Phosphoproteomic Analysis of the Bovine Herpesvirus Type 1 Virion

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Bovine herpesvirus (BHV-1) is an initiator of the bovine respiratory disease complex (BRDC), which costs the US cattle industry billions of dollars each year. Our study focuses on the effects of phosphorylation during BHV-1 infection. Evidence suggests that herpesviruses tightly regulate phosphorylation of proteins packaged in the virions. We have manipulated phosphorylation levels throughout infection to compare effects of over-phosphorylation on viral replication. We have confirmed patterns of increased phosphorylation with the use of phosphatase inhibitors (PI) and noted that infection alone seems to trigger significant phosphorylation of proteins. When quantifying extracellular viral particles, we observed no significant difference in viral production when phosphatase inhibitors were added after infection; however, we observed a slight decrease in viral production when inhibitors were added before infection. To determine variation in infectivity due to protein over-phosphorylation, we infected cells with wild-type virus and virus produced in the presence of PI, followed by viral progeny quantification. We observed no infectivity differences. These observations suggest the virus may not package over-phosphorylated proteins. We are currently purifying virions to determine the levels of phosphorylation and any differences seen in virion constituents produced in wild-type and over-phosphorylated virus.

INTRODUCTION

Bovine herpesvirus (BHV-1) is a member of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae*. BHV-1 is typically associated with other manifestations in cattle such as secondary bacterial infections, infectious pustular vulvovaginitis/balanoposthitis (IPV/IPB), encephalopathies, fetal infections, and infectious bovine rhinotracheitis (IBR), commonly known as shipping fever.[8] BHV-1 induced immune suppression and various bacterial infections can synergistically cause bovine respiratory disease complex (BRDC), which costs the United States an estimated 3 billion dollars annually.[4] BHV-1 typically establishes an acute infection in mucosal tissues of the oral and respiratory tract and then establishes a lifelong latency in the sensory neurons that can reactivate with stress to re-excrete active virus. Currently, several vaccines are in use, including inactivated, subunit, and modified live versions, but these available vaccines remain incapable of preventing latency and re-excretion of infectious virus due to reactivation.[8]

The post-translational modification of phosphorylation is necessary for BHV-1 infection. A few phosphoproteins have already been confirmed to significantly affect infection. For example, mature phosphorylated glycoprotein E (gE) is critical for viral pathogenicity.[10,11,12] Gene-deleted marker vaccines, such as the gE-deletion vaccine, are currently being used for immunization and diagnostic testing.[11] Other phosphoproteins that are essential for infection include VP22 (necessary virulence factor), the transactivating tegument protein (essential for growth), and the lamina and membrane proteins encoded by UL 31 and UL34, respectively (involved in primary envelopment of virus).[3,5,7,10]

Our goal is to further extend our knowledge of the role of phosphorylation during BHV-1 infection. By conducting the infection in an over-phosphorylated environment, we are able to monitor viral replication and determine whether the viral particles generated differ in infectivity. In addition, we will determine the phosphoproteome of the BHV-1 virion (Figure 1). With this research, viral protein

functions and the role of phosphorylation may be further deduced and exploited for the optimization of vaccinations.

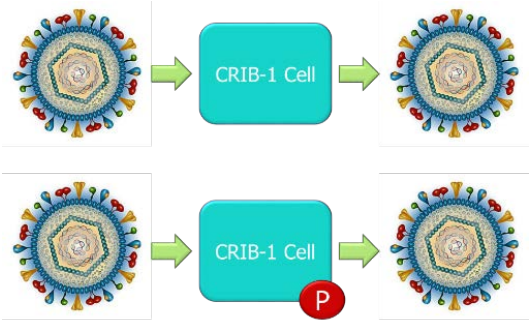

Figure 1. Research project goal. Our goal is to compare wild-type BHV-1 and over-phosphorylated BHV-1 to determine effects of over-phosphorylation during infection. Subsequent study of the virion and phosphoproteins will help to further elucidate the role of phosphorylation in BHV-1 infection.

MATERIALS AND METHODS

Cells and virus. CRIB-1 (ATCC CRL-11883) bovine kidney cells derived from Madin-Darby bovine kidney (MDBK) cell line were grown in HyClone's medium supplemented with 5% fetal bovine serum (FBS), Streptomycin (100 µg/mL), and Penicillin (10 U/mL). Confluent monolayers of CRIB-1 cells were infected with BHV-1 Cooper strain (ATCC VR864) at a multiplicity of infection (MOI) of 1.0 PFU per cell. At 24 hpi, the supernatant was collected. Cell debris was removed through light centrifugation at 4°C for 30 minutes. Supernatant containing viral particles were stored at -80°C. Remaining cell pellets were stored at -20°C.

Titration of virus particles. Supernatant was used to quantitate viral production using plaque assays. 10-fold serial dilutions of viral particles were made with serum-free medium. 70% confluent monolayers of CRIB-1 cells (in 6-well dishes) were infected with 1 mL aliquots of serially diluted viral particles. After 1 hour of incubation at 37°C, inoculum was removed and replaced with 2 mL of 1% agarose and enriched media. Once solidified, the plates were incubated at 37°C for approximately 48 hours. Cultures were fixed with 2% paraformaldehyde and stained with crystal violet.

Phosphatase inhibition studies. Monolayers of CRIB-1 cells were infected with a MOI of 0.1. PhosSTOP inhibitory cocktail (Roche Applied Science) was added in dilutions of 1:100 and 1:30 to samples four hours before or after infection. Supernatant and/or cells were collected at various time points during infection.

Cell lysis and Western blotting. Cells were lysed in 100 µL of Laemmli sample buffer (Bio-Rad) supplemented with β-mercaptoethanol. Samples were boiled for 5 minutes and used for SDS-polyacrylamide gel electophoresis. Immunodetection of phosphorylated proteins was performed using anti-phosphotyrosine (p-Tyr) (Santa Cruz no. sc-7020) and anti-phosphothreonine (p-Thr) (Santa Cruz no. sc-5267) monoclonal antibodies.

Infectivity assessment. CRIB-1 monolayers were infected with virus at a MOI of 1. Some infected monolayers were subjected to phosphatase inhibitor treatment (1:30) four hours before or after infection. Supernatants were collected 24 hpi and titrated. Virus concentration was normalized for wild-type and treated culture supernatants to infect a fresh set of cell monolayers with an MOI of 1. Supernatants were collected 24 hpi and titrated.

Viral purification. Virus from >30 (100mm^2) plates was pelleted through a 30% sucrose cushion using a Beckman L5-50B ultracentrifuge at 75,000 x g for 2 hours at 4°C. The viral pellet was resuspended in 5 mL of Tris-EDTA (TE) buffer and centrifuged on a 30-60% sucrose continuous gradient at 75,000 g for 2 hours at 4°C. Syringe extraction was utilized to remove two distinct bands. Virus from each band was pelleted through a 30% sucrose cushion and resuspended in 1 mL of TE for 24

hours at 4°C. Resuspended pellets were titrated and measured for protein concentration with the micro BCA protein assay (Thermo Scientific). Pellets were digested with 10 µg/mL of Proteinase K and 170 units of Sequencing Grade Modified Trypsin (Promega). Digested pellets were sent for transmission electron microscopy analysis.

Phosphoproteomic analysis. Virions in TE buffer were lysed with 4% SDS, boiled for 5 minutes, and stored at -80°C for future protein manipulations. Viral proteins will be digested and enriched via immobilized metal affinity chromatography (IMAC) and further analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). Data collected from LC-MS/MS will be filtered through the Mascot 2.2 search engine and analyzed using Proteome Discoverer 1.4.

RESULTS

We used a phosphatase inhibitor (PI) to study how phosphorylation levels effect viral infection. Very little increase in phosphorylation was seen in mock-infected samples; whereas, infection alone triggered phosphorylation, which was dose-dependent, as expected. This increase in phosphorylation correlated to an induced over-phosphorylated environment (Figure 2).

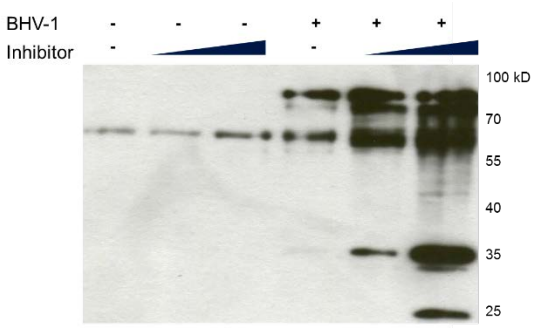

Figure 2. Effects of phosphorylation in BHV-1 infection. CRIB-1 cells were infected and treated with increasing PI (1:100, 1:30) added after infection, as indicated above. Cell lysates were collected at 48 hours post infection (HPI) and analyzed via western blot, using antibody against phosphorylated against p-Tyr.

To explore the effects of over-phosphorylation on viral production, we added the PI either before or after infection and compared the subsequent viral titers with wild-type virus. At twenty-four hours after infection, when inhibitor was added before infection, viral titers were reduced in varying degrees; whereas, only a small effect was seen when inhibitor was added after infection. This difference was not statistically significant; however, we observed a consistent trend. By forty-eight hours post infection, viral titers were relatively equal between inhibited and uninhibited samples. These results suggest a defect early in replication, but that the virus is able to accommodate for the environmental change and make up for this loss in productivity (Figure 3).

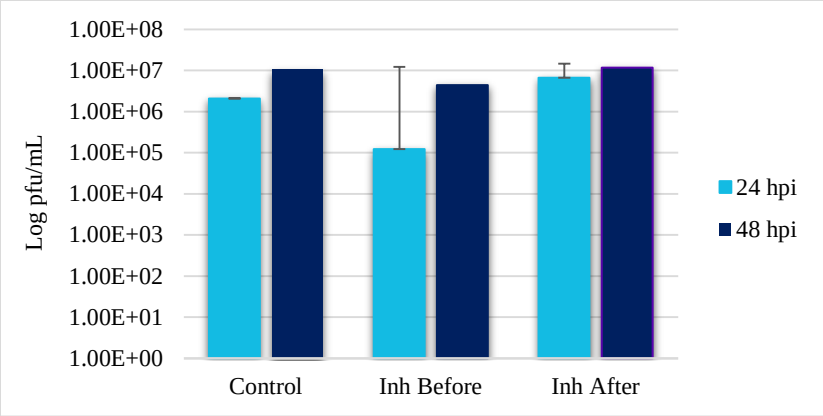

Figure 3. Effects of over-phosphorylation on virus replication. CRIB-1 cells were infected with a MOI of 0.1. Supernatant was collected at 24 and 48 HPI. Plaque assays were performed to obtain plaque forming units per milliliter (pfu/mL). Values are shown as mean ± SEM. Number of repetitions per sample: Control (n=11), Inhibitor Before (n=6), Inhibitor After (n=5).

To determine whether this replicative defect could be seen at earlier time points, we generated a one-step growth curve of infected CRIB-1 cells from eight to forty-eight hpi. Again, inhibited samples had slightly lower viral titer than wild-type (Figure 4).

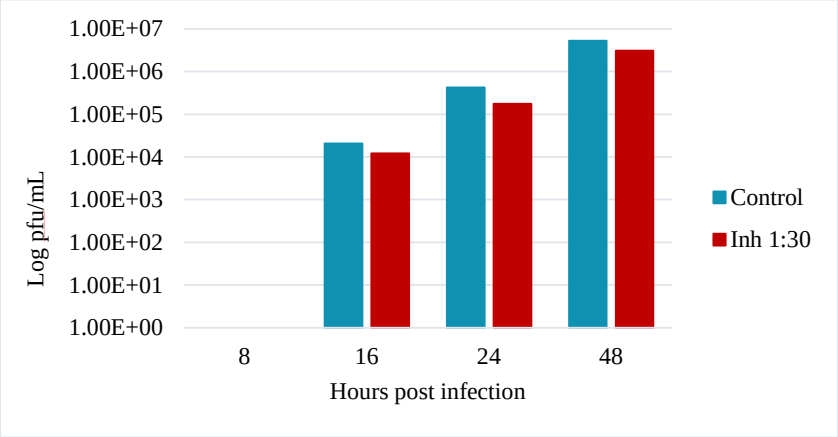

Figure 4. One-step growth curve. CRIB-1 cells were infected with a MOI of 0.1. Samples were collected at 8, 16, 24, and 48 hpi. Plaque assays were performed to obtain plaque forming units per milliliter (pfu/mL). Values are shown as mean ± SEM (n=7).

Next, to determine whether virus generated in either a normal or an over-phosphorylated environment differed in infectivity capacity. Virus grown in the absence or presence of PI was used to infect fresh CRIB-1 cells. The infectivity of the progeny virions was assessed using plaque assay. A slight decrease in infectivity was seen in the virions generated with inhibitor added before infection, which correlates to our previous findings, but no difference was seen when inhibitor was added after infection. These findings suggest BHV-1 is tightly regulating the phosphorylated proteins that are packaged into progeny virions to promote optimal viral infectivity (Figure 4). Simultaneously, the small decrease in

infectivity suggests that the BHV-1 virions generated in the two environments may differ slightly in their constituents.

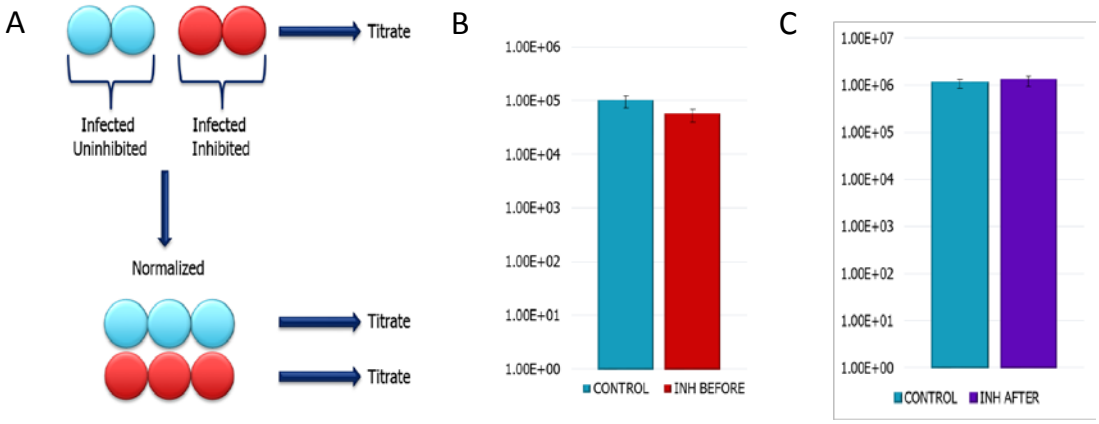

Figure 4. Effects of over-phosphorylation on viral infectivity. CRIB-1 cells were infected with a MOI of 1 in the presence/absence of PI (1:30) added before/after infection. Supernatant was collected at 24 hpi. Pfu/mL was calculated, and titers were normalized to infect fresh CRIB-1 cells with an MOI of 1. Supernatant was collected at 24 hpi, and virions were quantified via plaque assays. Values shown as mean ± SEM. (A) Serial infection protocol (B) Inhibitor added before infection (C) Inhibitor added after infection.

Next, we intend to explore the content of packaged phosphoproteins in wild-type virions and virions generated in the presence of PI. We purified BHV-1 virions in sucrose gradients and assessed the purity level via electronic microscopy Figure 6). Optimization of the purification protocol is ongoing to ensure no cellular contaminants are present.

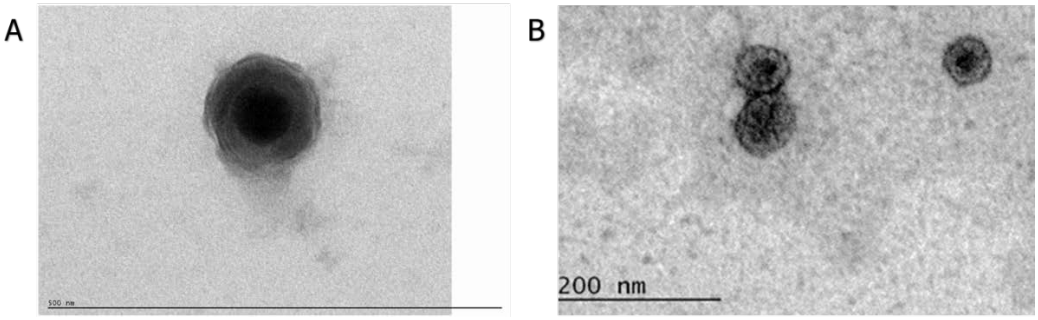

Figure 6. Analysis of an extracellular virions by EM. Purified virions from CRIB-1 cells were negatively stained and analyzed by EM. Bar represents A. 500 nm B. 200 nm.

Lastly, we have compiled a list of viral proteins that are predicted to be phosphorylated by NetPhos and KinasePhos software. More than seventy proteins were analyzed and 44 were predicted to be phosphorylated. Some of these phosphoproteins have been confirmed experimentally. The full list will help us analyze the data obtained from the phosphoproteomic analysis (partial list Table 1).

Table 1. Phosphorylation site predictions on BHV-1 virion proteins. Only the proteins that have been experimentally confirmed are shown. Individual viral sequences were retrieved from NCBI genomic database and analyzed using NetPhos2.0 and KinasePhos2.0.

Gene	Protein	Function	P Sites	Tyr	Ser	Thr	Reference
UL36	Large Tegument Protein	Capsid Protein	49	6	19	24	McNabb[6]
RS1 (?)	bICP4	Gene Regulation	25	4	14	7	Mettenleiter[7]
US3	US3 Ser/Thr Kinase	Protein Phosphorylation/ Apoptosis/Egress	21	1	16	4	Mettenleiter[7]
UL47	Tegument VP8	Possible Gene Regulation	18	6	6	6	Labiuk[5]
US8	Glycoprotein E	Cell-to-cell Spread	17	3	7	7	Shaw[12]
UL31	Nuclear Egress Lamina Protein	Nuclear Egress	14	1	9	4	Chang[1]
UL49	Tegument VP22	Virion Morphogenesis	13	4	8	1	Mettenleiter[7]
UL13	Tegument Ser/Thr Kinase	Protein Phosphorylation	9	1	3	5	Cunningham[2]
UL34	Nuclear Egress Membrane Protein	Nuclear Egress	2	0	1	1	Mettenleiter[7]

With a preliminary list of phosphoproteins and purified virions, we may now enrich for such phosphoproteins in both normal and over-phosphorylated virions. We will identify phosphorylated proteins and the degree of phosphorylation using LC-MS/MS. Data analysis will be provided by Proteome Discoverer.

CONCLUSION

In conclusion, we have shown that BHV-1 incorporates and highly regulates phosphorylation during infection. Over-phosphorylation induced by phosphatase inhibitors have little effect on both replication and infectivity. The only effects are seen when the inhibitor is added before infection. Consequently, the packaging of proteins, specifically phosphoproteins, into BHV-1 virions are tightly controlled. To further deduce which phosphoproteins are packaged into the virion, we have purified extracellular virions for further analysis using mass spectrometry. These proteins and their phosphorylation levels will be compared in wild-type and over-phosphorylated virions to determine the level of specificity of the BHV-1 virion.

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