, 261-268.
 Oakley, R.H. and J.A. Cidlowski, 2013. The biology of the glucocorticoid receptor: New
 392 signaling mechanisms in health and disease. *J Allergy Clin Immunol* 132, 1033-1044.
 Padgett, D.A., J.F. Sherida, J. Dorne, G.G. Berntson, J. Candelora, and R. Glaser, 1998. Social
 394 stress and the reactivation of latent herpes simplex virus type 1. *Proc Natl Acad Sci U S A* 95,
 7231-7235.
 396 Pearce, L.R., D. Komander, and D.R. Alessi, 2010. The nuts and bolts of AGC protein kinases.
Nature Reviews Molecular Cell Biology 11, 9-22.
 398 Prince, L.R., L. Allen, E.C. Jones, P.G. Hellewell, S.K. Dower, M.K. Whyte, and I. Sabroe 2004.
 The role of interleukin-1B in direct and toll-like receptor 4-mediated neutrophil activation and
 400 survival. *Am J Pathol* 165, 1819-1826.
 Rhen, T. and J.A. Cidlowski, 2005. Antiinflammatory action of glucocorticoids - new
 402 mechanisms of old drugs. *New England J of Medicine* 353, 1711-1723.
 Rock, D., J. Lokensgard, T. Lewis, and G. Kutish, 1992. Characterization of dexamethasone-
 404 induced reactivation of latent bovine herpesvirus 1. *J Virol* 66, 2484-2490.

Schonevild, O.J.L.M., I.C. Gaemers, and W.H. Lamers 2004. Mechanisms of glucocorticoid signalling. *Biochemica et Biophysica Acta* 1680, 114-128.

Sheffy, B.E. and D.H. Davies, 1972. Reactivation of a bovine herpesvirus after corticosteroid treatment. *Proc Soc Exp Biol Med* 140, 974-976.

Sherk, A.B., D.E. Frigo, C.G. Schnackenberg, J.D. Bray, N.J. Laping, W. Trizna, M. Hammond, J.R. Patterson, S.K. Thompson, D. Kazmin, J.D. Norris, and D.P. McDonnell, 2008. Development of a small-molecule serum- and glucocorticoid-regulated kinase-1 antagonist and its evaluation as a prostate cancer therapeutic. 68, 7475-7483.

Shimeld, C., T.J. Hill, W.A. Blyth, and D.L. Easty, 1990. Reactivation of latent infection and induction of recurrent herpetic eye disease in mice. *J Gen Virol* 71, 397-404.

Sinani, D., E. Cordes, A. Workman, P. Thunuguntia, and C. Jones, 2013. Stress induced cellular transcription factors expressed in trigeminal ganglionic neurons stimulate the herpes simplex virus type 1 (HSV-1) infected cell protein 0 (ICP0) promoter. *J Virol* 87, 1183-1192.

Smoak, K.L. and J.A. Cidlowski, 2004. Mechanisms of glucocorticoid receptor signaling during inflammation. *Mechanisms of Aging and Development* 125, 697-706.

Webster, M.K., L. Goya, Y. Ge, A.C. Maiyar, and G.L. Firestone, 1993. Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol Cell Biol* 13, 2031-2040.

Wirth, U.V., C. Fraefel, B. Vogt, C. Vlcek, V. Paces, and M. Schwyzer, 1992. Immediate-early RNA 2.9 and early RNA 2.6 of bovine herpesvirus 1 are 3' coterminal and encode a putative zinc finger transactivator protein. *J Virol* 66, 2763-2772.

Wirth, U.V., K. Gunkel, M. Engels, and M. Schwyzer, 1989. Spatial and temporal distribution of bovine herpesvirus 1 transcripts. *J Virol* 63, 4882-4889.

Wirth, U.V., B. Vogt, and M. Schwyzer, 1991. The three major immediate-early transcripts of bovine herpesvirus 1 arise from two divergent and spliced transcription units. *J Virol* 65, 195-205.

Workman, A., J. Eudy, L. Smith, L. Frizzo da Silva, D. Sinani, H. Bricker, E. Cook, A. Doster, and C. Jones, 2012. Cellular transcription factors induced in trigeminal ganglia during dexamethasone-induced reactivation from latency stimulate bovine herpesvirus 1 productive infection and certain viral promoters. *J Virol* 86, 2459-2473.

FIGURE LEGENDS

Figure 1. The SGK inhibitor inhibits BoHV-1 replication in a dose-dependent manner.

Panel A: Bovine kidney cells (CRIB) were incubated in 10% fetal calf serum and then subcultured 24 hours prior to infection. Three hours prior to infection, CRIB cells were pretreated with the designated concentration of GSK650394 (nM) and then infected with BoHV-1 at an MOI of 0.01. Medium containing 10% fetal calf serum (black columns) or 2% stripped fetal calf serum (hatched columns) was then added. Infectious virus was collected at 24 hours after

infection by three rounds of freeze-thawing and then plaque assays performed. The number of
444 plaques present in cells infected in the presence of DMSO (solvent control) was set at 100% and
the values in other samples normalized to this value.

446 **Panel B:** Monkey kidney (Vero cells) were incubated in 10% fetal calf serum and then sub-
cultured 24 hours prior to infection. Three hours prior to infection, Vero cells were treated with
448 the designated concentration of GSK650394 (nM) and then infected with HSV-1 (KOS strain) at
an MOI of 0.01. Medium containing 10% fetal calf serum (black columns) or 2% stripped fetal
450 calf serum (hatched columns) was then added. Infectious virus was collected at 24 hours after
infection by three rounds of freeze thawing, and plaque assays performed. The number of
452 plaques present in cells infected in the presence of DMSO (solvent control) was set at 100% and
the values in the other samples were normalized to this value. The studies in Panel A and B were
454 repeated 3 times and an asterisk denotes significant differences between the DMSO control ($P <$
0.05) as determined by the Student t test.

456 **Panel C:** The designated cell line was incubated with the DMSO or the designated
concentration of GSK650394 (nM) for 24 hours. The number of viable cells was quantified
458 based on ATP present in culture using the CellTiter-Glo Luminescent Cell Viability Assay
(Sigma; G7572) according to manufacturer's instruction as a measure of cell viability. These
460 studies are the average of three independent studies.

462 **Figure 2. Analysis of SGK-1 protein levels during productive infection.**

CRIB cells were mock infected or infected with BoHV-1 at an MOI of 2. Cells were incubated
464 in 2% "stripped" fetal bovine serum as described in Figure 1 after infection. At the designated
times after infection (hours), whole-cell lysate was prepared. Equivalent amounts of protein for

each sample (50 ug protein) were electrophoresed in a SDS-PAGE gel, and western blot analysis performed using a commercially available SGK1 antibody (Abcam; ab59337). The closed circle denotes the location of a novel SGK1 band detected in infected cells (middle panel is a longer exposure of the top panel). Tubulin protein levels were analyzed in the respective samples as a loading control. The relative levels of the SGK bands were estimated by comparing the intensity of these bands to tubulin using a BioRad ChemiDoc MP and PMI System. The SGK value was set at 100 and the other values normalized to this value. These results are representative of three independent experiments.

Figure 3. GSK650394 treatment reduces steady state levels of BoHV-1 proteins.

Cells were grown as described in Figure 1 prior to infection. Three hours prior to infection 25 nM of GSK650394 was added to the designated cultures. CRIB cells were infected with BoHV-1 (moi = 2) and then stripped FBS added. At the designated times after infection (hours), whole cell lysate (50 ug protein) was prepared and Western Blots performed using the designated antibodies directed against a viral protein. Arrows denote the position of bICP0 (97 kD), bICP4 (178 kD), bICP22 (45 kD), VP16 (65 kD), and the bracket denotes various gE forms. Tubulin was used as a loading control. The lane marked M are molecular weight markers: numbers to left are the approximate size of the respective bands. These results are representative of 4 independent experiments. The relative levels of the respective BoHV-1 proteins were estimated by comparing the intensity of the bands to tubulin using a BioRad ChemiDoc MP and PMI System. The value of the respective viral protein that was first detected after infection was set at 10 and the other values normalized to this value. ND refers to those lanes in which the viral protein was not detected. These results are representative of three independent experiments.

Figure 4. GSK650394 does not inhibit the ability of the synthetic corticosteroid dexamethasone to stimulate IETu1 or MMTV LTR promoter activity. Mouse neuroblastoma cells (Neuro-2A) were transfected with 2 ug DNA of a reporter construct containing the IETu1 promoter (Panel A) or the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) (Panel B). Five hours post-transfection, cells were incubated with stripped serum containing media. At 24 hours post-transfection, the designated cultures were treated with 10 nM dexamethasone (DEX) and 25 or 100 nM GSK650394. At 48 hours after transfection, promoter activity was measured as previously described (Kook et al., 2015b). These studies are the average of 3 independent experiments. An asterisk denotes significant differences between the DMSO control without DEX ($P < 0.05$) as determined by the Student *t* test.

Figure 1

Figure 2

Figure 3

Figure 4