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Investigation of Bovine Herpesvirus 1 (BHV-1) Encoded Infected Cell Protein 0 (bICP0)

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INVESTIGATION OF BOVINE HERPESVIRUS 1 (BHV-1) ENCODED INFECTED CELL PROTEIN 0 (BICP0)

by

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INVESTIGATION OF BOVINE HERPESVIRUS 1 (BHV-1) ENCODED INFECTED CELL PROTEIN 0 (BICP0)

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Bovine herpesvirus 1 (BHV-1) is a significant pathogen of cattle. Following acute infection, BHV-1 establishes a latent infection that persists for the life of the infected host. Stress induced factors cause the virus to reactivate from latency, resulting in virus transmission and transient immune suppression. BHV-1 encoded bICP0 is expressed early and constitutively throughout productive infection. bICP0 is critical for efficient viral replication, virulence, and reactivation in cattle because it stimulates viral transcription and interferes with innate immune responses. bICP0 potentially interacts with a variety of proteins to activate viral gene expression and inhibit innate antiviral defenses. bICP0 localizes to promyelocytic leukemia (PML) protein-containing nuclear domains, which are associated with antiviral activity and commonly targeted for disruption by a wide variety of viruses. The zinc RING finger motif within bICP0 plays a critical role in the biological functions of bICP0, and possesses intrinsic E3 ubiquitin ligase activity that is important for polyubiquitination and subsequent degradation of proteins.

Results from these studies demonstrated mutations within the zinc RING finger increased bICP0 protein levels, presumably due to disruption of the ability of bICP0 to induce its own ubiquitination. Sequences at its C-terminus are also important for

regulating the half-life of bICP0. BHV-1 infection and bICP0 expression alone reduced PML protein levels. Unexpectedly, bICP0 mutants that localized primarily in the cytoplasm also induced PML degradation. bICP0 was readily detected in the cytoplasm of low passage bovine cells at later times post infection, suggesting bICP0 induces degradation of cytoplasmic isoforms of PML to promote viral replication. Identification of bICP0-interacting proteins was also investigated to further elucidate the mechanisms underlying bICP0 functions. The ability of BHV-1 and a bICP0 zinc RING finger mutant to grow in oncogenic cells was also examined to determine if defects in viral growth of the mutant could be relieved in cells with potential defects in their interferon response. Collectively, studies presented in this dissertation determine that nuclear and cytoplasmic bICP0 have functions that promote productive infection.

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KEYWORDS & ABBREVIATIONS

aa amino acid

BHV-1 bovine herpesvirus type 1

bICP0 BHV-1 infected cell protein 0

bp base pair

E early

hpi hours post infection

HSV-1 herpes simplex virus type 1

HA hemagglutinin

IE immediate early

IETu immediate early transcription unit

IFN interferon

IRF interferon regulatory factor

kDa kilo Dalton

LR latency related

ND10 nuclear domains 10

NLS nuclear localization signal

PML promyelocytic leukemia protein

RING really interesting novel gene

SUMO small ubiquitin-like modifier

TAD transcriptional activation domain

Ub ubiquitin

wt wild type

CHAPTER 1

LITERATURE REVIEW

I. Introduction

Bovine herpesvirus type 1 (BHV-1) is a significant pathogen of cattle. Herpesviruses have linear, double-stranded DNA genomes and replicate in the nucleus of infected cells. The hallmark of all herpesviruses is their ability to establish a latent infection that persists for the lifetime of the infected host. Stress-induced factors cause the virus to reactivate from latency, resulting in recurrent lytic infection and transmission of the virus. Three subfamilies make up the family *Herpesviridae*: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Each herpesvirus subfamily is unique in the length of their replication cycle and the subset of cells in which latency is established. Members of the subfamily *Betaherpesvirinae* have long replication cycles compared to the other *Herpesviridae* subfamilies and establish latency in leukocytes. *Gammaherpesvirinae* members infect immune cells such as B-lymphocytes, and include Epstein-Barr virus (EBV), which causes mononucleosis, and Kaposi's sarcoma-associated herpesvirus (KSHV) commonly found in AIDS patients. *Alphaherpesvirinae* subfamily members are neurotropic, have a short replication cycle, and induce severe cytopathology. In addition to BHV-1, other clinically important *Alphaherpesvirinae* members include the pathogens: human Herpes simplex virus types 1 and 2 (HSV-1, HSV-2) and Varicella zoster virus (VZV) the causative agent of "chicken pox" and "shingles", Pseudorabies virus (PRV) which infects pigs, and equine herpesvirus 1 (EHV-1) of horses.

The *Alphaherpesvirinae* subfamily members listed above all encode infected cell protein 0 (ICP0)-related proteins that are characterized by the presence of a zinc RING

(which stands for "really interesting novel gene") finger domain and their ability to activate viral transcription (Everett et al., 2010). These proteins also have inherent E3 ubiquitin ligase activity and interact with promyelocytic leukemia (PML) protein-containing nuclear structures (Everett et al., 2010). The biology of BHV-1 encoded bICP0 and its role in infection are discussed in this literature review.

II. Overview of BHV-1 infection

A. Pathogenesis and immune evasion

BHV-1 is a pathogen of cattle that contributes to considerable economic losses to the cattle industry every year (Jones and Chowdhury, 2007; Turin et al., 1999). Clinical disease associated with BHV-1 infection includes conjunctivitis, respiratory infection and pneumonia, genital and reproductive disorders, abortions, and in rare cases encephalitis (Tikoo et al., 1995). BHV-1 is subdivided into three subtypes: BHV-1.1 is associated with respiratory disease, BHV-1.2a is most commonly linked to genital infections, and the less pathogenic BHV-1.2b strains (Jones and Chowdhury, 2007). BHV-1 infection causes suppression of the host's immune defenses and as a consequence can lead to secondary infections (Carter et al., 1989; Griebel et al., 1990; Griebel et al., 1987b; Griebel et al., 1987c). Bovine respiratory disease (BRD) or "Shipping Fever" is an upper respiratory infection complex that can be initiated by BHV-1 infection (Jones and Chowdhury, 2007; Muylkens et al., 2007; Yates, 1982). BRD and BHV-1 infections cost the United States cattle industry approximately 3 billion dollars in losses annually ((NASS), 1996; Bowland and Shewen, 2000; Carter et al., 1989; Griebel et al., 1987a;

Griebel et al., 1990; Griebel et al., 1987c; Ishmael, 2001; Kapil, 1997; Powell, 2005; Tikoo et al., 1995; Turin et al., 1999). BHV-1 modified live attenuated virus or killed virus vaccines that protect against clinical disease in adults are commercially available (Jones and Chowdhury, 2007). However, BHV-1 vaccination can cause immune suppression leading to serious disease in young calves or abortions in pregnant cows (Jones and Chowdhury, 2007; van Drunen Littel-van den Hurk, 2006). In addition, modified live vaccines can reactivate from latency, be transmitted and cause disease in immune suppressed animals (Ellis et al., 2005; Muylkens et al., 2007; van Drunen Littel-van den Hurk et al., 2001). The fact that BHV-1 establishes lifelong latent infection complicates the development of effective vaccines.

BHV-1 interferes with immune recognition and innate immune response pathways (Jones, 2009). CD4+ T cells are semi-permissive to BHV-1 and their infection by this virus results in apoptosis (Winkler et al., 1999). BHV-1 encoded glycoprotein N impairs immune recognition of infected cells by interfering with MHC I antigen presentation to CD8+ T cells (Hariharan et al., 1993; Hinkley et al., 1998; Koppers-Lalic et al., 2005; Nataraj et al., 1997). Glycoprotein G is secreted from BHV-1 infected cells and is able to bind a broad range of chemokines (Bryant et al., 2003); thus, potentially interfering with the activation of inflammatory and immune response pathways that lead to antiviral functions (Baggiolini, 1998). BHV-1 infected protein cell protein 0 (bICP0) inhibits induction of interferon beta (IFN-β) by interfering with transcriptional co-activators, interferon regulatory factors 3 and 7 (IRF3, IRF7) and p300 (Henderson et al., 2005; Saira et al., 2007; Saira et al., 2009; Zhang et al., 2006). The latency related (LR) gene products also have a protective effect against apoptosis (Ciacci-Zanella et al., 1999;

Henderson et al., 2004a) and inhibit infiltration of inflammatory cells into the trigeminal ganglia of calves (Perez et al., 2006), the site where latency is established.

B. Productive infection

BHV-1 productive infection occurs in mucosal surfaces of the eyes, respiratory tract, or genital tract. During the acute lytic stage of infection, high levels of virus are produced and shed from the infected mucosal tissues for up to 10 days after infection (Jones, 1998; Jones, 2003). The herpesvirus virion is composed of a DNA core surrounded by an icosahedral nucleocapsid, a tegument and an envelope. The tegument is a proteinaceous matrix located between the capsid and envelope and contains important factors for initiating viral transcription (Valicek and Smid, 1976). Glycoproteins of the viral envelope bind to receptors on the cell surface and fusion with the cellular membrane occurs. The viral capsid is released into the cytosol, transported to the nucleus, and the viral DNA ejected into the nucleus where viral replication takes place. During productive infection, viral genes are temporally expressed in three distinct phases: immediate early (IE), early (E), and late (L) (Jones, 1998; Jones, 2003; Wirth et al., 1989). IE viral gene expression is initiated by the BHV-1 the virion component trans-inducing factor (bTIF) and cellular transcriptional activator Oct-1 (Misra et al., 1994; Misra et al., 1995). IE proteins activate E gene expression, followed by viral DNA replication. Late genes include structural proteins such as glycoproteins, nucleocapsid and tegument proteins important for virion assembly. Subsequently, viral genomic DNA is packaged and encapsidated. Following assembly in the nucleus, viral capsids migrates from the nucleus

to the cell surface, acquiring envelope from nuclear budding and secondary envelopment from compartments within the cytoplasm (Mettenleiter et al., 2009). Finally, infectious virus particles are released.

The BHV-1 genome is 135.3 kbp, 72% G + C rich, and encodes at least 70 known proteins (Meyer et al., 1997; Plummer et al., 1969). The genome is divided into a unique long (L) and unique short (S) segment flanked with internal and terminal inverted repeats (Figure 1A). Located within the inverted repeats are two IE transcription units (IEtu1, IEtu2). IE genes encoded by IEtu1 and IEtu2 are alternatively spliced to give rise to three major IE proteins essential for regulating viral gene expression (Wirth et al., 1991). The IEtu1 transcript encodes BHV-1 infected cell protein 0 (bICP0) and bICP4 (Wirth et al., 1992). bICP0 acts as a promiscuous transcriptional activator that stimulates all three classes (IE, E, and L) of viral promoters, whereas, expression of bICP4 represses IEtu1 promoter activity (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1991). The bICP22 protein is encoded by IEtu2 and acts as a trans-repressor of viral promoters from each class (Koppel et al., 1997).

bICP0 is the major regulator of BHV-1 gene expression and is critical for efficient viral replication (Geiser et al., 2005; Saira et al., 2008). During productive infection bICP0 is constitutively expressed, in part because it can activate its own promoters. Expression of bICP0 is under the control of the IEtu1 and E promoters (Figure 1A). The bICP0 protein is translated from the alternatively spliced IE/2.9 transcript and the unspliced E/2.6 transcript (Wirth et al., 1991). These two transcripts share an identical open reading frame (ORF) that expresses a 676 amino acid protein that migrates at 97

kDa (Wirth et al., 1992). Although bICP0 is not required for viral replication, it is a critical factor of the BHV-1 infection cycle. The functions of bICP0 are reviewed and discussed in more detail in the following sections.

C. Latency and reactivation

Following productive infection, BHV-1 establishes a latent infection which allows the virus to persist for the life of the host. Virus particles enter sensory neurons of the peripheral nervous system via cell-to-cell spread. For acute infections that occur in the ocular or oronasal cavities, the trigeminal ganglion is the primary site for latency. For genital infections, latency is established in the neurons associated with the sacral ganglia. During latency, lytic viral gene expression and production of infectious virions ceases. However, viral genome can be detected in the sensory ganglia (Inman et al., 2002; Schang and Jones, 1997), as well as other non-neuronal sites such as the tonsils (Winkler et al., 2000), peripheral blood (Fuchs et al., 1999), spleen and lymph nodes (Mweene et al., 1996).

The latent stage of infection is marked by expression of the latency related (LR) gene and ORF-E, the only two transcripts known to be abundantly expressed during latency (Inman et al., 2004; Jones, 1998; Jones, 2003; Kutish et al., 1990). Both ORFE and the LR produce proteins during latency (Meyer et al., 2007; Perez et al., 2007). The LR gene contains two ORFs and two RFs that lack an initiating methionine (ATG). LR transcripts can be alternatively spliced which potentially gives rise to multiple fusion proteins (Devireddy and Jones, 1998; Hossain et al., 1995). The LR gene is anti-sense to

bICP0 and ORFE, and expression of the LR transcript is sufficient to inhibit bICP0 expression (Bratanich et al., 1992; Geiser et al., 2002). Two families of small non-coding RNAs encoded by LR were identified which contained putative viral micro RNAs that reduced bICP0 protein levels, but not steady-state levels of bICP0 RNA (Jaber et al., 2010). Inhibition of bICP0 subsequently extinguishes lytic viral gene expression.

Expression of LR and ORF-E is important not only for establishing, but also maintaining latency and allowing persistence of the viral genome in sensory neurons (Jones, 1998; Jones, 2003). Expression of LR proteins is necessary for inhibition of cell growth (Schang et al., 1996), apoptosis (Ciacci-Zanella et al., 1999; Henderson et al., 2004a), and infiltration of inflammatory cells to sites of latently infected neurons (Perez et al., 2006). ORF-E expression induces neurite-like outgrowth in murine neuroblastoma cells which suggests it may promote normal neuronal functions of latently infected cells (Perez et al., 2007). An LR mutant virus was able to grow to similar titers as wt BHV-1 in culture, but exhibited reduced clinical symptoms and viral shedding in cattle (Inman et al., 2001a; Inman et al., 2002; Perez et al., 2005). In addition, the LR mutant was unable to reactivate from latency in infected calves, suggesting expression of LR gene products is not critical for productive infection, but is required for reactivation (Inman et al., 2002; Jones, 2003; Jones and Chowdhury, 2007; Perez et al., 2005).

Stress-induced factors and immune suppression can lead to reactivation from latency. Upon reactivation, expression of LR and ORF-E genes is significantly reduced, lytic gene expression proceeds and infectious virions are produced (Jones, 1998; Jones, 2003; Jones and Chowdhury, 2007). Treatment with glucocorticoids or the synthetic

corticosteroid dexamethasone (DEX) can induce reactivation and is used for investigative studies in cattle (Inman et al., 2002; Rock et al., 1992). DEX treatment was shown to activate the bICP0 early promoter, and expression of bICP0 subsequently resulted in reactivation from latency (Workman et al., 2009). Furthermore, a bICP0 mutant BHV-1 strain able to establish latency, failed to reactivate in calves following treatment with DEX (Saira et al., 2008). In summary, the LR gene and bICP0 are critical regulators of the latency-reactivation cycle.

III. BHV-1 encoded bICP0

A. bICP0 mutant viruses

bICP0 plays a pivotal role during the BHV-1 infection cycle. bICP0 is not absolutely required for viral replication; however, it is critical for efficient viral growth and propagation. Transfection of BHV-1 genomic DNA into permissive cells results in inefficient, basal level of viral replication and formation of plagues. Co-expression of BHV-1 DNA and bICP0 significantly enhances viral replication and plaque formation, indicating bICP0 stimulates productive infection (Geiser and Jones, 2003; Inman et al., 2001b). bICP0-null or mutant viruses have reduced growth potential and are not stable. A BHV-1 recombinant virus that does not express the bICP0 protein (bICP0-null virus) does not produce plaques, yields reduced viral titers by at least 100 fold, and establishes a persistent-like infection in bovine cells (Geiser et al., 2005). Furthermore, the bICP0-null virus was replication-deficient in calves (Geiser et al., 2005). Another BHV-1 recombinant virus (51G) containing a single mutation within the C_3HC_4 zinc RING

finger, an important functional domain of bICP0, was able to induce plaques, but grew poorly compared to the rescued virus or wt BHV-1 (Saira et al., 2008). The 51G mutant also exhibited reduced growth kinetics, virulence, and was unable to reactivate from latency in infected calves (Saira et al., 2008). Collectively, these studies demonstrate that expression of the bICP0 protein is critical for BHV-1 growth in cultured cells and productive infection in the host.

B. Transcriptional activation

bICP0 stimulates productive infection by acting as a potent transcriptional activator of all three classes of viral genes; however, it is does not appear to be a DNA-binding protein (Wirth et al., 1992). Instead, bICP0 appears to interact with other transcriptional regulators in order to stimulate viral gene expression. bICP0 has been shown to promote activation of viral transcription in part by associating with chromatin remodeling enzymes. bICP0 interacts with histone deacetylase 1 (HDAC1) and this interaction leads to relief of transcriptional repression mediated by HDAC1 (Zhang and Jones, 2001). bICP0 also associates with the transcriptional co-activator p300, which possesses intrinsic histone acetyltransferase (HAT) activity (Zhang et al., 2006). Over-expression of p300 trans-activated a late viral promoter (gC) and enhanced productive infection (Zhang et al., 2006).

HATs and HDACs are key factors of transcriptional regulation because they modulate histones which are important components involved in packaging DNA into chromatin (Hassig and Schreiber, 1997; Kiermaier and Eilers, 1997; Struhl, 1998; Wade

and Wolffe, 1997; Wolffe, 1996). HATs promote transcription by acetylating histone tails which destabilizes the chromatin structure allowing transcription machinery to gain access to promoter sequences for gene expression. HDACs remove acetyl groups from histones returning chromatin to its tightly packaged structure and thereby repress transcription. Thus, the ability of bICP0 to interact with these types of chromatin remodeling enzymes and other putative transcriptional regulators plays a critical role in the ability of bICP0 to stimulate viral gene expression.

Several domains have been identified that are important for transcriptional activation by bICP0 (Figure 1B). An intact zinc RING finger motif was not required for bICP0 to interact with either HDAC1 or p300 (Zhang et al., 2006; Zhang and Jones, 2001); however, mutagenesis of the zinc RING finger did affect the ability of bICP0 to activate viral transcription (Zhang et al., 2006; Zhang and Jones, 2001). In addition to the zinc RING finger motif, two major transcriptional activation domains (TADs) have been identified which consist of the region between amino acids 78 and 256, and C-terminal residues at or near amino acid 457 of the bICP0 protein (Zhang et al., 2005). bICP0 also contains an acidic region which can be found in many transcriptional activators (Zhang et al., 2005). To date, it has not been shown to play a role in activating transcription (Zhang et al., 2005). Further studies are required to understand the complete molecular mechanism of transcriptional activation by bICP0 and the role of TAD residues for this activity.

C. Interferon inhibition

In addition to its ability to stimulate productive infection, bICP0 is able to regulate the interferon (IFN) response. IFN-β is part of the type I IFN response pathway that plays a pivotal role in amplification of innate immunity and antiviral defense (Katze et al., 2002; Randall and Goodbourn, 2008). bICP0 inhibits IFN-β transcriptional activation by affecting at least three major regulators of IFN-β expression: IRF3, IRF7, and p300 (Saira et al., 2007; Saira et al., 2009; Zhang et al., 2006). IRF3 and IRF7 are key components of the IFN response pathway because they act as co-activators of the IFN-β promoter (Hiscott, 2007). p300 is a co-activator of the IRF3-dependent IFN signaling pathway and other antiviral signaling cascades (Hiscott, 2007; Vo and Goodman, 2001; Weaver et al., 1998; Yoneyama et al., 1998). Upon viral recognition by innate immune sensors, IRF3 and IRF7 become activated through phosphorylation events and are translocated to the nucleus. In the nucleus, IRF3 and IRF7 associate with other co-activators including p300 to induce IFN-β gene expression. bICP0 inhibits IFN-β promoter activity by inducing proteasome-dependent degradation of IRF3 (Saira et al., 2007). In addition, bICP0 associates with IRF7 and this interaction also inhibits activation of the IFN-β promoter (Saira et al., 2009). The interaction between bICP0 and p300 may also interfere with p300-stimulated antiviral signaling (Zhang et al., 2006).

D. E3 ligase activity

Several functional domains of bICP0 have been identified, including a nuclear localization signal (NLS) located at its C-terminus, two transcriptional activation domains (TADs) separated by an acidic region, and a C_3HC_4 zinc RING finger motif (Figure 1B). The zinc RING finger domain located at the N-terminus contains seven conserved cysteine residues from the 13th to the 51st amino acid of the bICP0 protein. The zinc RING finger motif is conserved among *Alphaherpesvirinae* ICP0 homologues and plays a critical role in the biological functions of bICP0 (Everett et al., 2010). The C_3HC_4 zinc RING finger motif possesses intrinsic E3 ubiquitin ligase activity and the ICP0-related proteins encoded by HSV-1, VZV, EHV-1, PRV and BHV-1 were all shown to be active E3 ligases *in vitro* (Everett et al., 2010). E3 ubiquitin ligases can be divided into HECT- or really interesting novel gene (RING)-containing proteins, and proteins containing a zinc RING finger motif make up the largest class of potential E3 ubiquitin ligases (Deshaies, 2005a; Deshaies, 2005b; Pickart, 2001). E3 ubiquitin ligases are diverse and provide the specificity by which proteins are modified by ubiquitin (Nagy and Dikic, 2010; Pickart, 2001).

ICP0 is an established E3 ubiquitin ligase (Boutell et al., 2002; Hagglund and Roizman, 2002; Van Sant et al., 2001) that induces the ubiquitin mediated degradation of a number of proteins (Boutell and Everett, 2003; Chelbi-Alix and de The, 1999; Everett et al., 1999a; Everett et al., 1998; Fukuyo et al., 2011; Lomonte and Morency, 2007; Lomonte et al., 2001; Parkinson et al., 1999). bICP0 has also been shown to induce polyubiquitination and proteosome-dependent degradation of certain cellular proteins.

bICP0 directly catalyzes the ubiquitination of IκBα in order to activate the NF-κB signaling pathway (Diao et al., 2005), and reduces IRF3 protein levels in a proteasome-dependent manner (Saira et al., 2007). An intact zinc RING finger and sequences near the C-terminus were required for bICP0-mediated IRF3 proteolysis (Saira et al., 2007). Studies designed to identify targets of bICP0 for ubiquitination are discussed in Chapter 2, and the effect of bICP0 on cellular promyelocytic leukemia (PML) protein is examined in Chapter 3 of this dissertation.

E. Cytotoxicity of bICP0

Herpesviruses are cytolytic and their infection causes cytopathic effect. BHV-1 infection of calves or of cultured cells is known to lead to apoptosis (Devireddy and Jones, 1999a; Lovato et al., 2003; Winkler et al., 2000). Interestingly, transient expression of bICP0 is toxic to transfected cells (Inman et al., 2001b). bICP0 can induce cleavage of caspase 3 which leads to activation of the apoptotic pathway (Henderson et al., 2004b). Compared to a pro-apoptotic factor (Bax), bICP0 induced caspase 3 cleavage at later times after infection, suggesting the ability of bICP0 to activate caspase 3 is by an indirect mechanism (Henderson et al., 2004b). Mutations within the zinc RING finger motif of bICP0 reduced toxicity associated with expression of bICP0 (Henderson et al., 2004b). A bICP0 mutant lacking amino acids from 356 to 676 of the C-terminus of the bICP0 gene (referred to in the literature as ΔbICP0 or ΔC-terminus) localizes primarily to the cytoplasm and was more efficient at activating caspase 3 cleavage than wt bICP0 (Henderson et al., 2004b; Inman et al., 2001b), suggesting that interactions of bICP0 with

cytoplasmic factors could enhance cytotoxicity and apoptosis. Although, induction of apoptosis is usually believed to negatively regulate and control virus infection, there are examples to the contrary. For example, caspase 3 has been shown to enhance HSV-1 reactivation from latency (Hunsperger and Wilcox, 2003). The fact that bICP0 is critical for reactivation in cattle, suggests that the ability of bICP0 to activate caspase 3 may play a role in stimulating reactivation from latency and productive infection (Henderson et al., 2004b; Jones, 2003).

F. Localization of bICP0

bICP0 contains a basic NLS-like motif (KRRR) that is located between amino acids 622 and 625 at the C-terminus of the protein (Zhang et al., 2005). Deletion of fragments near the C-terminus including the NLS domain, result in an accumulation of bICP0 in the cytoplasm (Inman et al., 2001b; Zhang et al., 2005). During productive infection or in transiently transfected cells, bICP0 can be found at PML protein-containing subnuclear domains (Gaudreault and Jones, 2011; Inman et al., 2001b; Parkinson and Everett, 2000). A zinc RING finger mutant is also observed to localize to foci within the nucleus, however, its pattern is distinct from wt bICP0 (Inman et al., 2001b). In general, wt bICP0 can be found diffusely throughout the nucleus, or localized discretely to PML nuclear domains or nucleoli-like structures in infected or transiently transfected cells. Conversely, the zinc RING finger mutant primarily has a consistent punctate nuclear appearance, suggesting certain zinc RING finger-dependent modifications or interactions with other proteins may influence bICP0 localization.

bICP0 can also be found in the cytoplasm of low passage bovine cells at later times of BHV-1 infection (Gaudreault and Jones, 2011). This implicates a role for cytoplasmic bICP0 during infection, and suggests late viral proteins or induced factors may sequester bICP0 to the cytoplasm. Recently, bICP0 localized to the cytoplasm was shown to inhibit IFN-β promoter activity and induce degradation of IRF3 as efficiently as wt bICP0 (Da Silva and Gaudreault, unpublished). Thus, this data suggests a role for cytoplasmic bICP0 in repressing the innate immune response after infection.

IV. Cellular PML and viral infection

A. PML nuclear bodies

The PML protein is an essential component of PML bodies, also referred to as nuclear domain 10 (ND10), PML oncogenic domains (PODs), or Kruppel bodies, which are distinct nuclear structures that have a punctate appearance when examined by fluorescent microscopy (Lallemand-Breitenbach and de The, 2010). The size and number of these nuclear foci depend on the cellular context, but in general, they range from 1 to 30 structures with a diameter of approximately 0.2 to 1 μm (Dellaire and Bazett-Jones, 2004). The presence of the PML protein is essential for the formation of these unique domains and for recruitment of other proteins (Ishov et al., 1999; Zhong et al., 2000). Constitutive components of PML bodies include Sp100, DAXX, and SUMO (small ubiquitin-like modifier) (Negorev and Maul, 2001). Cell cycle, cellular stress, DNA damage, and other extrinsic factors can influence the composition of PML nuclear bodies (Dellaire and Bazett-Jones, 2004; Everett et al., 1999b; Maul et al., 2000). A

growing number of more than 160 proteins involved in a variety of cellular activities are known to traffic to and from PML nuclear domains (Lallemand-Breitenbach and de The, 2010; Van Damme et al., 2010). A common feature among proteins recruited to PML bodies is their ability to be SUMOylated (Bernardi and Pandolfi, 2007; Van Damme et al., 2010). PML bodies have been linked to such responses as DNA damage, stress, senescence, apoptosis, protein degradation, interferon, and viral infection (Bernardi and Pandolfi, 2003; Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Lallemand-Breitenbach et al., 2001; Regad and Chelbi-Alix, 2001; Tavalai and Stamminger, 2008). However, the underlying function of these distinct subnuclear structures is still not clearly understood and elucidation is complicated by their dynamic nature. A current model proposed is that PML acts as the glue which brings protein partners together at PML bodies to enhance post-translational modifications and thus yield activation, sequestration, or degradation of proteins (Lallemand-Breitenbach and de The, 2010).

B. The PML protein

The PML gene was first identified 20 years ago as the fusion partner with the retinoic acid receptor alpha (RARa) gene due to a translocation between chromosomes 15 and 17 found in patients with acute promyelocytic leukemia (APL) (de The et al., 1990; de The et al., 1991). PML is expressed in most cells of normal tissues and can also be found in tumors of various origins; however, loss of PML expression correlates with tumor progression (Gurrieri et al., 2004). PML knockout mice are viable, but are prone

to develop tumors and more susceptible to infections (Wang et al., 1998a; Wang et al., 1998b). Overexpression of PML can inhibit tumor formation in nude mice (Mu et al., 1994) and the replication of several viruses (Blondel et al., 2002; Bonilla et al., 2002). The human PML gene contains 9 exons and generates multiple isoforms due to alternative splicing designated as PML I-VII that differ in their C-termini (Jensen et al., 2001). PML expression is dependent on cell type and the stage of differentiation (Cho et al., 1998; Flenghi et al., 1995), as well as extrinsic factors such as infections and stress-induced responses (Maul et al., 2000). IFN, IRF3, and the tumor suppressor p53 have all been found to induce PML expression through distinct mechanisms (Ferbeyre et al., 2000; Kim et al., 2007; Stadler et al., 1995). Splice variants of PML differ by their expression level, have unique subcellular and subnuclear localizations, and are implicated in having distinct functions in a variety of cellular processes (Condemine et al., 2006).

PML undergoes extensive post-translational modifications which regulate the stability, localization, and activity of the protein (Nichol et al., 2009; Reineke and Kao, 2009). Modification of PML by SUMO is the most characterized and appears to be important for PML regulation at several levels (Lallemand-Breitenbach et al., 2001; Nichol et al., 2009). SUMO-modification of PML is required for the formation of nuclear body structures, maintaining their integrity, and recruiting other proteins (Bernardi and Pandolfi, 2007; Evdokimov et al., 2008; Nacerddine et al., 2005; Zhong et al., 2000). SUMOylation regulates arsenic-induced PML ubiquitination which leads to PML degradation (Tatham et al., 2008). Phosphorylation of PML appears to be required for modifications by SUMO, but is able to regulate PML activity independently of SUMOylation (Hayakawa and Privalsky, 2004). Acetylation of PML is implicated to

play a role in the PML-dependent apoptotic pathway (Hayakawa et al., 2008). PML is also reported to be modified by interferon-stimulated gene 15 (ISG15) another small ubiquitin-like peptide (Shah et al., 2008) which is conjugated to proteins following stimulation by type 1 interferons, and infection by viruses or bacteria (Welchman et al., 2005).

Most PML isoforms contain a common NLS at their C-terminus and are localized to the nucleus. However, PML is also known to be expressed in the cytoplasm either because of loss of the NLS due to alternative splicing or recruitment by other proteins (Condemine et al., 2006; Lin et al., 2004a). Cytoplasmic PML has been shown to be important for regulating apoptosis and in resistance to viral infection (Giorgi et al., 2010; McNally et al., 2008). Although the effect of PML on various cellular processes and response pathways has been documented {Reviewed in (Bernardi and Pandolfi, 2003; Lallemand-Breitenbach and de The, 2010; Pearson and Pelicci, 2001; Salomoni and Pandolfi, 2002)}, the underlying molecular mechanisms remain greatly unknown, and no unifying principle for the physiological effects of PML has been discovered (Culjkovic-Kraljacic and Borden, 2010).

C. Viral interactions with PML

It is well established that infection by a wide variety of viruses affects PML and PML-containing nuclear bodies [Reviewed in (Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Tavalai and Stamminger, 2008)]. A number of viruses target PML in order to promote viral replication and encode proteins that are able to regulate

PML by a variety of mechanisms. A summary of DNA and RNA viruses and their encoded proteins that are known to colocalize with PML and disrupt PML body organization are listed in Table 1. Several of these viral proteins induce PML degradation, and reduction of PML correlates with increased viral replication efficiency. For example, lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), rabies, and encephalomyocarditis virus (EMCV) have increased replication efficiency in PML knockout mice (Bonilla et al., 2002), or cells derived from PML knockout mice (Blondel et al., 2002; Djavani et al., 2001; El McHichi et al., 2010). Overexpression of PML also correlates with repression of replication of rabies virus, LCMV and VSV (Blondel et al., 2002; Bonilla et al., 2002). In contrast, replication of HSV-1 and human foamy virus (HFV) was not shown to be affected by knockdown of endogenous PML; however, in the absence of PML these viruses were less sensitive to IFN (Chee et al., 2003; Regad and Chelbi-Alix, 2001). These studies are in support of growing evidence that implicates PML as a mediator of IFN-induced antiviral response (Everett and Chelbi-Alix, 2007; Regad and Chelbi-Alix, 2001). PML belongs to the TRIM (TRIpartite Motif) family of proteins (Jensen et al., 2001). The TRIM proteins contain RBCC motif which consists of a RING-finger, two B-boxes, and an alpha-helical Coiled-Coil domain. These proteins are emerging as important players in IFN responses and antiviral defense (Ozato et al., 2008). Furthermore, PML isoforms I-IV and VI have been shown to independently have antiviral activity (Blondel et al., 2010; Chelbi-Alix et al., 1998; Cuchet et al., 2011; Doucas and Evans, 1996; Iki et al., 2005; Pampin et al., 2006).

The ICP0-related proteins of *Alphaherpesvirinae* members are known to interact with PML bodies. The proteins encoded by HSV-1, VZV, EHV-1, PRV, and BHV-1 (ICP0, ORF61P, EICP0, EP0 and bICP0, respectively) have been observed to affect PML bodies with varying outcomes (Everett et al., 2010; Kyratsous and Silverstein, 2009; Parkinson and Everett, 2000; Reichelt et al., 2009). HSV-1 encoded ICP0 was the first viral protein reported to modify PML nuclear structures and has been the most widely studied (Everett and Chelbi-Alix, 2007; Everett and Maul, 1994; Geoffroy and Chelbi-Alix, 2011; Maul et al., 1993). ICP0 disrupts PML bodies by inducing proteasome-dependent degradation of the PML and Sp100 proteins (Chelbi-Alix and de The, 1999; Everett et al., 1998). This effect of ICP0 correlates with increased viral gene expression and replication (Everett and Maul, 1994; Everett et al., 2008; Everett et al., 2006). In addition to HSV-1 ICP0, EHV-1 and PRV encoded ICP0-like proteins are able to cause changes in the abundance of certain SUMO-modified PML isoforms (Everett et al., 2010). Conversely, VZV encoded ORF61P does not appear to significantly alter PML and Sp100 protein levels; however, depletion of PML did enhance viral replication (Kyratsous and Silverstein, 2009). There is recent evidence that endogenous PML can form nuclear cages that entrap VZV nucleocapsids in the nucleus and consequently inhibit formation of infectious viral particles (Reichelt et al., 2011). PML IV was identified as the isoform responsible for sequestering VZV nucleocapsids to these nuclear PML cages (Reichelt et al., 2011). This distinct group of PML nuclear bodies appears to safely contain aggregation-prone aberrant proteins and viral capsids and thus contributes to the intrinsic antiviral host response against VZV (Reichelt et al., 2011). Although BHV-1 encoded bICP0 is known to localize to and disorganize PML nuclear bodies,

previous studies have failed to show that bICP0 affects PML steady state levels (Everett et al., 2010; Inman et al., 2001b; Parkinson and Everett, 2000). Studies investigating the regulation of PML by bICP0 are examined in Chapter 3 of this dissertation.

FIGURE 1

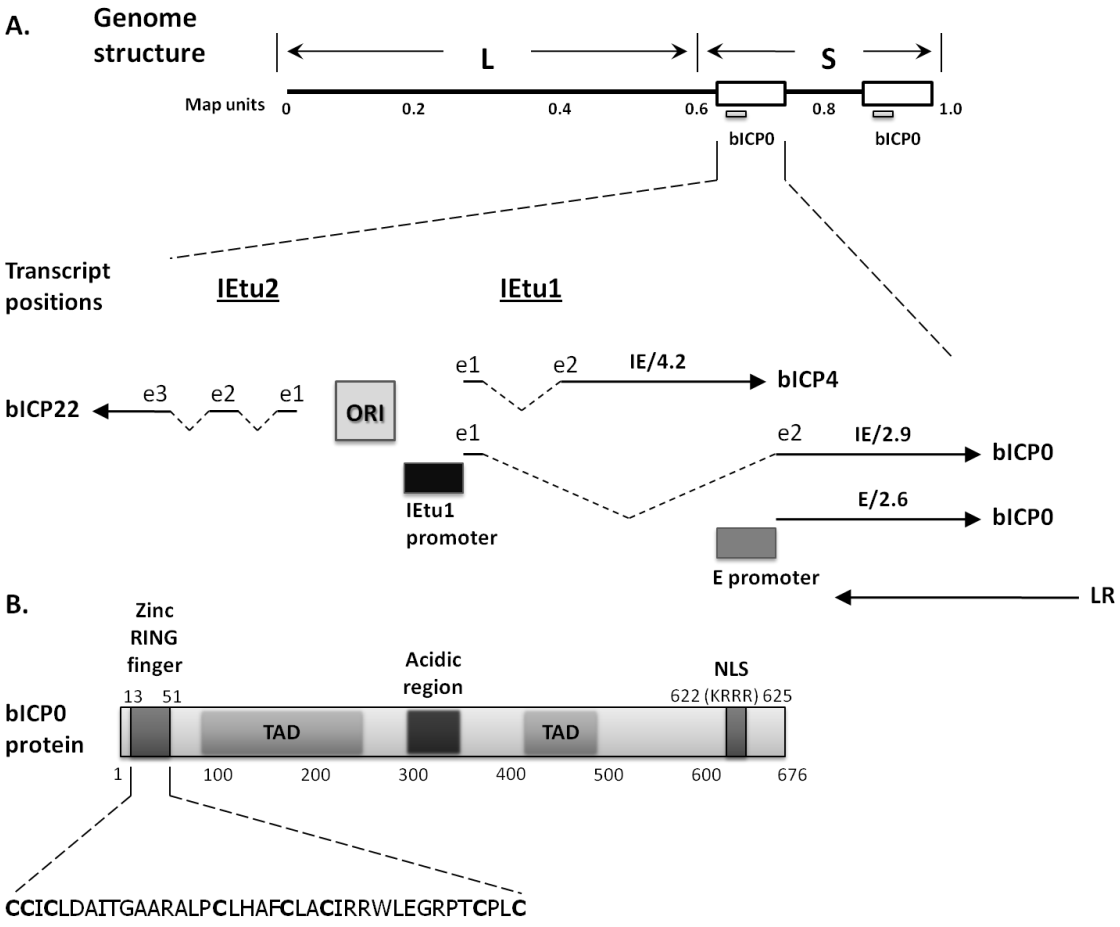

Figure 1. Map of the BHV-1 genome, location of bICP0 transcripts, and domains of the bICP0 protein.

Panel A: Schematic of the bICP0 gene within the BHV-1 genome and the position of transcripts encoded by immediate early transcription unit (IEtu1) and IEtu2. The Unique Long (L) and Unique Short (S) regions of the BHV-1 genome are indicated. IEtu1 encodes bICP4 (IE/4.2) and bICP0 (IE/2.9) and these immediate early (IE) transcripts are under the control of the IEtu1 promoter. The E/2.6 early transcript encodes bICP0 under the control of the early (E) promoter. IEtu2 encodes bICP22 in the opposite orientation to IEtu1. Exons (e) are illustrated by solid lines and the spliced intron sequences depicted by the dashed lines. The origin of replication (ORI) is denoted. The latency related (LR) transcript which partially overlaps bICP0 in the opposite orientation is also shown.

Panel B: Schematic of the bICP0 protein and its known functional domains. The functional domains include a nuclear localization signal (NLS) at the C-terminus, two transcriptional activation domains (TAD), an acidic domain, and the C3HC4 zinc RING finger at the N-terminus. The amino acid sequence (13-51) of the zinc RING finger domain is shown and the 7 highly conserved cysteine residues are highlighted in bold text.

Table 1. The affects of viral encoded proteins on PML nuclear bodies

Virus	*Viral protein*	*Affect on PML*	*References*
DNA viruses			
HSV-1	ICP0	Induces proteasome-dependent degradation of PML and disrupts PML NBs	(Everett et al., 1998; Everett and Maul, 1994; Maul et al., 1993)
KSHV	LANA-2	Disrupts PML NBs	(Marcos-Villar et al., 2009)
HCMV	IE1	Disrupts PML NBs	(Ahn and Hayward, 1997)
EBV	BZLF	Disrupts PML NBs	(Adamson and Kenney, 2001)
HPV	E6	Induces proteasome-dependent degradation of PML	(Louria-Hayon et al., 2009)
Adenovirus	E4Orf3, E1B-55 K	Disrupts PML NBs	(Doucas and Evans, 1996)
RNA viruses			
HCV	core	Interacts with PML IV and inhibits PML IV-induced apoptosis	(Herzer et al., 2005)
HDV	L-HDAg	Alters PML NB structures and distribution of associated proteins	(Bell et al., 2000)
LCMV	Z	Interacts with PML and reorganizes PML NBs	(Borden et al., 1998)
Rabies	P	Interacts with PML and reorganizes PML NBs	(Blondel et al., 2002)
EMCV	3Cpro	Induces proteasome- and SUMO-dependent degradation of PML	(El McHichi et al., 2010)

HSV-1, herpes simplex virus 1; KSHV, Karposi's sarcoma-related herpesvirus; HCMV, human cytomegalovirus; EBV, Epstein Barr virus; HPV, human papillomavirus; HCV, hepatitis C virus; HDV, hepatitis delta virus; LCMV, lymphocytic choriomeningitis virus; EMCV, encephalomyocarditis virus. {Table adapted from (Geoffroy and Chelbi-Alix, 2011)}

CHAPTER 2

The zinc RING finger domain of bICP0 regulates protein ubiquitination and stability of the bICP0 protein.

Part of the studies presented in this chapter was included in a previous publication:

Kazima Saira, Shafiqul Chowdhury, **Natasha Gaudreault**, Leticia da Silva, Gail Henderson, Alan Doster, and Clinton Jones (2008). The zinc RING finger of bovine herpesvirus 1-encoded bICP0 protein is crucial for viral replication and virulence. J Virol 82: 12060-12068.

ABSTRACT

Bovine herpesvirus 1 (BHV-1) encoded infected cell protein 0 (bICP0) contains a C_3HC_4 zinc RING (really interesting novel gene) finger motif that possesses intrinsic E3 ubiquitin ligase activity and is critical for its functions. The zinc RING finger domain is conserved among alphaherpesvirinae bICP0 homologues and is important for efficient polyubiquitination and degradation of certain cellular proteins. Studies with herpes simplex virus have demonstrated the zinc RING finger motif is also important for the stability of the ICP0 protein itself due to regulation by autoubiquitination. In this study, we examined what effect transiently expressed bICP0 had on the polyubiquitination of cellular proteins and attempted to identify novel proteins targeted by bICP0 for degradation. In addition, we examined what effect the zinc RING finger domain had on the stability of bICP0 in transiently transfected or productively infected cells. These studies provide further evidence that bICP0 is a functioning E3 ubiquitin ligase and suggest that this activity may be important for regulating bICP0 protein levels during productive infection.

INTRODUCTION

Ubiquitin-dependent proteolysis is a critical process for regulating cellular protein levels in order to maintain homeostatic cellular conditions (Hochstrasser, 1995). Ubiquitination is a post-translational modification event that generally targets proteins for degradation by the 26S proteosome (Sorokin et al., 2009). Ubiquitin-protein conjugation involves three families of enzymes. The highly conserved ubiquitin enzymes 1 (E1) and E2 are responsible for recruiting ubiquitin and catalyzing the initial steps of the pathway (Schulman and Harper, 2009; van Wijk and Timmers, 2010). The third family of enzymes in this pathway is the E3 ligases which catalyze the final step of the ubiquitin conjugation reaction. E3 ubiquitin ligases are diverse and provide the specificity by which proteins are ubiquitin modified (Nagy and Dikic, 2010; Pickart, 2001). Proteins containing a zinc RING finger motif make up the largest class of potential E3 ubiquitin ligases (Deshaies, 2005a).

BHV-1 bICP0 is a C_3HC_4 zinc RING containing multifunctional viral protein that is critical for viral replication and virulence (Saira et al., 2008). An intact zinc RING finger domain is required for many of bICP0's functions (Jones, 2009), including its ability to efficiently induce polyubiquitination and proteosome-dependent degradation of certain cellular proteins. bICP0 has been shown to directly catalyze polyubiquitination of IκBα in order to activate the NF-κB signaling pathway (Diao et al., 2005). In addition, bICP0 reduced protein levels of interferon regulatory factor 3 (IRF3) and promyelocytic leukemia protein (PML) (Gaudreault and Jones, 2011; Saira et al., 2007). Inhibition of

the proteosome interfered with bICP0 induced reduction of these proteins suggesting that proteolysis was ubiquitin mediated.

Viruses utilize ubiquitin-dependent proteolysis to influence both cellular and viral protein levels (Gao and Luo, 2006). Herpes simplex virus 1 (HSV-1) encoded infected cell protein 0 (ICP0) is an established E3 ubiquitin ligase (Boutell et al., 2002; Hagglund and Roizman, 2002; Van Sant et al., 2001) that induces the ubiquitin mediated degradation of a number of proteins (Boutell and Everett, 2003; Chelbi-Alix and de The, 1999; Everett et al., 1999a; Everett et al., 1998; Fukuyo et al., 2011; Lomonte and Morency, 2007; Lomonte et al., 2001; Parkinson et al., 1999). It is known that HSV-1 ICP0 also regulates its own expression by self-ubiquitination, and that zinc RING finger mutants are more stable due to their disabled E3 ligase activity (Boutell et al., 2002; Canning et al., 2004; Everett et al., 1995). The C_3HC_4 zinc RING finger is a conserved functional domain among *Alphaherpesvirinae* subfamily member ICP0 homologues, including BHV-1 encoded bICP0. Consequently, we hypothesize that bICP0 functions as an E3 ubiquitin ligase to induce ubiquitination of proteins and that it may also regulate its own expression levels by autoubiquitination.

bICP0 is a multifunctional protein that plays a pivotal role in the BHV-1 infectious cycle (Jones, 2009). Identifying novel ubiquitinated proteins targeted for degradation by bICP0 would provide insight to the functions and the underlying mechanisms of bICP0 during productive infection. We therefore compared the global level of ubiquitinated proteins in cells expressing wt bICP0 or a zinc RING finger mutant, and examined the protein stability of bICP0 in cells infected with a wt BHV-1

strain or a zinc RING finger mutant virus. We predicted that mutation of the bICP0 zinc RING finger domain would hinder its E3 ligase activity, and as a result affect its ability to induce ubiquitination of proteins and to regulate its own half-life. This study demonstrates that the zinc RING finger domain plays an important role in bICP0's ability to induce protein ubiquitination, and as a result influences its own stability.

MATERIALS AND METHODS

Cells and viruses

Bovine kidney (CRIB) cells and murine neuro-2A cells were grown in Earle's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin (10 U/ml) and streptomycin (100 ug/ml) in a humidified 5% CO2 chamber at 37 C. CRIB cells, derived from MDBK cells, are resistant to infection with bovine viral diarrhea virus and were obtained from Ruben Donis (Centers for Disease Control and Prevention, Atlanta, GA).

The wt BHV-1 Cooper strain was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services (Ames, IA). Construction of the BHV-1 bICP0 zinc RING finger domain mutant (51g) and the rescue virus (51gR) was described previously (Saira et al., 2008). BHV-1 strains were propagated and titrated in CRIB cells. For infection studies, cells were plated onto 60 or 100 mm culture dishes 16 to 24 hours prior to virus infection. Total cells per plate were counted and the multiplicity of infection (MOI) calculated. Cells were infected with the wt BHV-1, 51g, or 51gR at an MOI of 1.0, and were harvested at the indicated times after infection.

Expression plasmids

The wild type and mutated bICP0 plasmids are cloned into a pCMV2 expression vector downstream of a Flag tag and under the control of the human cytomegalovirus (CMV) promoter. The zinc RING finger mutant 13G/51A contains point mutations within two conserved amino acids of the C3CH4 zinc RING finger (Inman et al., 2001b). The ubiquitin plasmid contains a hemagglutinin tag (HA-Ub). For transfections, TransitIT-LT1 reagent (MIR 2300, Mirus) was used according to the manufacturer's protocol. Empty vector was used as a fill to ensure equal amounts of total DNA were introduced into the cells.

Western blot analysis

Cells were harvested at the indicated times after infection. Cells were washed with PBS and suspended in cell lysis buffer (100 mM Tris, pH 8.0, 1mM EDTA, 100 mM Na Cl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride) and one tablet of complete protease inhibitor (Roche Molecular Biochemicals) per 10 ml of buffer. The cell suspension was sonicated and incubated at 4°C for 20 min, then centrifuged at 13,000 rpm at 4°C for 15 min. Protein concentrations were then determined by the Bradford assay (Bio-Rad), and the lysate was boiled in SDS-PAGE buffer. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Membranes were blocked in Tris-buffered saline that contained 5% milk. The ubiquitin (sc-9133), HA (sc-805), and beta-actin (sc-1616) antibodies were

purchased from Santa Cruz Biotechnology. Expression of the bICP0 protein was detected using a polyclonal anti-bICP0 peptide antibody that was produced in rabbits. Membranes were then incubated overnight with the indicated primary antibody in 5% milk-containing 0.1% Tween 20–Tris-buffered saline. After washing with 0.1% Tween 20–Tris-buffered saline, membranes were incubated with donkey anti-goat (sc-2020; Santa Cruz Biotechnology) or donkey anti-rabbit (NA934V, Amersham Biosciences) horseradish peroxidase-conjugated immunoglobulin G secondary antibodies diluted in blocking buffer. Immunodetection was performed with enhanced chemiluminescence western blotting detection reagents (Perkin-Elmer, MA) in accordance with the manufacturer's protocol. All figures are representative of three or more independent experiments.

Ubiquitin Enrichment Assay

For several experiments we used the Ubiquitin Enrichment Kit (Pierce 89899) to isolate intracellular polyubiquitin-modified proteins through the use of a high-binding affinity resin provided by the kit. Cell extracts were collected as described, protein concentrations measured and equal amounts used for ubiquitin enrichment according to the manufacturer's protocol. Extracts collected after enrichment were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis.

RESULTS

bICP0 induces ubiquitination of cellular proteins.

The C3HC4 zinc RING finger domain located in bICP0 possesses intrinsic E3 ubiquitin ligase activity (Diao et al., 2005), and previous studies have demonstrated bICP0 is able to induce the proteasome-dependent degradation of specific proteins (Gaudreault and Jones, 2011; Saira et al., 2007). We therefore attempted to identify novel proteins targeted for polyubiquitination and consequently proteolysis by bICP0. Ubiquitination of cellular proteins was examined following co-transfection of 2A cells with a hemagglutinin-tagged ubiquitin (HA-Ub) plasmid and bICP0, 13G51A, or empty expression vector. Cells were collected and lysed 48 hours post-transfection and analyzed by western blot with anti-HA antibody. As expected, over-expression of ubiquitin resulted in a ladder of ubiquitinated proteins that resemble a smear (Figure 1). Increased accumulation of ubiquitinated proteins in cells expressing bICP0 was consistently observed (Figure 1, lane 2) suggesting bICP0 was a functionally active E3 ubiquitin ligase that increased protein ubiquitination. However, we were unable to consistently detect unique ubiquitinated bands in cells transfected with wt bICP0. A bICP0 mutant containing substitutions of two critical cysteine residues of the zinc RING finger domain (13g51a) was also tested. Cells expressing the 13g51a mutant consistently produced lower levels of ubiquitinated proteins than wt bICP0, and resulted in similar or less levels than empty vector expressing cells (Figure 1, lane 3). In summary, these

studies suggest bICP0 was able to induce the ubiquitination of cellular proteins and that an intact zinc RING finger domain is important for this activity.

Analysis of ubiquitinated proteins during productive infection.

Ubiquitinated protein levels were examined in cells that were productively infected with wt BHV-1 or the 51g mutant virus. The 51g mutant contains a single amino acid substitution that results in compromised growth in cultured cells, and reduces virulence in cattle (Saira et al., 2008). Because this mutation is a disruption of the bICP0 zinc RING finger domain, we predicted that the E3 ubiquitin ligase activity would also be impaired and ubiquitinated protein levels would be reduced compared to wt BHV-1 infected cell levels. CRIB cells were infected for 1 hour at 1 or 5 MOI. The media was then replaced and lactacystin added to prevent proteosome-dependent degradation of proteins. Cellular extracts were collected and ubiquitin levels analyzed by western blot 8 hours post infection. We were unable to consistently detect a significant difference between BHV-1 infected cells and mock (Figure 2, A), or the 51g mutant (Figure 2, B).

Polyubiquitination of IRF3 induced by bICP0.

Expression of bICP0 has been shown to reduce IRF3 protein levels in transiently transfected cells in a proteasome-dependent manner (Saira et al., 2007). We therefore attempted to demonstrate polyubiquitination of IRF3 induced by bICP0 in transfected cultured cells. In order to purify and evaluate only ubiquitin modified proteins we used

an ubiquitin enrichment kit purchased from Pierce which uses a high-affinity resin to isolate polyubiquitinated proteins from cell extracts. Neuro-2A cells transfected with bICP0, IRF3, and/or HA-Ub were collected and analyzed by western blot with IRF3 or ubiquitin antibodies. Cells were treated with lactacystin prior to collection in order to prevent proteasome-dependent degradation of proteins. After ubiquitin enrichment, slightly higher levels of ubiquitinated IRF3 were detected in cells that expressed bICP0 (Figure 3). Polyubiquitinated proteins have an added mass of at least 32 kDa compared to the mass of a non-modified protein of interest. Consistent with the above results, an increased accumulation of ubiquitinated proteins was observed in cells expressing bICP0 following ubiquitin enrichment (data not shown). These experiments provide further evidence that bICP0 is a functional E3 ubiquitin ligase.

bICP0 protein levels during productive infection.

bICP0 expression was examined following infection of CRIB cells with the 51g mutant or 51gR at an MOI of 1. Cell lysate was collected at different times after infection, and Western analysis was performed with an anti-bICP0 peptide antibody. Higher levels of bICP0 protein expression were consistently observed following infection of bovine cells with the 51g mutant than following infection with 51gR or wt BHV-1 (Figure 4 and data not shown). Previous studies demonstrated that HSV-1 ICP0 mutants with reduced E3 ubiquitin ligase activity have increased stability as a result of decreased autoubiquitination (Boutell et al., 2005; Canning et al., 2004). We predicted that the cysteine-to-glycine mutation in the 51st amino acid of bICP0 disrupted the putative E3

ubiquitin ligase activity of bICP0 and, consequently, that the 51g mutant version of bICP0 would have a longer half-life. To test this prediction, CRIB cells were infected with wt BHV-1, the 51gR strain, or the 51g mutant, and at 6 h after infection, cells were treated with 100 ug/ml cycloheximide treatment. The bICP0 protein was readily detected after treatment with cycloheximide when cells were infected with the 51g mutant. Conversely, bICP0 was not readily detected in cells infected with wt BHV-1 or the 51gR strain after treatment with cycloheximide. As expected, the bICP0-specifec antiserum did not recognize a protein in mock-infected cells. Similar levels of beta-actin were present in each lane, confirming that similar protein levels were loaded for each sample (Figure 4, bottom). In summary, these studies suggested that the 51g mutant bICP0 had increased stability during productive infection and was consequently expressed at higher levels.

DISCUSSION

In this study, we provide evidence that bICP0 is an active E3 ubiquitin ligase, which is able to induce ubiquitination of proteins and regulate its own expression levels by self-ubiquitination. We also attempted to demonstrate the ubiquitination of IRF3 (Figure 3), a known protein targeted for degradation by bICP0 (Saira et al., 2007). In cells expressing bICP0, we consistently observed slightly higher levels of ubiquitinated proteins (Figure 1). More specifically, we also observed an increase in polyubiquitinated IRF3 in cells expressing bICP0. Compared to transient transfection, productively infected cells expressing bICP0 generally appeared to have less of a significant effect on ubiquitinated protein levels (Figure 2). This may be attributed to the expression level and half-life of bICP0 which is more abundant and stable in the absence of other viral proteins, suggesting bICP0 levels are regulated by other viral encoded or induced factors (Gaudreault and Jones, 2011). Furthermore, we were not able to identify novel ubiquitinated targets in cells infected with BHV-1 or expressing bICP0.

Transient-transfection assays and cell-free assays have indicated that the bICP0 protein possesses E3 ubiquitin ligase activity which can induce ubiquitin dependent proteolysis of proteins and that the zinc RING finger is crucial for this activity (Diao et al., 2005). The 51g mutation is predicted to disrupt the structure of the zinc RING finger and the putative E3 ubiquitin ligase activity of bICP0 because mutation of a single amino acid within HSV-1 ICP0 disrupts the secondary and tertiary structures of the zinc RING finger (Everett et al., 1995). This conformational disruption interferes with many of the functions attributed to bICP0 including its ability to activate productive infection (Inman

et al., 2001b; Saira et al., 2008). As we predicted, levels of ubiquitinated proteins were reduced in cells expressing a bICP0 zinc RING finger mutant compared to wt bICP0. Furthermore, during transient transfection or productive infection (Figure 3), high levels of bICP0 were detected in cells expressing bICP0 proteins containing zinc RING finger mutants (Inman et al., 2001b; Zhang et al., 2005). It is well established that HSV-1 ICP0 zinc RING finger mutants have increased half-lives because they cannot induce their own ubiquitination (Boutell et al., 2002; Canning et al., 2004; Everett et al., 1995). Consequently, we suggest that in addition to other viral encoded or induced factors, bICP0 also regulates its own half-life by self-ubiquitination. Additional experiments are required to confirm this hypothesis.

FIGURE 1

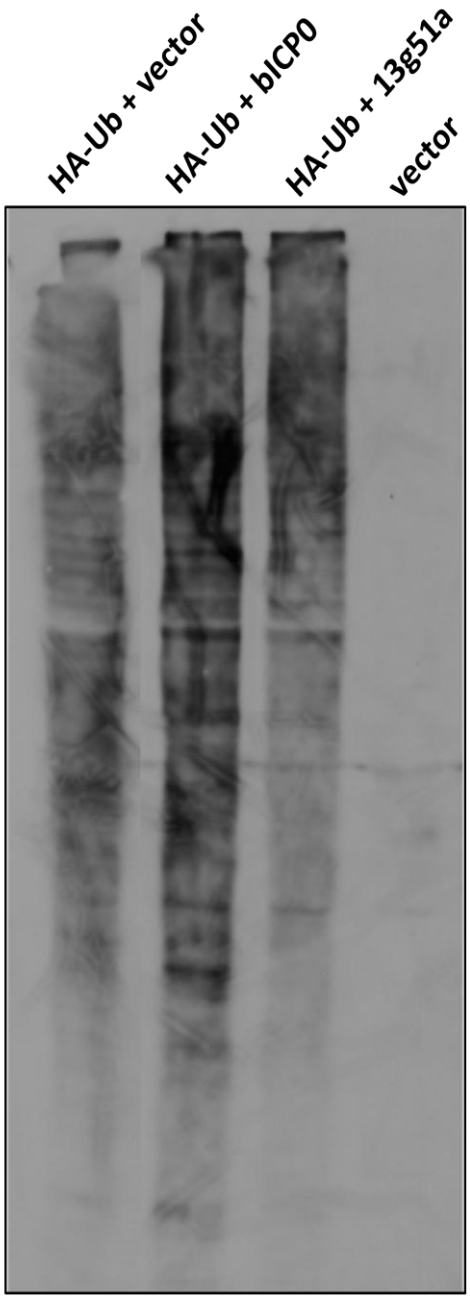

Figure 1. Ubiquitinated proteins from cells expressing bICP0. Neuro-2A cells were co-transfected with HA-tagged ubiquitin (HA-Ub) and bICP0, 13g51a, or empty expression vector. The 13g51a contains single substitution mutations of two conserved cysteine residues within the zinc RING finger domain of bICP0. Whole cell extracts were collected 48 h after transfection and subjected to western blot analysis. Ubiquitin levels were monitored using an HA antibody.

FIGURE 2

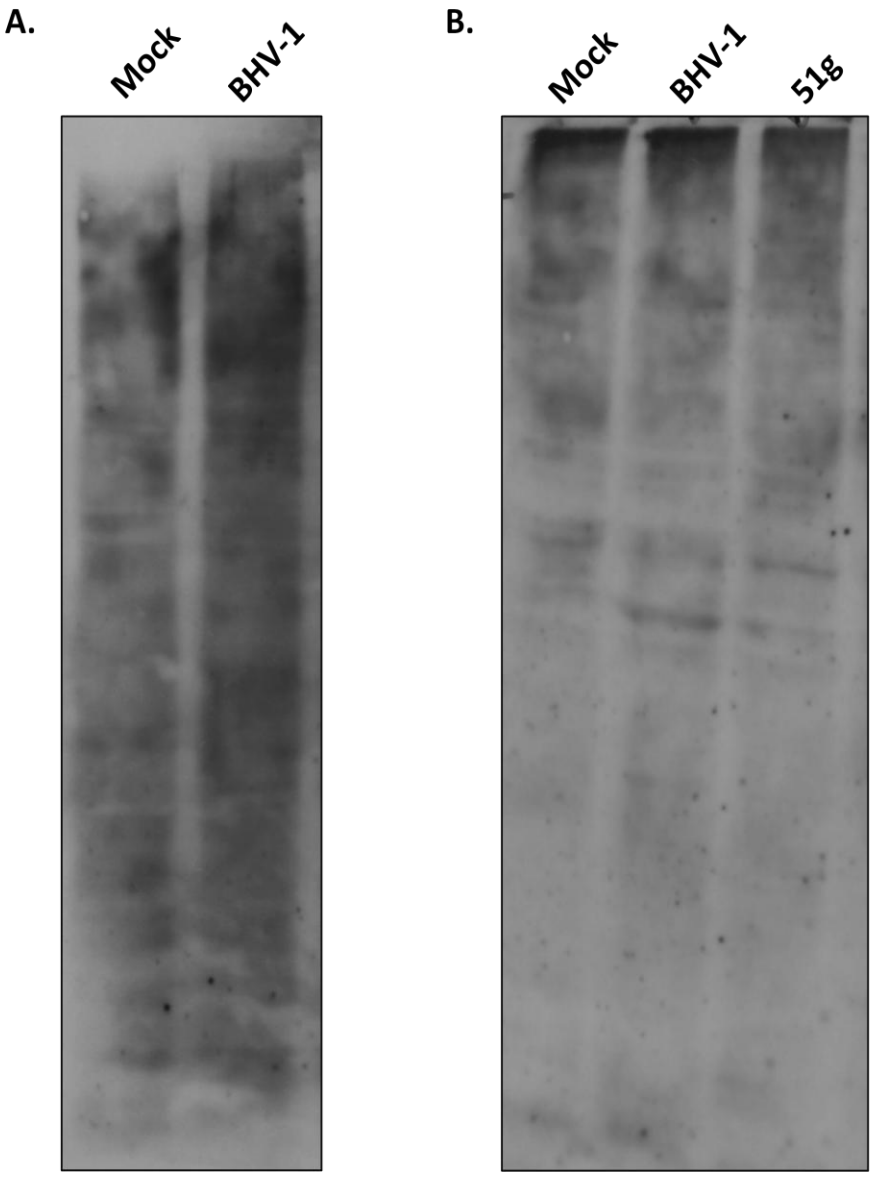

Figure 2. Ubiquitinated proteins from productively infected cells. CRIB cells were mock infected, or infected with wt BHV-1 or 51g mutant at 1 MOI (**A**) or 5 MOI (**B**). After 1 h, media was replaced with media containing 0.015mM of the proteasome inhibitor lactacystin to prevent degradation of proteins. Whole cell extracts were collected at 8 h post infection and analyzed by western blot. An antibody recognizing full length ubiquitin was used to monitor ubiquitinated protein levels.

FIGURE 3

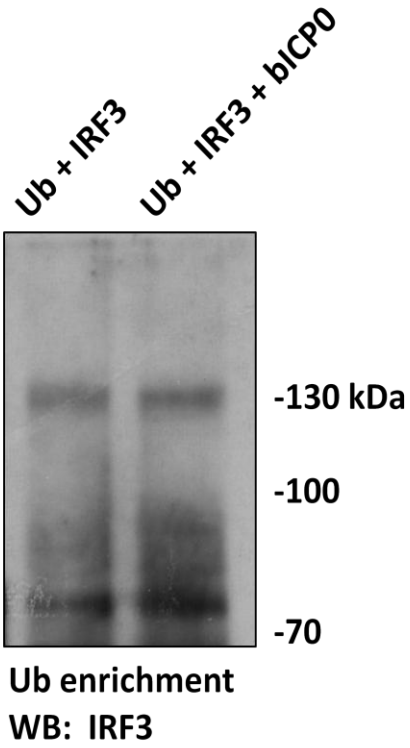

Figure 3. Polyubiquitinated IRF in cells expressing bICP0. Neuro-2A cells were co-transfected with HA-Ub, IRF3, and bICP0 or empty expression vector. Cells were treated with lactacystin prior to collection to prevent proteasome-dependent proteolysis. Whole cell extracts were collected at 48 h after transfection and there protein concentrations measured by Bradford assay. Equal amounts of each sample were used for ubiquitin enrichment, a high-affinity resin system that isolates polyubiquitinated proteins from cell extracts (Pierce). Ubiquitin (data no shown) and IRF3 proteins were detected using a ubiquitin or IRF3 antibodies, respectively. The IRF3 protein migrates at approximately 50 kilo Daltons (kDa). Ubiquitin migrates at approximately 8 kDa, and polyubiquitinated proteins have an added mass of at least 32 kDa.

FIGURE 4

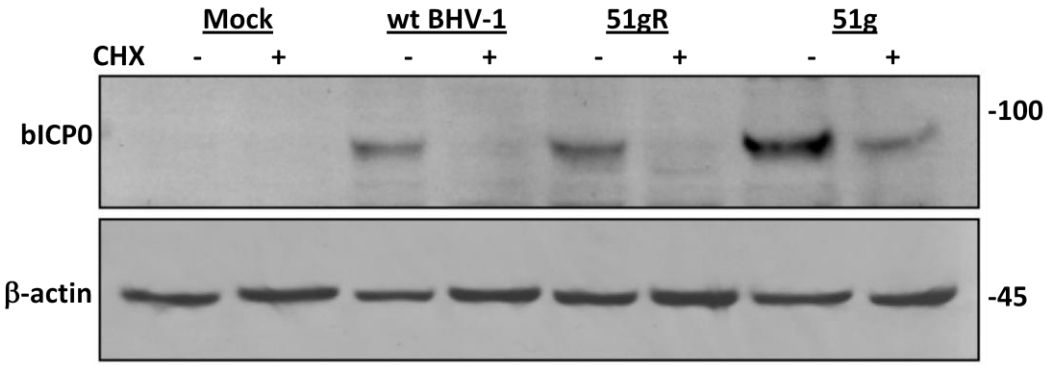

Figure 4. bICP0 protein levels during productive infection. CRIB cells were mock infected, or infected with wt BHV-1, the 51g mutant, the 51g rescued virus (51gR) at an MOI of 1. At 6 h after infection, some cultures were treated with 0.1 mg/ml cycloheximide (Sigma) (CHX; + lanes) to inhibit protein synthesis. Two hours later (8 h after infection), whole cell extracts were collected and subjected to western blot analysis. bICP0 levels were detected using bICP0-specific polyclonal antibodies raised in rabbits. The bICP0 protein migrates at approximately 97 kDa, and beta actin migrates at approximately 42 kDa (marked at right).

CHAPTER 3

Regulation of promyelocytic leukemia (PML) protein levels and cell morphology by bovine herpesvirus 1 infected cell protein 0 (bICP0) and mutant bICP0 proteins that do not localize to the nucleus.

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ABSTRACT

BHV-1 is an important pathogen of cattle. The infected cell protein 0 (bICP0) encoded by BHV-1 is an important regulatory protein because it is constitutively expressed and can activate all viral promoters. The mechanism by which bICP0 activates viral promoters is not well understood because bICP0 does not appear to be a sequence specific binding protein. A C_3HC_4 zinc RING (really interesting novel gene) motif at the N-terminus of bICP0 has E3 ubiquitin ligase activity, which is important for activating viral gene expression and inhibiting interferon dependent transcription. Like other *Alphaherpesvirinae* ICP0 homologues, bICP0 is associated with promyelocytic leukemia (PML) protein-containing nuclear domains. During productive infection of cultured cells, BHV-1 induces degradation of the PML protein, which correlates with efficient productive infection. In this study, we demonstrated that a plasmid expressing bICP0 reduces steady state levels of the PML protein, and the C_3HC_4 zinc RING finger is important for PML degradation. Surprisingly, bICP0 mutants with an intact C_3HC_4 zinc RING finger that lack a nuclear localization signal also reduces steady PML protein levels. In addition, mutant bICP0 proteins that primarily localize to the cytoplasm induced morphological changes in transfected cells. During productive infection, bICP0 was detected in the cytoplasm of low-passage bovine kidney, but not established bovine kidney cells. These studies demonstrated that bICP0, even when not able to efficiently localize to the nucleus, was able to induce degradation of the PML protein and alter the morphology of transfected cells.

INTRODUCTION

BHV-1 infections can lead to conjunctivitis, pneumonia, genital disorders, abortions, and an upper respiratory infection known as bovine respiratory disease (BRD) or "Shipping Fever" (Saira et al., 2008; Tikoo et al., 1995). BHV-1 can initiate BRD by immunosuppressing cattle (Carter et al., 1989; Griebel et al., 1987a; Griebel et al., 1990; Griebel et al., 1987c; Yates, 1982), which leads to secondary bacterial infections and life-threatening pneumonia (Yates, 1982). BRD costs the cattle industry more than $1 billion/year in the United States ((NASS), 1996; Bowland and Shewen, 2000; Carter et al., 1989; Griebel et al., 1987a; Griebel et al., 1990; Griebel et al., 1987c; Ishmael, 2001; Kapil, 1997; Powell, 2005; Tikoo et al., 1995). Modified live vaccines are available, and in general, they prevent clinical disease in adults. However, vaccine strains are immunosuppressive, can cause serious disease in young calves or abortions in pregnant cows, and can reactivate from latency.

Infection of permissive cells (Devireddy and Jones, 1999b) or calves (Winkler et al., 1999) leads to rapid cell death, in part due to apoptosis. Viral gene expression is temporally regulated in 3 distinct phases: immediate early (IE), early (E), or late (L). IE gene expression is stimulated by a virion component, bTIF, which interacts with a cellular transcription factor (Oct-1) and stimulates transcription {reviewed in (Jones, 1998; Jones, 2003)}. Two IE transcription units exist: IEtu1 and IEtu2. IEtu1 encodes homologues of two HSV-1 proteins, ICP0 and ICP4. IEtu2 encodes a protein similar to the HSV ICP22 protein. bICP0 is crucial for productive infection because it activates all viral promoters (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1991). The bICP0 E

promoter is activated by dexamethasone (Workman et al., 2009), a stimulus that induces reactivation from latency suggesting activation of the bICP0 E promoter is an important step during reactivation from latency {reviewed in (Jones, 1998; Jones, 2003)}.

bICP0 (Inman et al., 2001b), HSV-1 encoded ICP0 (Everett et al., 1995; Everett and Maul, 1994; Lium and Silverstein, 1997; Parkinson and Everett, 2000), and equine herpesvirus 1 encoded ICP0 (Bowles et al., 1997; Bowles et al., 2000) contain a C_3HC_4 zinc RING finger near their N terminus that activates productive infection. ICP0 (Everett and Maul, 1994; Maul et al., 1993) and bICP0 (Inman et al., 2001b; Parkinson and Everett, 2000) localize with and disrupt promyelocytic leukemia (PML) protein-containing nuclear domains. The C_3HC_4 zinc RING finger domains located in bICP0 (Diao et al., 2005) and ICP0 (Boutell and Everett, 2003; Boutell et al., 2002; Van Sant et al., 2001) possess intrinsic E3 ubiquitin ligase activity. Thus, bICP0 and ICP0 can induce ubiquitin dependent proteolysis of specific proteins (Everett and Maul, 1994; Parkinson et al., 1999). A single point mutation within the C_3HC_4 zinc RING finger of bICP0 reduced the growth potential of BHV-1 in cultured cells and this recombinant virus has reduced virulence in cattle (Saira et al., 2008). bICP0 also contains two transcriptional activation domains and a nuclear localization signal (NLS) located near its C-terminus (Zhang, 2005). Deletion of sequences encompassing the NLS reduces, but does not eliminate, the ability of bICP0 to trans-activate a simple promoter (Saira et al., 2008; Saira et al., 2009; Zhang, 2005).

In this study, we examined the association of bICP0 with PML bodies during productive infection, and in transfected cells. As expected, bICP0 reduced PML levels.

Surprisingly, bICP0 mutant proteins that do not localize to the nucleus also reduced PML levels in transiently transfected cells, and had a profound effect on the morphology of transfected cells. Since bICP0 was detected in the cytoplasm of infected cells during productive infection, we suggest that cytoplasmic localization of bICP0 is important for certain functions ascribed to bICP0.

MATERIALS AND METHODS

Cells and viruses

Rabbit skin (RS) cells, and bovine kidney (CRIB) cells were grown in monolayer cultures in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (10 U/ml), and streptomycin (100 mg/ml) in a humidified 5% CO_2 atmosphere at 37°C. Low passage bovine kidney cells were prepared from a healthy calf that was 7 months old.

The BHV-1 wt Cooper strain was propagated and titrated in CRIB cells. For infection studies, cells were plated onto 60 or 100 mm culture dishes 16 to 24 hours prior to virus infection. Total cells per plate were counted and the multiplicity of infection (MOI) calculated.

Expression plasmids

The wild type and mutated bICP0 plasmids are cloned into a pCMV2C expression vector downstream of a Flag tag and under the control of the human cytomegalovirus (CMV) promoter. The zinc RING finger mutant 13G/51A contains point mutations within two conserved amino acids of the C_3HC_4 zinc RING finger (Inman et al., 2001b). bICP0 C-terminus deletion mutants (ΔC-terminus and ΔNcoI) were generated by deleting the SalI-XhoI fragment (amino acids 356 to 676) and NcoI-XhoI fragment (amino acids

607 to 676), respectively, from the Flag-tagged bICP0 construct (Inman et al., 2001b). The hemagglutinin (HA) tagged mouse PML plasmid encodes the equivalent of the human PML isoform I and was provided by Dr. Paul Ling (Baylor College of Medicine, Houston, Texas) (Plummer et al., 1969). The HA-tag is at the C-terminus of the PML cDNA. Generation of the bICP0 transposon insertion mutants were previously described (Zhang et al., 2005). The transposon insertion sites were first mapped by restriction endonuclease digestion, and then precise insertion sites were identified by DNA sequencing.

Western blot analysis

CRIB or RS cells were plated in 60 mm culture dishes 16 to 24 hours prior to infection or transfection, respectively. Cells were infected at the specified MOI and transfected using TransIT-LT1 (MIR 2300, Mirus) or Lipofectamine 2000 (11668, Invitrogen) reagents according to the manufacturer's protocol. At the indicated times, cells were washed with PBS and suspended in RIPA lysis buffer (1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride in PBS, and one tablet of complete protease inhibitor (Roche Molecular Biochemicals) per 10 ml). Cell lysate was incubated at 4°C with rotation for 25 minutes, sonicated briefly, and then clarified by centrifugation at 13,000 rpm at 4°C for 15 minutes. Protein concentrations were quantified by the Bradford assay, and SDS polyacrylamide gel electrophoresis performed. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore).

Membranes were blocked in Tris-buffered saline that contained 5% milk. The following antibodies were purchased from Santa Cruz Biotechnology: PML (sc-5621), HA (sc-805, sc-7392), or β-actin (sc-1616). bICP0 was detected using a specific bICP0 polyclonal antibody or monoclonal anti-Flag (Sigma, F1804). Membranes were then incubated overnight with the indicated primary antibody in 5% milk-containing 0.1% Tween 20–Tris-buffered saline. After washing with 0.1% Tween 20–Tris-buffered saline, membranes were incubated with donkey anti-goat (sc-2020; Santa Cruz Biotechnology), donkey anti-rabbit (NA934V, Amersham Biosciences), or sheep anti-mouse (NXA931, Amersham Biosciences) horseradish peroxidase-conjugated immunoglobulin G secondary antibodies diluted in blocking buffer. Immunodetection was performed with enhanced chemiluminescence western blotting detection reagents (Perkin-Elmer, MA) in accordance with the manufacturer's protocol. All figures are representative of three or more independent experiments.

Immunofluorescence

RS cells were plated in 60 mm culture dishes containing a sterilized cover slip at 16 hours prior to transfection. Cells were transfected as described above. For infection studies, RS cells were infected with BHV-1 at 1 or 5 MOI 24 hours after transfection, and processed for confocal microscopy at the indicated times. Cells were washed twice with plain MEM and fixed with 4% para-formaldehyde for 10 minutes, followed by three washes with PBS. Cells were permeabilized by incubating with 100% ethanol (-20°C) for 5 minutes. Slides were then washed three times and blocked in 3% BSA in PBS for 1

hour. The designated primary antibody was incubated for 2 hours at room temperature. After three washes with 0.05% Tween 20 in PBS, slides were incubated with the secondary antibody for 1 hour in the dark. Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit (A21050), Alexa Fluor 633 goat anti-mouse (A11008), and Alexa Fluor 647 donkey anti-rabbit (A31573) from Invitrogen. After slides were washed three times with 0.05% Tween PBS, cover slips were mounted on slides using Gel-mount aqueous mounting medium (G0918, Sigma). Images were obtained with a Bio-Rad confocal laser-scanning microscope (MRC-1024ES) with excitation/emission at 488/520 nm. Images are representative of three or more independent experiments.

Coimmunoprecipitation

Cell lysates were pre-cleared by incubating with rabbit serum (0.05 ml per 1 ml) for 1 hour on ice, followed by addition of washed protein A beads (0.05 ml slurry per sample) and incubated with rotation at 4°C for an additional 30 minutes. Beads were pelleted and the supernatants collected for immunoprecipitation. Samples were incubated with approximately 2 μg bICP0 or HA polyclonal antibodies per 0.5 mg of protein overnight with rotation at 4°C. PML (sc-5621) and HA (sc-805) polyclonal antibodies were purchased from Santa Cruz Biotechnology. To each sample 0.1 ml of protein A agarose bead slurry was added and incubated at 4°C with rotation for 4 hours. Beads were washed three times with lysis buffer and the protein eluted by adding 0.05 ml 2X loading buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, 0.1% Bromophenol Blue, 10% Glycerol) and boiling samples for 5 min.

Proteins were separated on an 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Extraction of viral genomic DNA for b-galactosidase assay

The BHV-1 gC blue virus has the Lac Z gene inserted downstream of the gC promoter, a late viral gene. CRIB cells were infected with the BHV-1 gC blue virus for 1 h, rinsed with PBS, and replaced with fresh medium. Cells with medium were collected after approximately 80% visible cytopathic effect, followed by three freeze/thaw cycles at -80 C. The supernatant was clarified by centrifugation (3600 rpm 4 °C, 30 min). Virus was pelleted using 5ml 30% sucrose cushion to 25ml virus, by centrifugation (25000 rpm for 3 h in a Beckman L7-65 ultracentrifuge using an SW28 rotor at 4 °C). The pellet was suspended in 900 ml of DNase I-free TE. The virions were disrupted by adding 50ml 20% SDS, 7.5ml of 1mg/ml RNase, and incubating the solution at 37 °C for 20 min. Proteinase K was added (50ml of a 10 mg/ml solution) and the solution was incubated at 60 °C for 30 min. Three phenol:Chloroform:isoamyl alcohol (25:24:1) extractions were performed, followed by two extraction with Chloroform:isoamyl alcohol (24:1). The resulting aqueous layer was subjected to two extractions with ether. DNA was precipitated with 2.2 volumes 100% ethanol and sodium acetate (0.3M) at -80 °C for 1 h. Viral DNA was pelleted by centrifuging13000 rpm for 15 min at rm temp and washed with 80% ethanol. After final spin at 1300 rmp for 5 min at rm temp, pellet was resuspended in 100ml TE and quantified using Bio-Rad SmartSpec 3000.

Beta-galactosidase assay

A similar protocol was followed as previously described. RS cells were split to 6-well culture plates and 16 h later transfected with a total of 2 ug DNA using Lipofectamine 2000 1:1 (11668, Invitrogen). BHV-1 gC blue viral DNA (1 ug) was cotransfected with bICP0 at a 64:1 ratio (mg BHV-1 DNA to mg bICP0 plasmid DNA), and specified ratios of HA-PML plasmid. Blank expression vector was used to maintain equivalent amounts of DNA. After 24 h transfection, cells were rinsed with PBS and fixed with 2% formaldehyde, 0.2% glutaraldehyde in PBS for 5 min. Cells were washed twice with PBS and stained overnight at 4°C with 0.1% Bluo-Gal (15519-028, Invitrogen), 5 mM K3FE(CN)6, 5 mM K4FE(CN)6, 2 mM MgCl2 in PBS. Cells were rinsed with PBS and number of blue cells counted.

RESULTS

BHV-1 infection affects endogenous PML levels.

BHV-1 productive infection disrupts and degrades PML nuclear body-associated proteins in a bICP0 dependent fashion (Inman et al., 2001b; Parkinson and Everett, 2000). To confirm and expand these findings, we initially examined the effect of BHV-1 productive infection on cellular PML protein levels in bovine kidney cells (CRIB). CRIB cells infected with BHV-1 at an MOI of 5 were collected at the designated times after infection and whole cell lysate was subjected to western blot analysis. Several high molecular weight bands were recognized by the PML antiserum, which was expected because PML undergoes extensive post-translational modifications (Everett and Maul, 1994). By 8 hours after infection, endogenous PML protein levels were reduced in BHV-1 infected cells compared to mock infected cells (Figure 1A). Sumoylation of PML targets it for proteasome-dependent degradation (Lallemand-Breienbach, 2001), and ICP0 preferentially degrades PML that is sumoylated (Boutell and Everett, 2003; Everett et al., 1998; Parkinson et al., 1999). Treatment of cultures with lactacystin, a proteasome inhibitor, partially restored PML levels in infected bovine cells (Figure 1B). Longer exposure of cells to lactacystin was not possible in CRIB cells because of toxicity complications (data not shown). Cellular PML protein levels were also analyzed by confocal microscopy at the designated times after infection. Reduced endogenous PML levels were observed in rabbit skin (RS) cells productively infected with BHV-1 (Figure 1C). These studies confirmed that BHV-1 infection reduced PML protein levels during

productive infection, and demonstrated that a functional proteasome played a role in regulating PML protein levels.

bICP0 induced proteasome-dependent degradation of PML.

To test whether bICP0 had an effect on PML protein levels, transient transfection assays were performed. RS cells were used for these studies because they are permissive for BHV-1 and approximately 50% of RS cells can be transfected. Cellular PML levels in RS cells transiently expressing bICP0 were analyzed by western blot and confocal microscopy (Figure 2A, B). Transient expression of bICP0 consistently had a modest effect on endogenous PML levels in rabbit skin (RS) cells (Figure 2A) or mouse neuroblastoma cells (data not shown). However, the effect was not as dramatic as in observed in productively infected cells (Figure 1). Consequently, we cotransfected RS cells with a plasmid that expresses HA-tagged PML and bICP0, and then examined PML levels using an anti-HA antibody. The HA-tagged PML expression vector contains a mouse cDNA that encodes the equivalent of human PML isoform I (Plummer et al., 1969). Whole cell lysate was collected 48 hours after transfection and subjected to western blot analysis. bICP0 consistently reduced HA-PML levels compared to cells transfected with the empty expression vector (Figure 2C). bICP0 is an E3 ubiquitin ligase that targets certain proteins for proteasome-dependent degradation (Diao et al., 2005). To test whether the proteasome was necessary for PML degradation, RS cells co-expressing HA-PML and bICP0 were treated with a proteasome inhibitor, lactacystin. Lactacystin treatment of cells transfected with the bICP0 expression plasmid has

consistently increased HA-PML levels (Figure 2C, lane 4). In summary, these results indicated that bICP0, in the absence of other viral proteins, induced proteasome-dependent PML degradation.

To determine if a direct interaction could be detected between bICP0 and PML, coimmunoprecipitation and western blot analysis were performed. RS cells were cotransfected with bICP0 or HA-PML expression plasmids. HA-PML was detected by western blot with the HA antibody following immunoprecipitation with the HA antibody (Figure 2D, lanes 3 and 4), as expected. However, we did not detect HA-PML from bICP0 immunoprecipitates of cells cotransfected with bICP0 and HA-PML (Figure 2D, lane 2). Thus, we were unable to provide evidence of a direct interaction between bICP0 and PML.

Localization of bICP0 to the nucleus was not required for PML degradation.

The effect of three bICP0 mutants on PML protein levels was analyzed by western blot analysis. The 13G/51A mutant contains substitutions of two conserved cysteine residues within the C_3HC_4 zinc RING finger of bICP0 (Inman et al., 2001b) (Figure 3A). The zinc RING finger is a critical functional domain of bICP0 that controls many of bICP0 functions (Saira et al., 2009). The ΔC-terminus and ΔNcoI mutants do not contain aa sequences spanning the bICP0 NLS (Zhang, 2005). We predicted that disruption of the zinc RING finger domain and deletion of the NLS would interfere with the ability of bICP0 to reduce PML protein levels. The 13G/51A mutant reduced HA-PML levels less efficiently than wild type bICP0 (Figure 3B). Other domains of bICP0

may also be important for inducing PML degradation because the 13G/51A mutant reduced HA-PML levels relative to the empty vector. Surprisingly, the ΔNco1 and ΔC mutants both reduced HA-PML levels at least as efficiently as wt bICP0 (Figure 3C).

In this study (Figure 3B and 3C) and in a previous study (Inman et al., 2001b), it appeared steady state levels of the truncated ΔC protein were higher than wt bICP0 or other mutants examined. All of the constructs were prepared in the same expression vector ruling out the possibility that the vector contributed to this difference. To estimate the ½ lives of the respective bICP0 protein levels, RS cells were transfected with the respective bICP0 expression plasmids and 24 hours later the respective cultures were treated with cycloheximide (CHX), an inhibitor of protein synthesis. Whole cell extract was collected at 2, 4, or 8 hours after CHX treatment, and bICP0 protein levels analyzed by western blot analysis. Wt bICP0 in transfected cells had a ½ life of 3-4 hours, but during productive infection the ½ life of bICP0 was less than 2 hours suggesting that viral encoded or induced factors regulated bICP0 levels during productive infection. The 13G/51A, and ΔNco1 mutant had a ½ life between 4-6 hours (Figure 3A). In contrast, the ΔC mutant protein had a ½ life greater than 8 hours, which confirmed that the ΔC-terminus protein was more stable than wt bICP0.

Localization of bICP0 mutants and their effect on PML expression.

To test whether bICP0 and the various mutant forms of bICP0 colocalized with PML in transfected cells, confocal microscopy was performed. Since the ΔNco1 mutant

primarily localizes to the cytoplasm and the ΔC-terminus mutant is present throughout cells, it was possible that PML re-localized these truncated mutants to the nucleus, and consequently the mutants efficiently reduced PML levels. RS cells were cotransfected with HA-PML and bICP0, 13G/51A, ΔNco1, or ΔC and processed for confocal microscopy 24 or 48 hours later. As expected, wt bICP0 and the 13G/51A mutant co-localized with HA-PML (Figure 4A). Consistent to what was observed in BHV-1 infected cells (Figure 4B), HA-PML appeared to form aggregates in cells expressing bICP0. HA-PML in cells transfected with 13G/51A, ΔNco1, or ΔC-terminus displayed a similar effect as wt bICP0. However, we saw no evidence for PML recruiting the ΔNcoI and ΔC-terminus mutants to the nucleus suggesting these mutants: 1) reduced PML levels by an indirect mechanism, or 2) bICP0 interacted with cytoplasmic PML prior to nuclear translocation.

We have consistently observed that RS cells transfected with the ΔNco1 or ΔC mutants displayed altered morphology, multiple nuclei or loss of nuclear integrity for example (Figure 4C and D). In general, the morphological effects caused by ΔC expression were more severe relative to the mutant bICP0 protein encoded by the ΔNco1 protein. For example, 60% of cells transfected with the ΔC-terminus deletion mutant contained fragmented cells, in which a nucleus was not obvious, as judged by DAPI staining (Figure 4D). More than 50% of cells transfected with the ΔNcoI mutant appeared to contain multi-lobed cells, and approximately 30% of the cells appeared to be fragmented. In contrast, nearly 60% of cells transfected with wt bICP0 had a normal cellular morphology. Cells that were not transfected contained more than 80% of the

cells that appeared to be normal, which was expected. In summary, these studies provided evidence that non-nuclear forms of bICP0 altered the morphology of transfected cells, which may have led to reduced PML levels.

Identification of bICP0 domains important for reducing PML protein levels.

To test whether other bICP0 functional domains were important for reducing PML protein levels, several bICP0 transposon mutants (Zhang et al., 2005) were selected for analysis (Figure 5A). The transposon mutants designated C, I, K, N, and O were selected based on their position throughout the bICP0 coding sequence, as well as their attenuation of certain bICP0 functions. The C, I, K, N, and O transposon mutants were shown previously to attenuate the ability of bICP0 to activate a minimal HSV-1 thymidine kinase promoter (Zhang et al., 2005). The mutants I, N, and O lost their ability to inhibit IRF7 or IRF3-induced IFN-β promoter activity, and the O mutant reduced IRF3 protein levels less efficiently compared to wt bICP0 (Saira et al., 2007).

For these studies, RS cells were cotransfected with wt bICP0 or the designated transposon mutants and HA-PML, followed by western blot analysis. All of the transposon mutants tested reduced HA-PML levels as analyzed by western blot (Figure 5B, top). Although there were slight differences between the transposon mutants and their ability to reduce HA-PML as compared to wt bICP0, this variation was most likely relative to the expression level of these proteins (Figure 5B, middle). The transposon mutants had previously been shown to have similar expression levels in human 293 cells (Zhang et al., 2005); however, differences in expression were evident throughout the

course of these experiments and this was most likely attributed to their ability to reduce PML levels. Lower expression levels of mutants I and K were confirmed by confocal microscopy studies. The mutants I, K, and O localized to the nucleus and appeared to have similar effects on PML organization which was consistent with what was observed for wt bICP0 (Figure 5C). These studies indicate that the ability of I, K, and O to localize with and reduce HA-PML was not disrupted.

Overexpression of PML reduced BHV-1 replication.

Given that PML is implicated to play a role in antiviral defenses, we investigated whether overexpression of PML inhibited productive infection. For this study, a BHV-1 strain containing the Lac Z gene inserted downstream of the gC promoter was used to analyze expression of the BHV-1 genome. bICP0 is a potent transcriptional activator that is critical for stimulating productive infection. Therefore, we used bICP0 to induce viral expression. A constant amount of viral genomic DNA was cotransfected with bICP0 and increasing amounts of HA-PML. Cells were fixed and stained for beta-gal expression 24 hours after transfection, and total positive cells were counted. Overexpression of PML reduced the ability of bICP0 to stimulate BHV-1 expression (Figure 6, black bars). The effect of ΔNcoI on viral expression in cells overexpressing PML was also analyzed. The ΔNcoI mutant activated expression of the BHV-1 genome comparable to wt bICP0 as determined by the number of beta-gal positive cells (Figure 6, grey bars). Similar to wt bICP0, overexpression of PML also appeared to have an inhibitory effect on ΔNcoI-induced viral genomic expression.

bICP0 localization during productive infection.

A recent study demonstrated that HSV-1 ICP0 localizes to the cytoplasm during productive infection (Kalamvoki, 2010; Kawaguchi et al., 1997; Van Sant et al., 2001) suggesting bICP0 may also localize to the cytoplasm during productive infection. To test this possibility, we examined the localization of bICP0 in an established bovine kidney cell line (CRIB) and in low passage bovine kidney cells. In CRIB cells (Figure 7) or in RS cells (data not shown), at least 90% of the bICP0+ cells contained nuclear bICP0, even at late times after infection. In low passage bovine kidney cells, we found that approximately ½ of the bICP0+ cells contained cytoplasmic bICP0 at 12 or 24 hours after infection (Figure 7). At 4 hours after infection, bICP0 was detected in the nucleus of infected low passage bovine kidney cells. As expected, bICP0 was not detected in uninfected cells.

DISCUSSION

In this study, we provided evidence that bICP0, in the absence of other viral genes, reduced PML protein levels and a functional proteasome was important for this reduction (Figure 2). When bICP0 was cotransfected with the HA-PML plasmid, lactacystin restored PML levels to levels comparable to cells cotransfected with the empty vector and HA-PML. Conversely, lactacystin treatment only partially restored PML protein levels during productive infection (Figure 1) suggesting additional virus-encoded or -induced factors influence PML protein levels. Although colocalization of wt bICP0 and HA-PML was observed by confocal microscopy (Figure 4A) suggesting a direct interaction, we did not detect a direct association between bICP0 and HA-PML by coimmunoprecipitation (Figure 2D). However, this finding was not unexpected. Since bICP0 is an E3 ubiquitin ligase, when bICP0 is associated with PML, PML becomes polyubiquitinated and is degraded. Thus, the association between PML and bICP0 is transient. Furthermore, the ability of the ΔNcoI and ΔC mutants that lack the nuclear localization signal to affect PML levels and nuclear organization similar to wt bICP0 raises the possibility that bICP0 influences PML by an indirect mechanism.

Although the zinc RING finger was important for PML degradation, additional domains of bICP0 appear to play a role because the 13G/51A mutant could still reduce PML protein levels when compared to the empty vector control (Figure 3B). Thus, we explored other domains of bICP0 that might be important for inducing degradation of PML by testing several bICP0 transposon insertion mutants. Transposon insertion near the N terminus downstream of the zinc RING finger domain (C and I), within the acidic

domain (K), or near the C-terminus (N and O) did not appear to affect the ability of bICP0 to localize with or reduce HA-PML levels (Figure 5). A previous study demonstrated the O mutant reduced IRF3 protein levels less efficiently than wt bICP0 (Saira et al., 2007). However, in this study the O mutant appeared to behave similar to wt bICP0 with respect to its affect on PML. Surprisingly, deletion of sequences containing the NLS also reduced PML protein levels in transfected cells. Several studies have demonstrated that most, if not all, PML isoforms can be detected in the nucleus and cytoplasm (Condemine et al., 2006; Giorgi et al., 2010; Lin et al., 2004a; McNally et al., 2008) suggesting bICP0 mutant proteins that do not localize to the nucleus (ΔC-terminus or ΔNcoI) can interact with PML proteins in the cytoplasm and potentially induce their degradation.

PML has a negative effect on HSV-1 productive infection and expression of ICP0 is necessary for overcoming this repression induced by PML (Everett et al., 2008; Everett et al., 2006). We therefore predicted that PML may also reduce the efficiency of BHV-1 productive infection. Induced expression of bICP0 in a stably transfected cell line de-represses a quiescent HSV-1 genome and this ability correlates with preventing the recruitment of PML body associated proteins, including PML, to replication complexes (Everett et al., 2010). The BHV-1 genome is not efficient at producing plaques when transfected into permissive cells unless bICP0 or other positive cellular factors are cotransfected with the genome (Geiser et al., 2002; Geiser and Jones, 2003; Inman et al., 2001b; Meyer, 2007). In this assay, the ΔC-terminus mutant stimulated plaque forming efficiency similar to wt bICP0 (Inman et al., 2001b). However, the ΔC-terminus mutant does not stimulate a simple thymidine kinase promoter construct (Inman

et al., 2001b) suggesting that reduction of PML protein levels by the ΔC-terminus mutant was important for increasing the efficiency of productive infection. The ΔNcoI mutant also had reduced transcriptional activation potential relative to wt bICP0 (Zhang et al., 2005); however, ΔNcoI was still able to activate viral replication (Figure 6). Overexpression of PML appeared to have a negative effect on BHV-1 genomic expression in the presence of ΔNcoI or wt bICP0 (Figure 6). Taken together, these data suggest PML represses viral replication, and bICP0 subsequently relieves this repression by inducing PML degradation.

The morphological changes induced by plasmids expressing the ΔC-terminus or ΔNcoI deletion mutants suggested these cells would not be viable for long periods of time. A previous study demonstrated that bICP0 and the ΔC-terminus mutant indirectly induced apoptosis, as judged by casapse 3 activation, in mouse neuroblasoma cells (neuro-2A) (Henderson, 2004) and (data not shown). In contrast, we were unable to detect activated caspase 3 in RS cells expressing either the ΔNco1 or ΔC-terminus mutants (data not shown) indicating bICP0-induced apoptosis in neuro-2A cells, but not RS cells. We further suggest that degradation of non-nuclear PML by the ΔNco1 or ΔC-terminus mutants in RS cells may affect apoptosis because non-nuclear PML promotes apoptosis at the endoplasmic reticulum by modulating calcium release (Giorgi et al., 2010). In addition to non-nuclear PML being enriched at the endoplasmic reticulum, PML is also enriched at mitochondria-associated membranes, and non-nuclear PML is associated with a protein kinase (AKT) that inhibits apoptosis (Giorgi et al., 2010). These observations suggested that bICP0 expression has different effects on cell survival.

For example, bICP0 can have a toxic effect on cells, but by reducing PML bICP0 may also interfere with specific apoptotic stimuli. There is precedence for bICP0 regulating cell type dependent cell death because BHV-1 induces cell type dependent cell death and deletion of bICP0 influences the outcome (Geiser, 2008). Clearly, additional studies are needed to understand how bICP0 regulates cell survival.

HSV-1 ICP0 is consistently detected in the cytoplasm of infected cells, regardless of whether the cells are low-passage or established (Kalamvoki, 2010; Kawaguchi et al., 1997; Van Sant et al., 2001). Although bICP0 was localized to the cytoplasm during late stages of infection in low-passage bovine kidney cells, this was not the case in established bovine kidney cells (CRIB) or RS cells (data not shown). Whether all low passage bovine cells contain bICP0 in the cytoplasm is unknown. Regardless of whether bICP0 is localized in the cytoplasm during productive infection, we predict cytoplasmic localized bICP0 plays a role during productive infection. For example, it is known that HSV-1 ICP0 blocks activation of the interferon response factor 3 only when it is in the cytoplasm (Paladino, 2010), and cytoplasmic ICP0 reorganizes microtubules (Liu, 2010). Recent work from our lab has shown cytoplasmic bICP0 is able to inhibit IFN-β promoter activity and induce degradation of IRF3 as efficiently as wt bICP0 (Da Silva and Gaudreault, unpublished). These data suggest a role for cytoplasmic bICP0 in repressing the innate immune response and altering cell fate after infection. Studies to address how bICP0 is moved to the cytoplasm in low-passage cells and what role cytoplasmic bICP0 plays during productive infection are in progress.

ACKNOWLEDGEMENTS

This research was supported by grants from the USDA and Agriculture and Food Research Initiative Competitive Grants Program (08-00891 and 09-01653). A grant to the Nebraska Center for Virology (1P20RR15635), in particular the confocal microscopy core facility, also supported certain aspects of these studies. Natasha Gaudreault was partially supported by a fellowship from a Ruth L. Kirschstein National Research Service Award 1 T32 AIO60547 (National Institute of Allergy and Infectious Diseases).

FIGURE 1

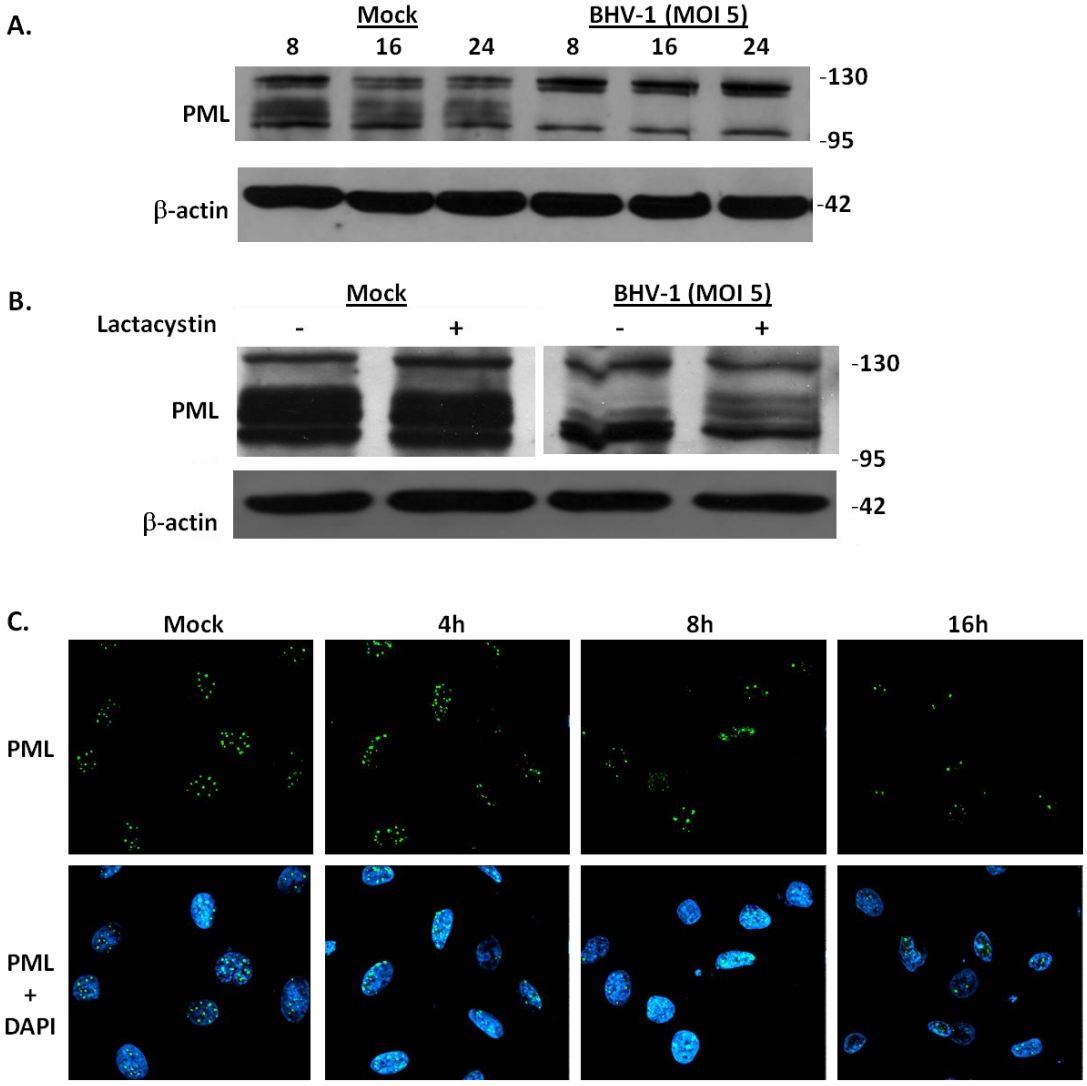


Figure 1. BHV-1 productive infection reduces PML protein levels.

Panel A: CRIB cells were mock infected or infected with BHV-1 (MOI=5) and whole cell lysate collected at the indicated time points after infection (hours) as described in the materials and methods. Anti-PML or anti-β-actin antibodies were used for western blot analysis of endogenous protein levels.

Panel B: CRIB cells were infected (MOI=5) for 8 hours followed by treatment with 15 uM lactacystin (70980, Cayman Chemical) for 4 h. Whole cell lysate was collected for western blot analysis at 12 hours after infection as described in the materials and methods. Anti-PML or anti-β-actin antibodies were used for western blot analysis of endogenous protein levels.

Panel C: RS cells were infected (MOI=5) and processed for confocal microscopy at the indicated times after infection. Primary anti-PML and secondary Alexafluor-488 (green) antibodies were used to visualize endogenous protein expression. DAPI (blue) was used to stain nuclear DNA.

FIGURE 2

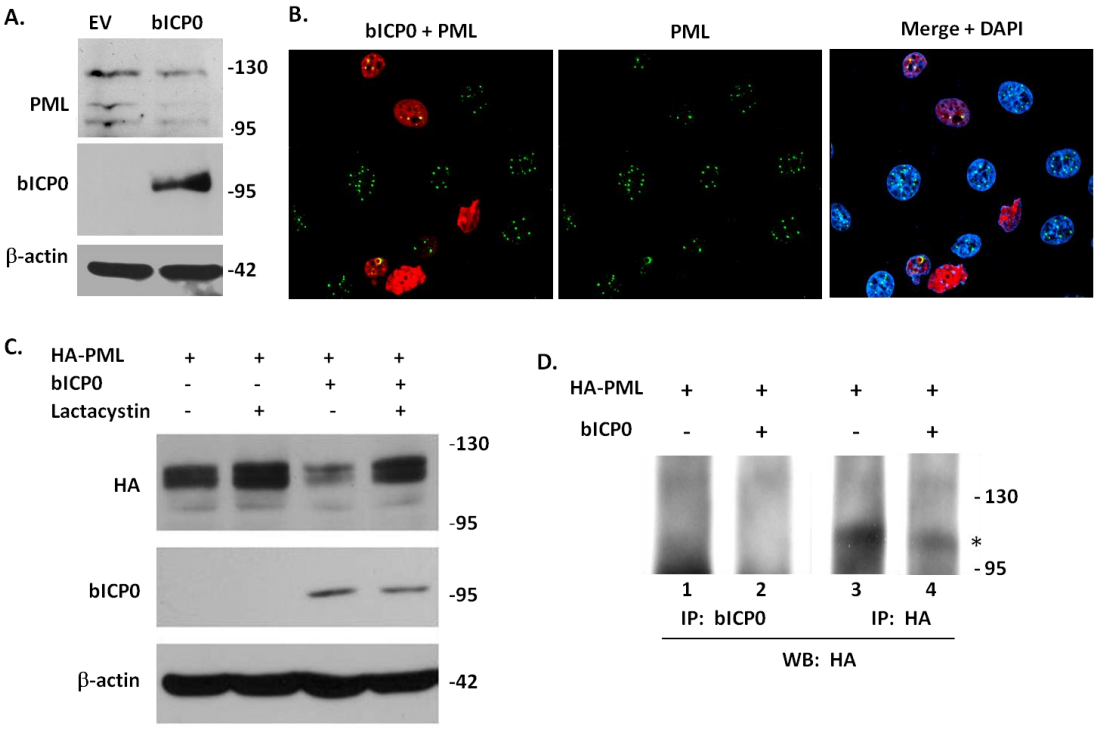


Figure 2. bICP0 induces proteasome-dependent degradation of PML.

Panel A: RS cells were transfected with the plasmid expressing wt bICP0 or the empty vector (pcDNA3.1). At 40 hours after transfection, PML, bICP0, or β-actin levels were examined by Western Blot analysis.

Panel B: RS cells were transfected with bICP0 and processed for confocal microscopy 24 h later. Cells were stained with anti-PML and anti-bICP0 antibodies, followed by secondary labeling with Alexafluor-633 and -488 for visualization of PML and bICP0 expression, respectively.

Panel C: RS cells were transfected with the HA-PML expression plasmid and the wt bICP0 plasmid or blank expression vector. Where indicated, cells were treated with 15 uM lactacystin (70980, Cayman Chemical) for 8 hours before preparation of whole cell lysate. Whole cell lysate was prepared at 48 hours after transfection and western blot analysis performed. Anti-PML anti-β-actin antibodies were used to detect endogenous protein levels. Transiently expressed HA-PML and bICP0 were detected using anti-HA and anti-bICP0 antibodies.

Panel D: RS cells were cotransfected with HA-PML, and bICP0 or empty vector. Whole cell lysates were collected 40 hours after transfection and subjected to immunoprecipitation (IP) with HA or peptide specific bICP0 polyclonal antibodies. Immunoprecipitated proteins were analyzed by western blot (WB) with the HA antibody. The HA-PML protein migrates between 95 and 100 kilo Daltons (kDa).

FIGURE 3

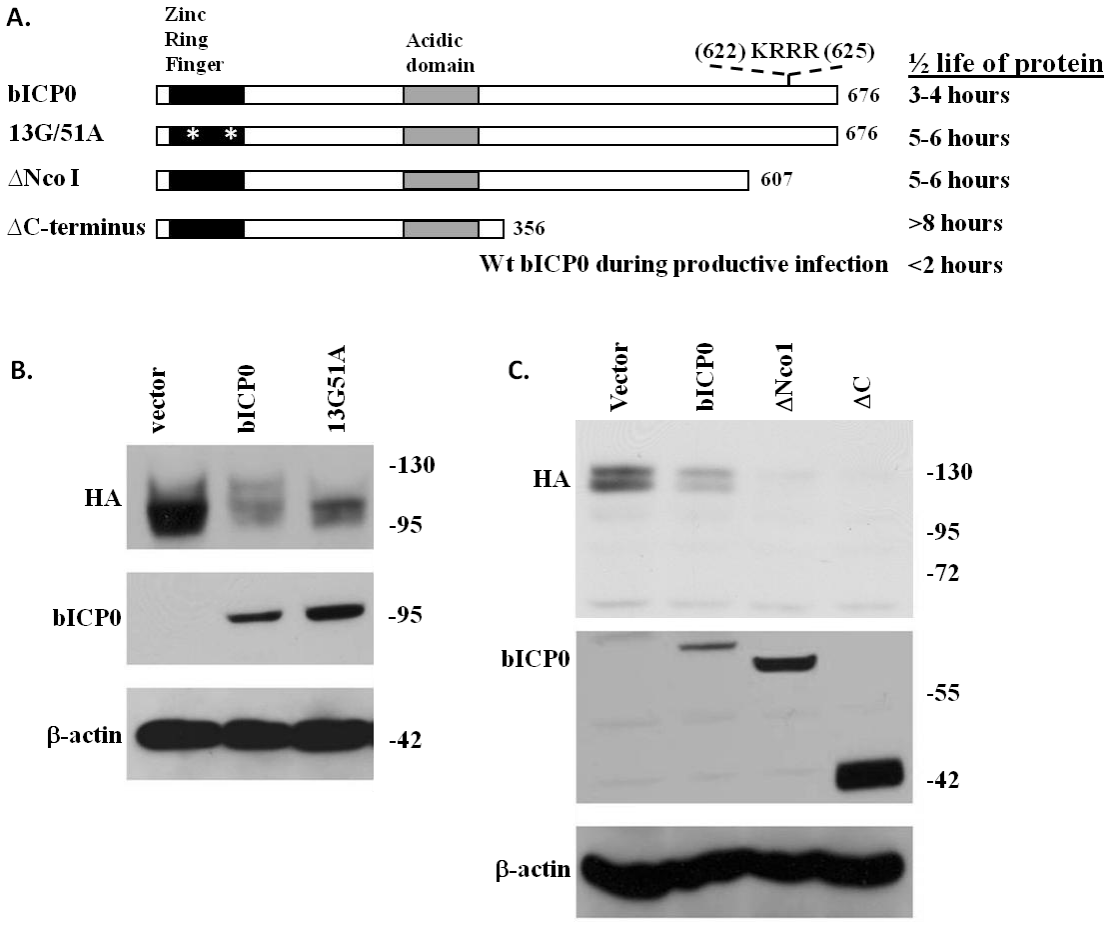


Figure 3. Effect of bICP0 mutants on PML levels.

Panel A: Schematic of wt bICP0 and C-terminal deletion mutants. Positions of the zinc RING finger and acidic domain are indicated. The NLS core sequence is from amino acid 622-625. The zinc RING finger mutant (13G/51A) has two well-conserved C's that were mutagenized to G and A respectively (Inman et al., 2001b). The ΔNcoI mutant has aa sequences from 607-676 deleted (Zhang, 2005). The ΔC-terminus construct contains a large deletion after amino acid 356 (Inman et al., 2001b). To estimate the ½ life of the various bICP0 mutants, RS cells were transfected with the indicated bICP0 plasmids and 24 hours later cultures were treated with cycloheximide (100 ug/ml) for 2, 4, 6, or 8 hours. Cells were then collected, whole cell lysate was prepared, and bICP0 protein levels determined by western blot analysis. bICP0 protein levels were estimated using the Quantity One volume analysis program after obtaining images of the gels using a BioRad Molecular Imager FX. The results from 3 independent experiments were averaged.

Panels B and C: RS cells were cotransfected with the indicated bICP0 expression plasmid or blank expression vector and the HA-PML expression vector, and processed for Western Blot analysis at 48 hours after transfection. Transiently expressed HA-PML and bICP0 were detected using anti-HA and anti-bICP0 antibodies. These studies are representative of three or more independent experiments.

FIGURE 4

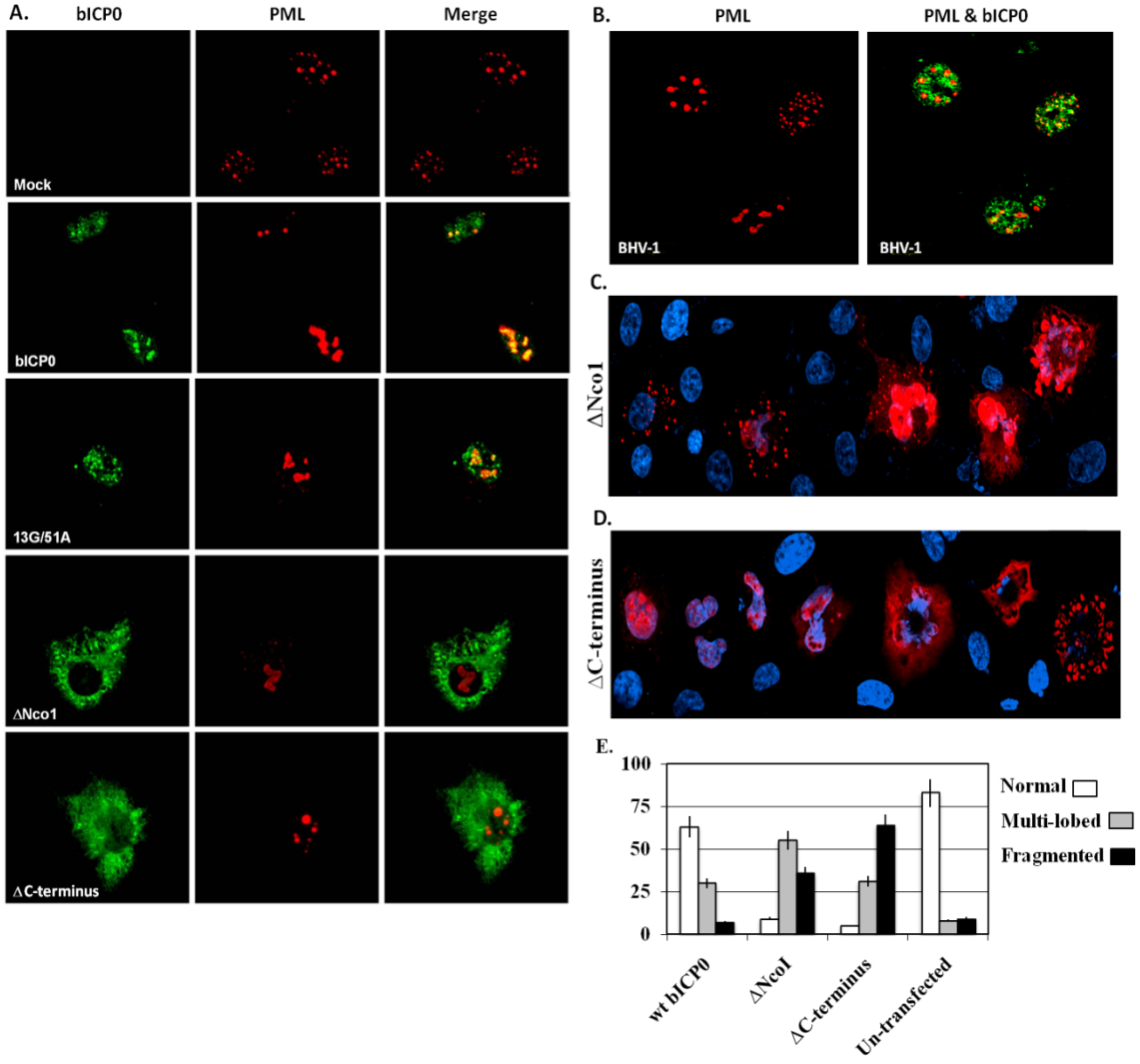


Figure 4. ΔNcoI and ΔC-terminus mutants do not co-localize with PML in the nucleus of transfected cells.

Panel A: RS cells were cotransfected with the designated bICP0 plasmids and the HA-PML expression vector. Cells were stained with anti-HA and anti-bICP0 antibodies, followed by secondary labeling with Alexafluor-633 and -488 for visualization of PML and bICP0 expression, respectively. The studies were repeated at least 4 times and the results were consistent.

Panel B: RS cells were transfected with HA-PML. After 24 h cells were infected with BHV-1 (MOI=5) and processed for confocal microscopy 8 h later. Cells were stained with anti-HA and anti-bICP0 antibodies, followed by secondary labeling with Alexafluor-633 and -488.

Panel C and D: RS cells were transfected with the ΔNco1 or ΔC-terminus plasmids and processed for confocal microscopy 24 hours later. RS cells were stained with propidium iodide (PI) for 15 minutes at room temperature prior to fixation. An anti-flag antibody was used to detect bICP0 expression. DAPI was used to stain nuclear DNA.

Panel E: From transfection studies described in panels A and B, the % of bICP0+ cells that appeared normal, contained multi-lobed cells, and those that contained a fragmented cell were estimated. As a control, untransfected cells in the same dish were also counted. For each sample, at least 300-400 cells were counted from 4 different transfections.

FIGURE 5

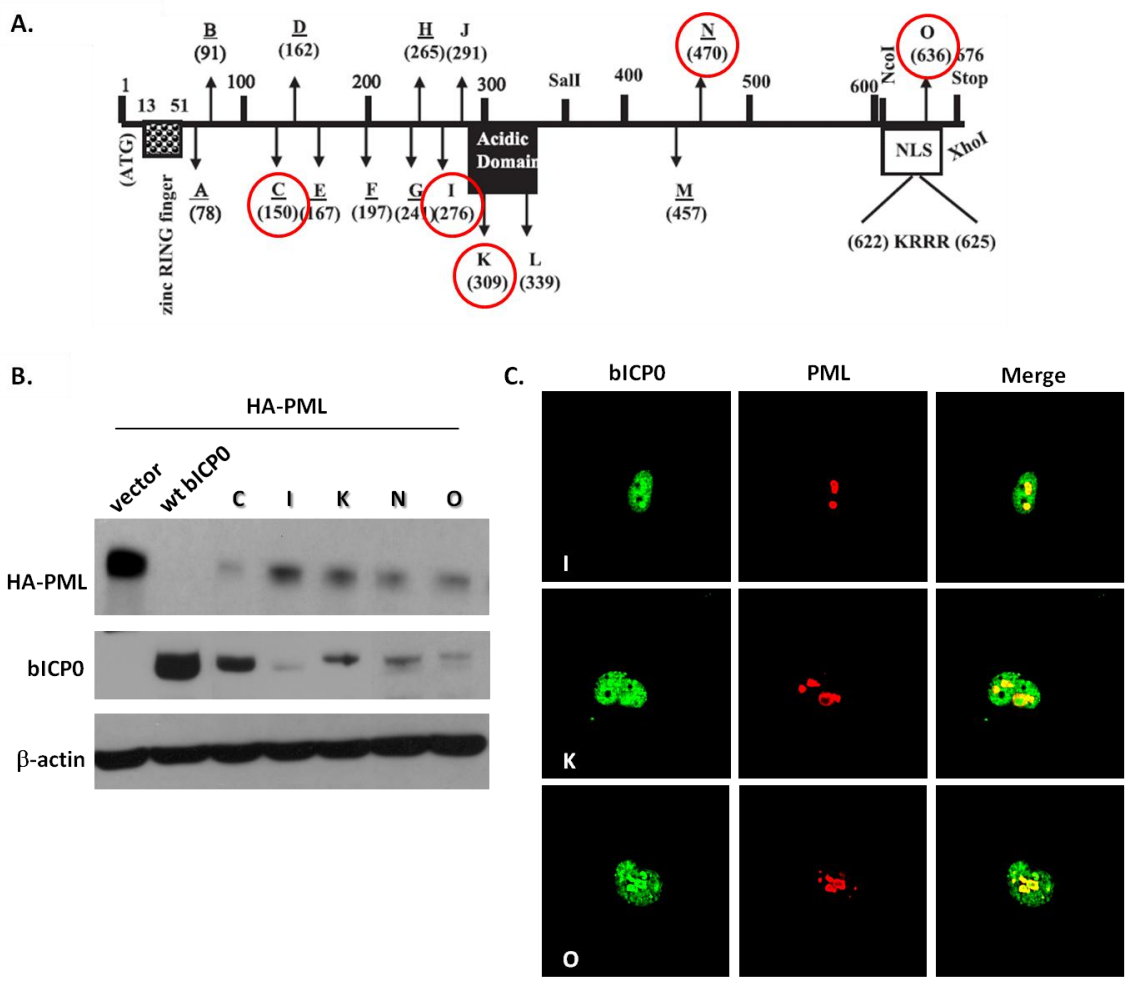


Figure 5. Identification of bICP0 domains important for inducing PML degradation.

Panel A: Schematic of bICP0 transposon insertion mutants. Construction and identification of the transposon insertion mutants were previously described (Zhang et al., 2005). The mutants were designated A to O, and the numbers in parentheses denote the amino acid number that was disrupted by transposon insertion. The positions of the zinc RING finger, acidic domain, consensus NLS (KRRR), ATG, and bICP0 stop codon are shown. The transposon mutants that had a dramatic effect on activating the thymidine kinase promoter are underlined. The mutants selected for analysis in this study are circled in red. RS cells were cotransfected with the designated bICP0 plasmids and the HA-PML expression vector.

Panel B: RS cells were processed for western blot analysis 40 h after transfection. Transiently expressed HA-PML and bICP0 were detected using anti-HA and anti-bICP0 antibodies.

Panel C: RS cells were processed for confocal microscopy 40 h after transfection. Cells were stained with anti-HA and anti-bICP0 antibodies, followed by secondary labeling with Alexafluor-633 and -488 for visualization of PML and bICP0 expression, respectively.

FIGURE 6

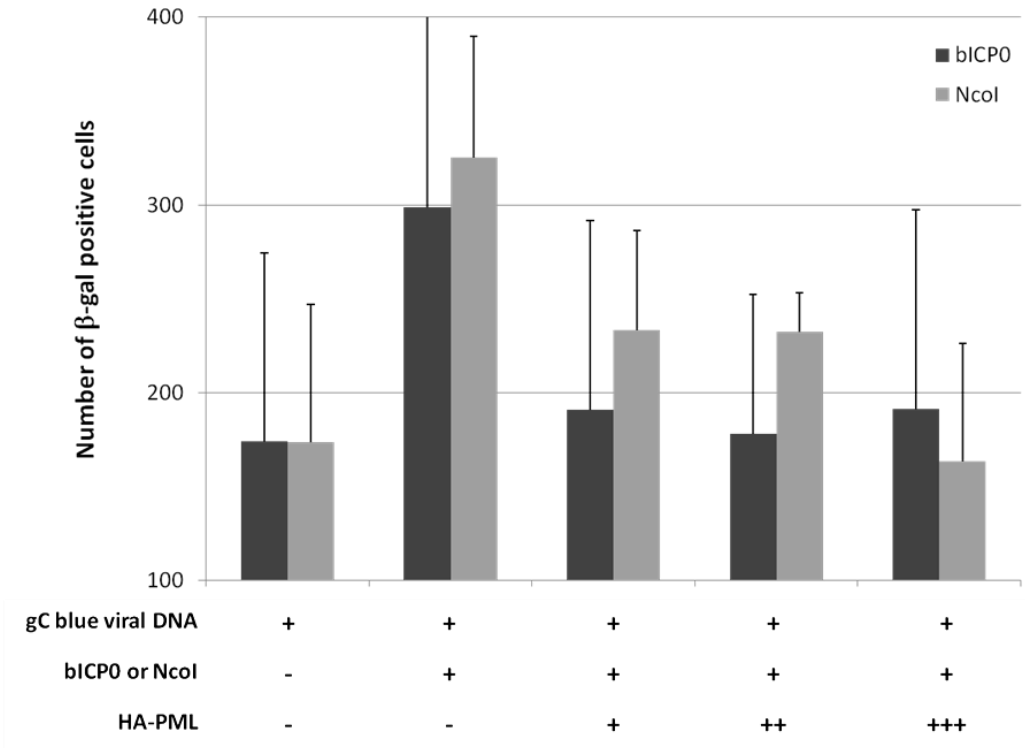


Figure 6. Overexpression of PML reduced BHV-1 replication.

RS cells were transfected with a constant amount of viral genomic DNA isolated from gC blue BHV-1 infected cells, a constant amount of bICP0 (black bars) or ΔNcoI (grey bars), and increasing amounts of HA-PML. Blank expression vector was used to ensure each reaction contained equal amounts of DNA. Cells were fixed 24 hours after transfection and beta-galactosidase assay performed. Total beta-gal positive cells were counted for each sample and values of 3 independent experiments were averaged.

FIGURE 7

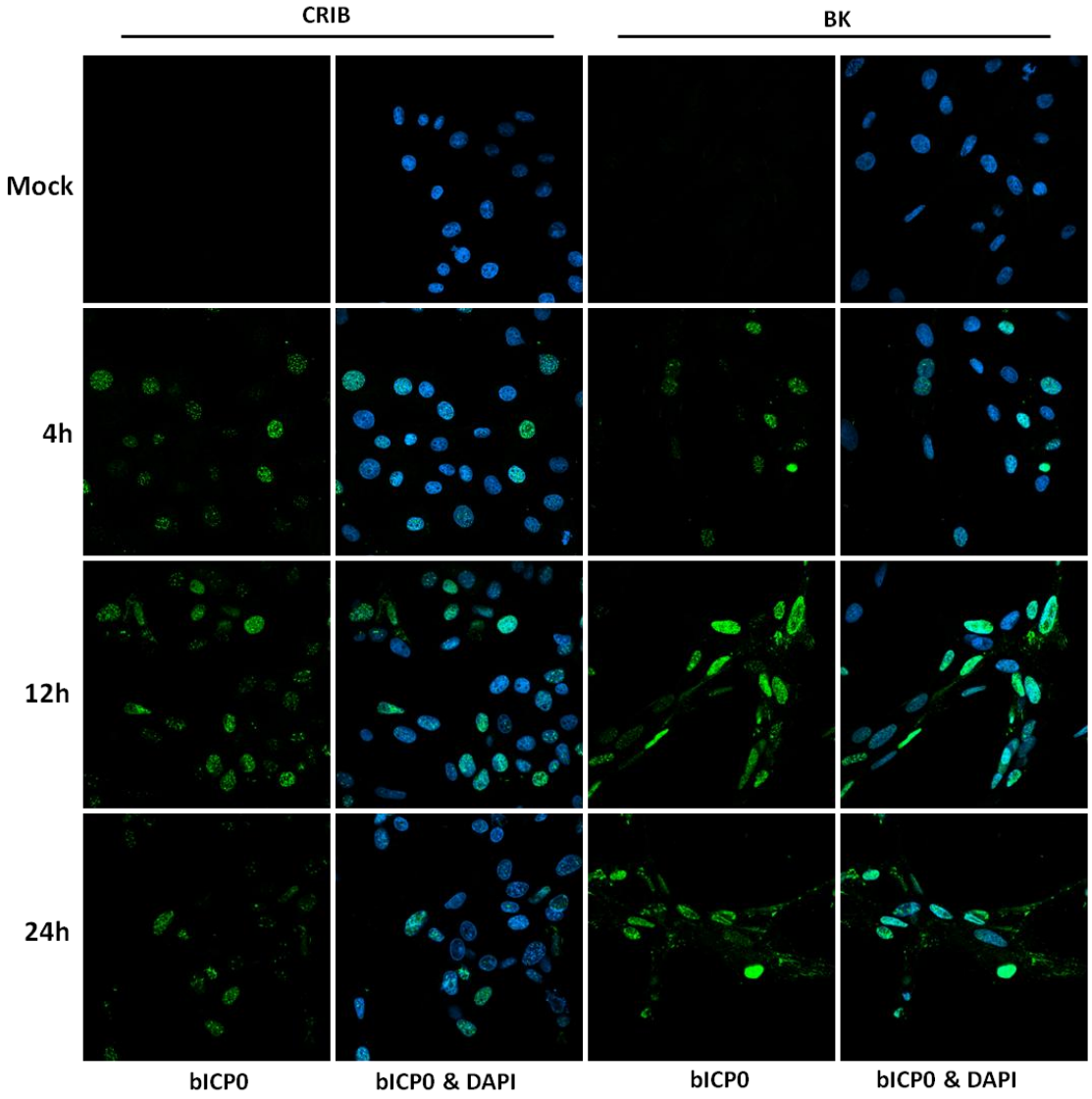


Figure 7. Localization of bICP0 during productive infection.

Established bovine kidney cells (CRIB) or low passage bovine kidney cells (BK) were infected with BHV-1 (MOI=5). At the designated times after infection (hours), confocal microscopy was performed using the bICP0 antibody. To visualize nuclei, DAPI staining was performed.

CHAPTER 4

Studies designed to identify novel bICP0 interacting proteins.

ABSTRACT

Bovine herpes virus type 1 (BHV-1) encoded infected cell protein 0 (bICP0) plays a critical role during the BHV-1 infectious cycle. bICP0 is a promiscuous transcriptional activator and inhibitor of the host interferon response. The bICP0 protein has been shown to directly associate with the chromatin remodeling enzyme histone deacetylase 1 (HDAC1) to promote expression of viral genes, and interferon regulatory factor 7 (IRF7) and histone acetyltransferase p300 to inhibit interferon beta (IFN-β) transcription. In this study, we attempted to identify other proteins that are associated with bICP0 by coaffinity purification and mass spectrometry analysis. Identification of bICP0 interacting proteins would aid in the elucidation of the underlying mechanisms of this important viral protein.

INTRODUCTION

BHV-1 encoded bICP0 is a multifunctional protein that is critical for viral replication and virulence (Jones, 2009). During productive infection, viral genes are temporally expressed in three distinct phases: immediate early, early, and late (Jones, 1998; Jones, 2003; Wirth et al., 1989). Expression of bICP0 is activated by an immediate early as well as an early promoter, and constitutive levels are expressed throughout productive infection (Wirth et al., 1992; Wirth et al., 1989; Wirth et al., 1991). bICP0 is a known promiscuous activator of viral gene expression and is required for efficient viral replication (Fraefel et al., 1994; Wirth et al., 1992; Wirth et al., 1991). Not only is bICP0 a potent activator of all three classes of viral genes, it also represses interferon dependent transcription (Henderson et al., 2005; Saira et al., 2007; Saira et al., 2009). Its ability to stimulate productive infection and regulate innate immune responses makes bICP0 a critical component of the BHV-1 infectious cycle.

During productive infection bICP0 localizes to and disrupts promyelocytic leukemia (PML) protein containing domains (Gaudreault and Jones, 2011; Inman et al., 2001b). bICP0 contains a C_3HC_4 zinc RING finger motif which possesses intrinsic E3 ubiquitin ligase activity and has been shown to induce the proteasome dependent degradation of specific cellular proteins, including PML and interferon regulatory factor 3 (IRF3) (Diao et al., 2005; Gaudreault and Jones, 2011; Saira et al., 2007). The bICP0 protein directly associates with histone deacetylase 1 (HDAC1) to promote viral gene expression (Zhang and Jones, 2001). It has also been shown to have a direct interaction with the histone acetyltransferase p300 and IRF7 to inhibit IFN-β activation (Saira et al.,

2009; Zhang et al., 2006). It is clear bICP0 plays multiple roles during productive infection; however, the specific mechanisms underlying bICP0 functions are not well understood. Due to its multiple roles and influence on host responses during infection, it is likely that bICP0 interacts with a number of cellular as well as BHV-1 encoded proteins.

The bICP0 protein is known to be a nuclear localizing protein, yet during the course of infection bICP0 can shuttle between the nucleus and cytoplasm. We recently demonstrated that bICP0 localizes to the cytoplasm of low passage bovine cells at later times of infection (Gaudreault and Jones, 2011). These findings suggest a role for cytoplasmic bICP0 during productive infection. Therefore, it is probable that bICP0 associates with proteins in the cytoplasm in addition to nuclear proteins. Identifying proteins that interact with bICP0 would provide insight into the molecular mechanisms, as well as, additional functions of bICP0 during productive infection. We therefore set forth to identify bICP0 binding partners by coaffinity purification and mass spectrometric analysis.

MATERIALS AND METHODS

Cells and virus

Bovine kidney (CRIB) cells and rabbit skin cells (RS) were grown in monolayer cultures in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 mg/ml) in a humidified 5% CO2 atmosphere at 37°C. The BHV-1 Cooper strain was propagated and titrated in CRIB cells. For infection studies, cells were plated onto 60 or 100 mm culture dishes 16 to 24 hours prior to virus infection. Total cells per plate were counted and the specified multiplicity of infection (MOI) calculated.

Expression plasmid and transfection

The wild type bICP0 plasmid is cloned into a pCMV2 expression vector downstream of a Flag tag and under the control of the human cytomegalovirus (CMV) promoter. Empty vector was included as a negative control. RS cells were plated onto 60 mm culture dishes 16 to 24 hours prior to transfection. RS cells were transfected using the TransitIT-LT1 reagent (MIR 2300, Mirus) according to the manufacturer's protocol.

Metabolic labeling of cells with [35]S-methionine

CRIB cells were infected with BHV-1 at an MOI of 5 for 3 hours. Cells were then washed with PBS and incubated with 2% dialyzed FBS methionine-cysteine free EMEM starvation media for 1 hour (Sigma). For radiolabeling of proteins 0.1 mCi [35]S-methionine was added to cultures and incubated for an additional 2 hours. Cells were collected for lysis with RIPA buffer (1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride in PBS, and one tablet of complete protease inhibitor [Roche] per 10 ml). Cell lysate was incubated at 4°C with rotation for 25 min, sonicated briefly, and then clarified by centrifugation at 13,000 rpm at 4°C for 15 min.

Coimmunoprecipitation

Cell lysates were pre-cleared by incubating with rabbit serum (0.05 ml per 1 ml) for 1 hour on ice, followed by addition of washed protein A agarose beads (0.05 ml slurry per sample) and incubated with rotation at 4°C for an additional 30 minutes. Beads were pelleted and the supernatants collected for immunoprecipitation. Samples were incubated with approximately 2 μg bICP0 polyclonal antibody per 0.5 mg of protein overnight with rotation at 4°C. To each sample, 0.1 ml of protein A agarose bead slurry was added and incubated at 4°C with rotation for 4 hours. Beads were washed three times with lysis buffer and the protein eluted by adding 0.05 ml 2X loading buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 50 mM dithiothreitol, 0.1% Bromophenol Blue, 10% Glycerol) and boiling

the samples for 5 min. Proteins were separated on an 8 or 10% SDS poly acrylamide gel electrophoresis (SDS-PAGE).

Western blot analysis

Protein concentrations were quantified by the Bradford assay, and SDS-PAGE. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). Membranes were blocked in Tris-buffered saline that contained 5% milk. The beta-actin antibody (sc-1616) was purchased from Santa Cruz Biotechnology. bICP0 was detected using a bICP0 peptide specific rabbit polyclonal antibody. Membranes were then incubated overnight with the indicated primary antibody in 5% milk-containing 0.1% Tween 20–Tris-buffered saline. After washing with 0.1% Tween 20–Tris-buffered saline, membranes were incubated with donkey anti-goat (sc-2020; Santa Cruz Biotechnology), donkey anti-rabbit (NA934V, Amersham Biosciences), or sheep anti-mouse (NXA931, Amersham Biosciences) horseradish peroxidase-conjugated immunoglobulin G secondary antibodies diluted in blocking buffer. Immunodetection was performed with enhanced chemiluminescence western blotting detection reagents (Perkin-Elmer, MA) in accordance with the manufacturer's protocol. Experiments were repeated three or more times.

Mass spectrometric analysis

The protein gel was stained with coomassie blue and bands of interest were excised for mass spectrometric analysis at the Nebraska Center for Mass Spectrometry facility located at the University of Nebraska-Lincoln (UNL). Further processing of the samples to be analyzed was performed by the sequencing facility.

RESULTS

Identification of bICP0 binding proteins.

In an effort to identify novel bICP0 interacting proteins in BHV-1 infected cells, we performed affinity purification of bICP0 with its putative binding partners which we sent for mass spectrometric analysis. Initially, to determine if we could detect unique bands that could be excised and submitted for sequencing, we metabolically labeled proteins of BHV-1 infected cells with radioactive [35]S-methionine. To maximize bICP0 protein levels, cells were infected at 5 MOI and whole cell extracts collected 5 hours post infection to avoid cytopathic effect of the virus on the cells. Protein extracts were immunoprecipitated with a peptide-specific bICP0 polyclonal antibody, the proteins separated by SDS-PAGE, and then visualized by autoradiography. We reproducibly detected multiple distinguishable bands from bICP0 immunoprecipitates of BHV-1 infected cell extracts that were not evident in mock samples that are denoted by the asterisks (Figure 1A). A tenth of the protein extracts used for immunoprecipitation were loaded as input (Figure 1B).

With regards to sample submission guidelines of the Nebraska Center for Mass Spectrometry at UNL, we prepared non-radiolabeled proteins from BHV-1 infected or mock infected cells to send for mass spectrometry. Previous experimental conditions were scaled up in order to achieve visualization of unique protein bands by coomassie blue staining of the protein gel. In addition, a set of bICP0 immunoprecipitates from extracts of RS cells transfected with bICP0 or empty expression vector were also

analyzed (Figure 2). RS cells were chosen for bICP0 overexpression because higher transfection efficiency can be achieved in these cells compared to CRIB cells. Distinct bands in bICP0 immunoprecipitates from BHV-1 infected or bICP0 transfected cell extracts were consistently observed by coomassie blue gel staining (Figure 2). When RS cells were transfected with a bICP0 expression construct, novel coomassie stained bands were also detected (Figure 3).

Bands of interest were excised and sent for further processing and mass spectrometric analysis at the UNL mass spectrometry facility. The same regions from the mock or empty vector samples were also analyzed as a negative control to rule out non-specific binding to the resin during the purification process. Four independent sets of samples were submitted for sequencing. A fraction of the bICP0 immunoprecipitated sample was analyzed by western blot with bICP0 antiserum to confirm the efficiency of the immunoprecipitation. bICP0 was easily detected by western blot in bICP0 immunoprecipitates of BHV-1 infected cell extracts and absent in mock samples, as expected (Figure 4, top). Equal amounts of mock infected and BHV-1 infected protein samples were subjected to immuno-purification as demonstrated by input levels of beta actin (Figure 4, bottom). Although differences between mock and infected samples were visibly observed, sequencing results did not clearly distinguish between proteins of mock and infected samples. Furthermore, proteins unique to BHV-1 infected or bICP0 transfected samples were not consistently observed.

DISCUSSION

Because of its multiple roles during productive infection, we predicted that bICP0 interacts with a variety of cellular proteins. Thus far, bICP0 has been shown to directly associate with HDAC, p300, and IRF7 (Saira et al., 2009; Zhang et al., 2006; Zhang and Jones, 2001). Although bICP0 has been shown to affect other cellular proteins such as PML and IRF3, a direct interaction was not evident (Gaudreault and Jones, 2011; Saira et al., 2007). Furthermore, bICP0 is able to activate viral transcription (Inman et al., 2001b; Zhang et al., 2006; Zhang and Jones, 2001; Zhang et al., 2005), yet there is no evidence to indicate that bICP0 binds directly to DNA . This implies bICP0 interacts with other regulatory factors to induce these effects and to stimulate viral gene expression.

Co-affinity purification and mass spectrometry analysis of proteins has been a successful system for identifying novel protein-protein interactions. At the present, no bICP0-protein interactions have been confirmed from the sequencing results obtained from these experiments. The mass spectrometry data revealed virtually identical results from extracts of cells infected with BHV-1 or transiently expressing bICP0, and the mock controls. Furthermore, proteins that were unique from infected or bICP0 expressing extracts were not consistently observed. It is possible that similar proteins were present in both mock and infected samples, but at different concentrations which was not evident from the mass spectrometry results. Therefore it is difficult to discern what is purely non-specific background and what protein interactions are specific. The bICP0 protein was easily detected by western blot from bICP0 immunoprecipitates of BHV-1 infected cell extracts, but not from mock infected samples (Figure 4) suggesting sufficient

isolation of bICP0 was achieved. It is possible that the isolation and purification conditions were not optimal for retaining weak interacting proteins, or that putative binding proteins were unstable and degraded by proteases. However, this is unlikely since a cocktail of protease inhibitors was used throughout the affinity purification process. Furthermore, unique bands were detected in bICP0 immunoprecipitates prepared from BHV-1 infected or bICP0 transfected cells compared to mock or empty vector expressing samples (Figures 1, 2 and 3). This suggests complications may have occurred during processing of the samples for analysis by mass spectrometry, or perhaps due to the search parameters used for generating the protein analysis data.

Other affinity-purification systems, such as biotin-streptavidin tagging of proteins could enhance binding and purification of bICP0 interacting proteins. Separation of bICP0 immunoprecipitated proteins on a two-dimensional gel would allow better isolation of proteins and perhaps easier identification. Alternative approaches such as yeast two-hybrid screening or protein microarrays have also been successful approaches to identify viral interacting proteins in other infectious virus systems. Although we have not confirmed any novel bICP0 binding proteins from these mass spectrometry results, our initial results clearly showed visibly unique proteins. Identification of interacting factors is a worthwhile endeavor and potential results could provide insight to the underlying mechanisms of bICP0 functions and enhance our understanding of bICP0's role during the BHV-1 infectious cycle.

FIGURE 1

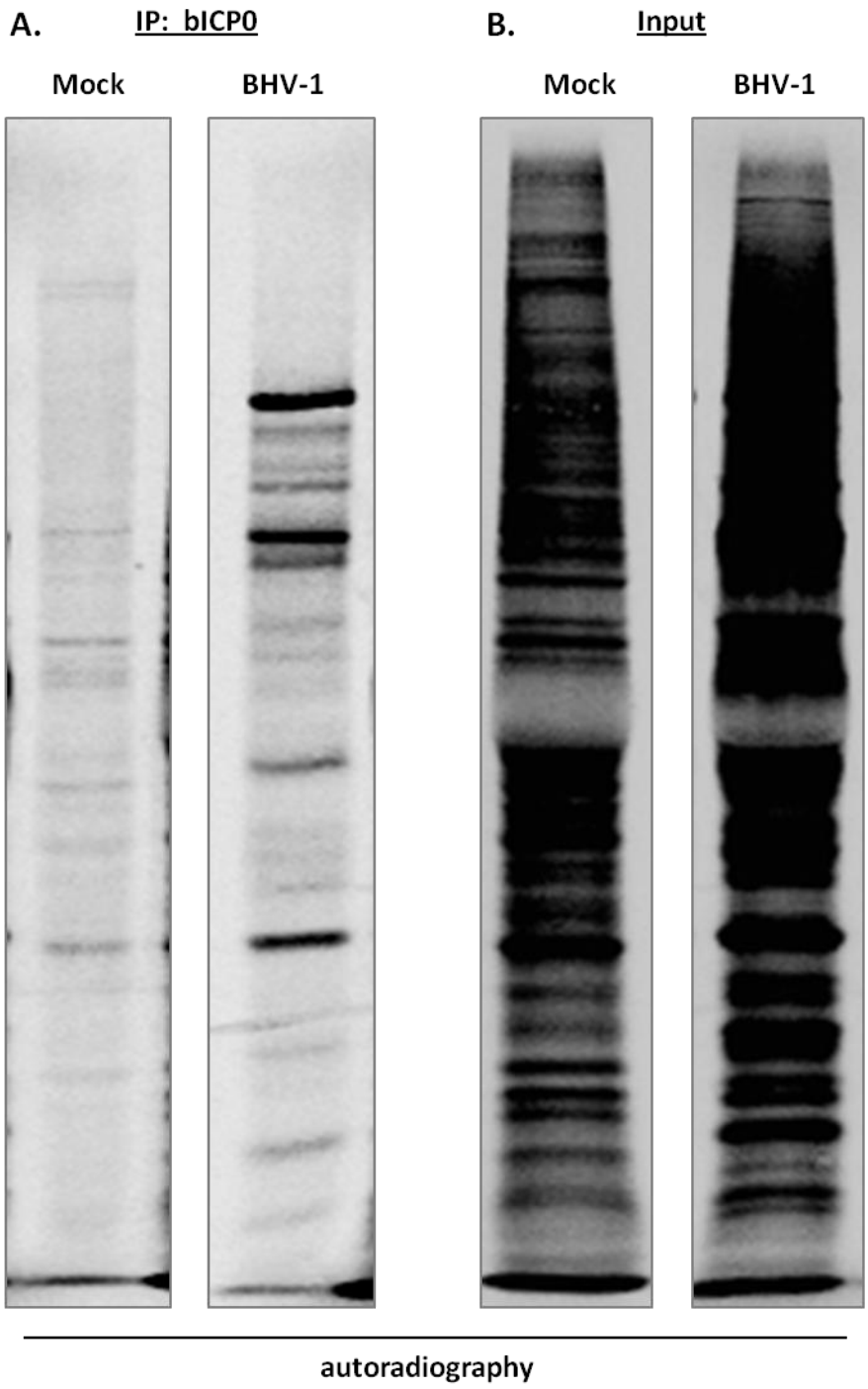


Figure 1. Metabolically labeled proteins of bICP0 immunoprecipitates from BHV-1 infected cells. CRIB cells were infected at an MOI of 5. After 3 hours of infection, cells were washed and incubated with 2% dialyzed FBS methionine-cysteine free EMEM starvation media for 1 hour. Proteins were radiolabeled with 0.1 mCi [35]S-methionine for an additional 2 hours. Whole cell protein extracts were collected and immunoprecipitated with a bICP0 peptide specific polyclonal antibody. Following separation by SDS-PAGE, the gel was dried and exposed to a phosphorimaging screen and analyzed using a Bio-Rad Molecular Imager FX.

FIGURE 2

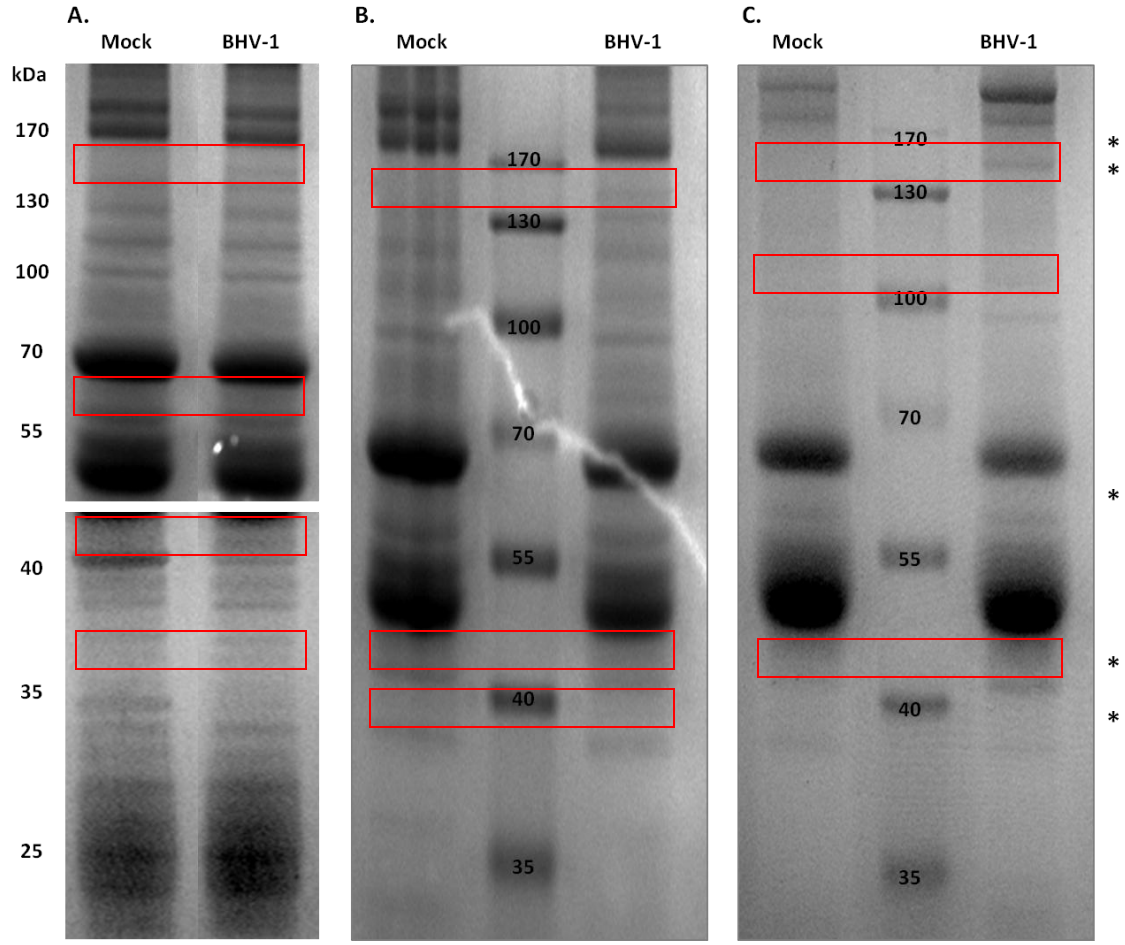
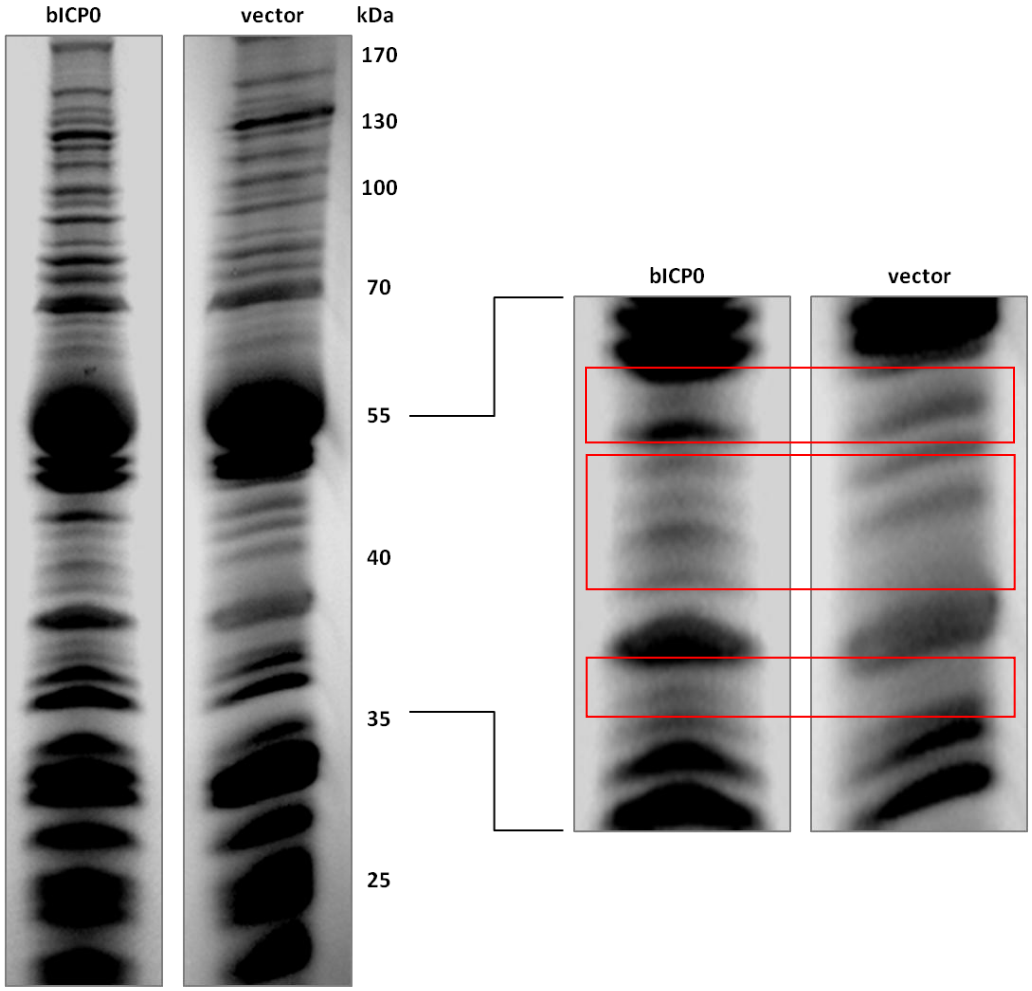


Figure 2. bICP0 immunoprecipitated proteins from BHV-1 infected cells. CRIB cells were infected at an MOI 5 and whole cell extracts collected 5 hours post infection. Extracts from infected or mock infected cells were used for immunoprecipitation with a bICP0 specific antibody. Proteins were separated by SDS-PAGE and visualized by coomassie blue stain. Sections from the gel outlined in red were excised from the gel and sent to the UNL sequencing facility for further processing and analysis by mass spectrometry. The asterisk (*) denotes regions where unique bands were consistently observed. Panels A, B and C are coomassie blue stained gels from independent experiments.

FIGURE 3



Coomassie blue stained gel

Figure 3. bICP0 immunoprecipitated proteins from bICP0 transfected cells. RS cells were transfected with bICP0 or empty vector and whole cell lysates were collected 40 hours after transfection. Protein extracts from transfected cells were used for immunoprecipitation with a bICP0 specific antibody. Proteins were separated by SDS-PAGE and visualized by coomassie blue stain. The panels to the right are magnified to exhibit more clearly the unique banding patterns in this region. Sections from the gel outlined in red were excised from the gel and sent to the UNL sequencing facility for further processing and analysis by mass spectrometry.

FIGURE 4

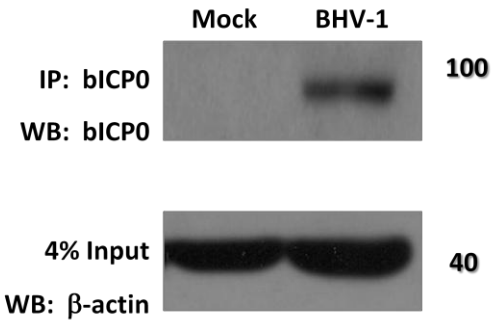


Figure 4. Isolation of bICP0 proteins from BHV-1 infected cells. A portion of the protein extracts recovered from immunoprecipitation and beta actin levels from the input prior to immunoprecipitation were analyzed by western blot using a bICP0 specific antibody or antibodies recognizing beta actin, respectively. The bICP0 protein migrates at approximately 97 kilo Daltons (kDa) and beta actin at approximately 42 kDa.

CHAPTER 5

Evaluation of the infectivity of wt BHV-1 and a bICP0 zinc RING finger mutant virus in cancer cells.

ABSTRACT

Type 1 interferons (IFN), IFN-α and IFN-β, play a major role in the recognition and response against viral infections. In many types of cancers, the IFN pathway has been disrupted, allowing certain viruses that are inhibited by IFN to grow in these types of transformed cells. BHV-1 encoded bICP0 plays a key role in controlling the IFN response by inhibiting the induction of IFN-β expression. Previous work demonstrating restriction of BHV-1 by the IFN signaling pathway and a recent study which investigated the oncolytic properties of BHV-1, prompted us to examine BHV-1 expression in cancer cells which possess potential defects in their IFN pathway. To learn more about how BHV-1 infection may be regulated by IFN, and furthermore, the importance of bICP0 in the context of control of IFN, we tested the growth of wt BHV-1 and a bICP0 zinc RING finger mutant virus (51g) in cancer cells. We hypothesized that potential defects in the IFN pathway of these transformed cell lines could relieve repression of BHV-1 replication and the restricted growth properties of the 51g virus. Due to the instability of the 51g virus, reversion of the mutation to wt was a complication during the course of these experiments.

INTRODUCTION

The type 1 IFN response plays a major role in the recognition and defense against viral infections (Katze et al., 2002; Kawai and Akira, 2006; Samuel, 2001; Uematsu and Akira, 2006). Type 1 interferons (IFN-α, IFN-β) secreted by activated cells bind to specific receptors on the surface of nearby cells to induce expression of interferon stimulated genes (ISGs), which subsequently stimulate a cascade of innate immune signaling pathways. BHV-1 and human herpes simplex virus type 1 (HSV-1) share similar life cycle and biological properties, and replication of both viruses appears to be inhibited by IFN. As a countermeasure, these viruses encode analogous ICP0 proteins which have important biological functions that act against the IFN antiviral defense (Eidson et al., 2002; Lin et al., 2004b; Mossman and Smiley, 2002) Jones, 2009). HSV-1 infection is regulated by IFN pathways in mice (Halford et al., 2006; Leib et al., 1999), and HSV-1 genomes that do not express ICP0 are more sensitive to IFN treatment in cultured cells (Harle et al., 2002; Mossman et al., 2000). Expression of the HSV-1 encoded ICP0 protein can inhibit the repressive effects of ISGs on HSV-1 and improve replication efficiency (Everett and Orr, 2009).

BHV-1 encoded bICP0 plays a key role in controlling the IFN response by inhibiting the induction of IFN-β. bICP0 inhibits IFN-β transcriptional activation by affecting at least three major regulators of IFN-β expression: Interferon Regulatory Factor 3 (IRF3), IRF7, and p300 (Saira et al., 2007; Saira et al., 2009; Zhang et al., 2006). IFN-β is a branch of the type I IFN response pathway that plays a pivotal role in amplification of innate immunity and antiviral defense (Katze et al., 2002; Randall and

Goodbourn, 2008). IRF3 and IRF7 are key components of the IFN response pathway, because they act as co-activators of the IFN-β promoter (Hiscott, 2007). p300 is a co-activator of IRF3-dependent IFN signaling and other antiviral signaling cascades (Hiscott, 2007; Vo and Goodman, 2001; Weaver et al., 1998; Yoneyama et al., 1998). bICP0 is able to inhibit IFN-β promoter activity by inducing proteasome-dependent degradation of IRF3 (Saira et al., 2007). In addition, bICP0 associates with IRF7 and this interaction also inhibits activation of the IFN-β promoter (Saira et al., 2009). The interaction between bICP0 and p300 may also interfere with p300-stimulated antiviral signaling (Zhang et al., 2006). Furthermore, mice infected with BHV-1 do not display clinical symptoms; however, mice lacking type I and type II IFN receptors in the context of a *RAG-2* gene deletion do not survive BHV-1 infection (Abril et al., 2004).

In many types of cancers, the IFN signaling pathway does not function properly (Hanahan and Weinberg, 2000). This allows certain viruses that are inhibited by IFN to grow in certain tumor cell lines. This observation has given rise to the development of oncolytic viruses as viral vaccines that can be used for the treatment of cancers (Bourke et al., 2011; Vaha-Koskela et al., 2007). Oncolytic virotherapy is a promising means to treat certain non-operable tumors. Consequently, a number of viruses are being specifically engineered for this purpose (Cervantes-Garcia et al., 2008; Kelly and Russell, 2007). HSV-1 ICP0-null oncolytic vectors are being generated for the use of cancer therapy (Hummel et al., 2005), and more recently BHV-1 has been implicated as a novel oncolytic virus with promising application for cancer virotherapy (Rodrigues et al., 2010). Compared to HSV-1, BHV-1 has a very strict host range (Murata et al., 1999). BHV-1 does not grow in normal rodent or human cells, but can replicate in certain

immortalized and transformed human cell types (Hammon et al., 1963; Murata et al., 1999; Rodrigues et al., 2010). This property has made BHV-1 a promising candidate for use as an oncolytic viral vector which was recently proposed (Rodrigues et al., 2010). The ability of BHV-1 to propagate in a number of normal, immortalized, and transformed human cell lines was investigated, and BHV-1 was shown to preferentially replicate in cells that were either immortalized or transformed (Rodrigues et al., 2010).

In this study, we examined the replication of BHV-1 in a cancer cell line (U2OS) that have putative defects in its IFN pathway. We wanted to compare the growth potential of a bICP0 zinc RING finger mutant virus (51g) that has restricted growth properties in calves and cultured cells (Saira et al., 2008), with wt BHV-1 in atypical cells in which the IFN system was defective. We hypothesized that the restricted growth properties of the 51g mutant may, in part, be due to the inability of the virus to inhibit the repressive effects of the IFN pathway. We investigated whether the potential defects in the IFN pathway of these transformed cell lines could relieve inhibition of the 51g virus to replicate efficiently. Therefore, we set forth to examine the viral growth kinetics and gene expression profiles of wt BHV-1 and the 51g mutant virus in normal and cancer derived cells.

MATERIALS AND METHODS

Cells and virus

P19 murine embryonic teratocarcinoma cells, U2OS human osteocarcinoma cells, bovine kidney (CRIB) cells, and low passage bovine turbinate (BT) cells were grown in monolayer cultures in Earle's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), penicillin (10 U/ml), and streptomycin (100 mg/ml) in a humidified 5% CO2 atmosphere at 37°C. The wt BHV-1 Cooper strain was obtained from the National Veterinary Services Laboratory, Animal and Plant Health Inspection Services (Ames, IA). Construction of the BHV-1 bICP0 zinc RING finger domain mutant (51g) was described previously (Saira et al., 2008). BHV-1 Strains were propagated and titrated in CRIB cells. For infection studies, cells were plated onto 60 or 100 mm culture dishes 16 to 24 hours prior to virus infection. Total cells per plate were counted and the specified multiplicity of infection (MOI) calculated. Cells were infected with the wt BHV-1 or 51g at an MOI of 1.0, and were harvested at the indicated times after infection.

RNA extraction and reverse transcription (RT) PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Cat# 15596-018) as described by the manufacturer. RNA was treated with DNase I (Invitrogen) and subjected to reverse transcription using SuperScript III reverse transcriptase (Invitrogen) according to manufacture protocol. RNA was reverse transcribed using oligo(dT)

primers (Invitorogen). Ten percent of the generated cDNA was used as template for PCR using GoTaq DNA polymerase (Promega). The following primer sequences were used to amplify viral transcripts: bICP0 forward primer 5'-TGC AGT CTC TCA TCC ACA GC-3' and reverse primer 5'-GCG ACC CGG TCA ATA AAC T-3'; bICP4 forward primer 5'-CTG CAG GAG GAA CAG CTT CT-3' and reverse primer 5'-GTG TTC GTG CCG GAG ATG-3'; bICP22 forward primer 5'-GCG CTG GTC CTC CGG CTC C-3' and reverse primer 5'-CTC GCT GGC GGG GCT TGG-3'; thymidine kinase (TK) forward primer 5'-GCC GCC GTA CTG GAC ATG CG-3' and reverse primer 5'-GCC GAG TCC CCG TAA GGC GAT-3'; bTIF forward primer 5'-CCC AAG CCC CGT TCG CAG C-3' and reverse primer 5'-TGC CCG CCC GCC CTT AGA A-3'; glycoprotein C (gC) forward primer 5'-GAG CAA AGC CCC GCC GA AGG A-3' and reverse primer 5'-TAC GAA CAG CAG CAC GGG CGG-3'. Cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer 5'-CCA TGG AGA AGG CTG GGG-3' and reverse primer 5'-CAA AGT TGT CAT GGA TGA CC-3' was used as a control to insure equal loading. PCR products were analyzed on a 1.2% agarose gel.

One-step growth kinetics

U2OS or BT cells were plated to 100 mm dishes 16 to 24 hours prior to infection. Cells were infected at MOI 1, followed by absorption for 1 hour at 37 C in serum-free media. Cells were then washed with phosphate buffered saline (PBS) and replaced with 10% serum containing media. Supernatants containing virus were collected at 8, 16, 24,

and 48 hours after infection and subjected to three rounds of freeze-thaw cycles. Supernatants were clarified by centrifugation and titered on CRIB cells for plaque assay.

RESULTS

Expression profiles of wt and a mutant BHV-1 in normal or cancer cells.

To examine the kinetics of wt BHV-1 and the 51g mutant virus in these cultured cell subsets, we analyzed viral gene expression by measuring transcript levels of viral genes. During BHV-1 productive infection, viral genes are temporally regulated and classified as immediate early (IE), early (E), or late (L) genes. Genes representative of each gene class were selected and mRNA transcript levels detected by RT-PCR. Primers from previous studies were used to detect transcripts of the IE genes bICP0, bICP4, and bICP22, E gene TK, and L genes bTIF and gC. Primary bovine turbinate (BT) cells and human osteocarcinoma (U2OS) cells were infected at an MOI of 1 and RNA was extracted after 4, 8, 16, or 24 hours of infection. Reverse transcription PCR was performed and expression of viral transcripts normalized to cellular GAPDH levels. Viral transcripts were readily detected in both U2OS and BT cells infected with wt BHV-1 or the 51g virus (Figure 1). Transcript levels of the genes analyzed appeared to be generally higher in U2OS cells infected with wt BHV-1 compared to 51g infected cells, especially at 4 and 8 hours after infection (Figure 1A). This result was opposite to what was observed in BT cells at these time points (Figure 1B). At 16 and 24 hours post infection (hpi) in U2OS and BT cells, all transcript levels analyzed, with the exception of bTIF, were expressed at comparable levels by both viruses (Figure 1A and 1B). Viral genes bICP22, gC, and to a slightly lesser extent TK were expressed at similar levels by both viruses at all time points in U2OS and BT cells (Figure 1A and 1B). Results from

these experiments did not appear to particularly agree with our hypothesis, but were inconclusive due to reversion or contamination of the 51g mutant virus. Upon noticing phenotypic growth changes of the 51g mutant, viral genomic DNA was extracted, the region containing the mutation amplified by PCR, and sent for sequencing. Sequencing results revealed loss of the 51g mutation, explaining the inconsistency in our results. Thus, we are unable to draw definitive conclusions from these experiments at the current time. In addition to BT and U2OS cells, a murine teratocarcinoma cell line (p19) was also examined; however, viral transcripts were not detected from these cells infected with either virus, indicating these cells were not permissive for BHV-1 infection (data not shown).

Growth kinetics of BHV-1 and 51g in normal and cancer cells.

The growth kinetics of wt BHV-1 and the 51g mutant virus in normal or cancer cells was then evaluated by plaque assay. U2OS and BT cells were infected at MOI of 1 in serum-free media followed by replacement with media containing 10% serum after one hour incubation. Supernatants containing virus produced from the infected cells were collected at 8, 16, 24, and 48 hpi and subjected to three rounds of freeze-thaw cycles. Supernatants were cleared by centrifugation and titers for plaque assay were performed in CRIB cells. Results of titers from two independent experiments in U2OS and BT cells are shown in Figure 2. We observed that the 51g mutant grew at slightly higher titers in U2OS cells than BT cells compared to wt BHV-1 at earlier times after infection (Figure 2A). By 24 hpi, the 51g mutant and wt BHV-1 produced similar titers in U2OS cells

(Figure 2A). Conversely, in BT cells, higher titers of wt BHV-1 were observed compared to the 51g virus (Figure 2B). This observation suggests that the 51g mutant may replicate more efficiently in U2OS cells than wt BHV-1. In both cell lines tested, the 51g virus had reduced growth potential compared to wt BHV-1 which had increased titers over time (Figure 2A and 2B). The 51g virus produces well-defined plaques that fail to spread (Saira et al., 2008). Therefore, impaired cell-to-cell spread of the 51g virus could partially explain the reduced growth kinetics observed in these experiments (Figure 2). Because we do not know exactly when the 51g mutant virus reverted, these experiments must be repeated to confirm these results. Nonetheless, these experiments do confirm that BHV-1 can grow in U2OS cells, but at reduced levels compared to infected permissive bovine BT cells.

DISCUSSION

The restriction of BHV-1 replication in bovine cells (Murata et al., 1999), the observation that disruption of type I and type II IFN signaling in mice abolishes this restriction (Abril et al., 2004), and the ability of BHV-1 to preferentially replicate in transformed cells with defects in their IFN pathway (Hammon et al., 1963; Rodrigues et al., 2010) suggest that the IFN response serves as an effective barrier to infection by BHV-1 and may contribute to its ability to infect transformed cells outside of its host range. A recent study failed to correlate cellular IFN signaling status and permissiveness for BHV-1, and instead suggested that BHV-1 replication may be particularly sensitive to cell cycle regulation (Rodrigues et al., 2010). What determines BHV-1 host restriction and the mechanism by which BHV-1 induces oncolysis of human cells remains to be elucidated, but these phenomena likely depended on a collection of different factors including both IFN signaling and cell cycle regulators.

BHV-1 genomes that fail to express the bICP0 protein or contain mutations within the zinc RING finger domain of bICP0 (51g) have defective replication and virulence properties in cattle, and are not stable in culture (Geiser et al., 2005; Saira et al., 2008). The 51g mutant virus grows poorly in permissive bovine cells under culture conditions (Saira et al., 2008). Calves infected with 51g produced 2 to 3 logs lower levels of infectious virus, exhibited reduced clinical symptoms, and did not reactivate from latency following dexamethasone infection which induces reactivation in cattle 100 percent of the time (Saira et al., 2008). BHV-1 viruses that are less virulent and that do not reactivate from latency are attractive candidates for the development of better BHV-1

vaccines and safer oncolytic viral vector-related therapies. Propagation of growth defective viruses, such as 51g, remains to be a challenge. While wt BHV-1 is readily propagated in culture, it is difficult to obtain high titers of the 51g mutant virus and consequently, repeated passage and concentration of the virus is necessary. We were interested to see if cells with known defects in their IFN signaling could alleviate the reduced growth potential of the 51g virus. These studies confirm that BHV-1 and the 51g mutant virus can grow in U2OS cells and viral gene expression could be readily detected, albeit at reduced levels compared to infected permissive bovine (BT) cells (Figures 1 and 2).

The impaired growth ability of 51g increases the selective pressure for mutations that rescue this growth defect. During the course of these studies, the phenotypic growth properties of the 51g mutant became less apparent; therefore, sequencing of the viral genomic DNA of the mutant was performed to verify the presence of the 51g mutation. The sequencing results revealed loss of the 51g mutation on two separate occasions. Reversion of the 51g mutation to wild type was an impediment to the progress of these experiments and thus, additional experiments are warranted to validate the results obtained and presented in this chapter. The instability of the mutation within this highly conserved functional motif clearly indicates that the preservation of this cysteine residue is crucial for the integrity of the zinc RING finger domain. Moreover, the phenotype of the 51g virus further underscores the significant role bICP0 plays in the success of viral infection and the requirement of this particular motif for the critical functions of bICP0.

FIGURE 1

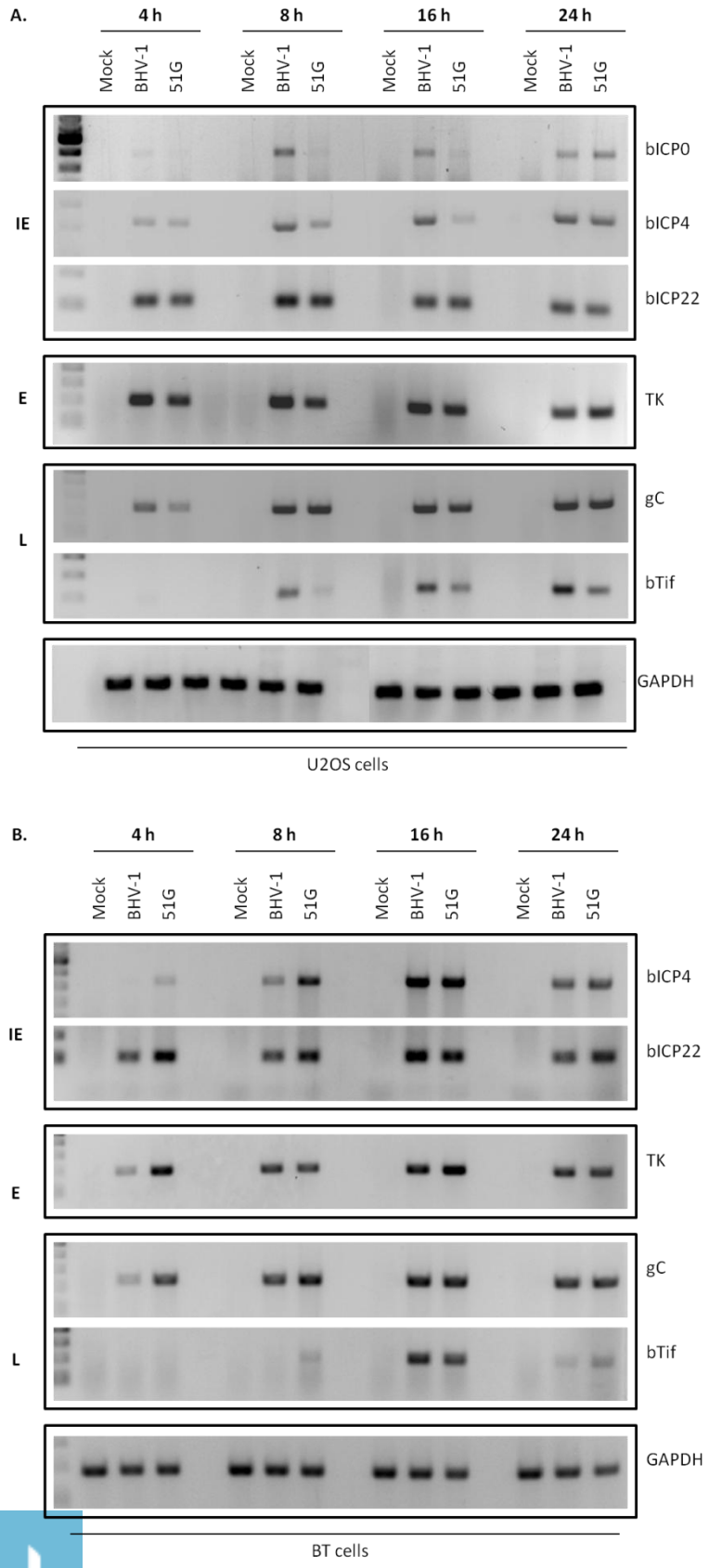


Figure 1. Expression profiles of wt and a mutant BHV-1 in cancer or normal cells. Human osteocarcinoma (U2OS) cells (**A**) and primary bovine turbinate (BT) cells (**B**) were infected with wt BHV-1 or the 51g virus. RNA was extracted from infected and mock infected cells at the indicated times after infection and RT-PCR performed. PCR products were analyzed on a 1.2% agarose gel. The viral transcripts evaluated are denoted on the right, and the class of each gene is indicated on the left. Cellular GAPDH was used to insure equal loading.

FIGURE 2

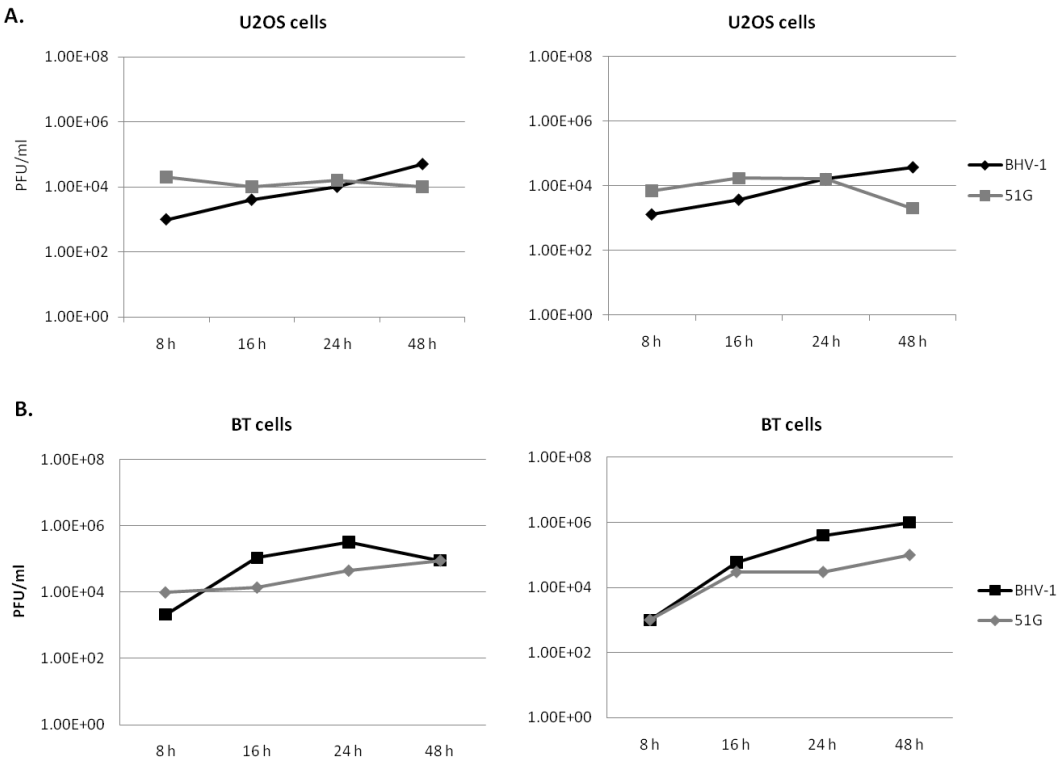


Figure 2. Growth of BHV-1 and the 51g mutant in normal and cancer cells. U2OS cells (**A**) and BT (**B**) were infected at 1 MOI with wt BHV-1 or the 51g virus. Supernatants containing virus produced from the infected cells were used for viral titration and plaque assay. Graphs represent two independent experiments for each cell type.

GENERAL CONCLUSIONS

BHV-1 is an economically important pathogen of cattle and although vaccines are available, better vaccines are warranted. Current modified live vaccines cause immune suppression in cattle and can lead to serious disease in young calves and abortions in pregnant cows (Jones and Chowdhury, 2007; van Drunen Littel-van den Hurk, 2006). Furthermore, these vaccines can reactivate from latency and be transmitted to susceptible cattle (Ellis et al., 2005; Muylkens et al., 2007; van Drunen Littel-van den Hurk et al., 2001). There has also been interest to develop BHV-1 as a vector to deliver antigens to cattle for vaccine purposes, and for use as an oncolytic vector for targeting treatment of human cancers (Rodrigues et al., 2010). Although *Alphaherpesvirinae* subfamily members share similar biological properties, BHV-1 has evolved its own mechanisms by which it is able to establish a successful infection. Further evaluation of the biology of BHV-1 is necessary to understand the underlying molecular events related to infection by this virus. Thus, the reasons stated above make BHV-1 biology a relevant area of study.

BHV-1 encoded bICP0 is a multifunctional protein that is critical for efficient replication, virulence, and reactivation from latency (Geiser et al., 2005; Saira et al., 2008). A number of important functions have been attributed to bICP0 (Jones, 2009); however, the underlying mechanisms by which bICP0 carries out these functions are not well understood. In order to gain insight into the molecular mechanisms and other potential functions of bICP0, coaffinity purification of bICP0 and mass spectrometry analysis of bICP0 bound proteins was performed. Although we have not confirmed any

novel bICP0 binding proteins from these mass spectrometry results, our initial results clearly showed visibly unique protein bands. This is promising for identifying bICP0 associating proteins and would contribute to our understanding of the underlying functional mechanisms of bICP0.

The C_3HC_4 zinc RING finger motif is a critical functional domain of bICP0 that possesses intrinsic E3 ubiquitin ligase activity (Diao et al., 2005; Everett et al., 2010). Although these studies did not identify any novel proteins targeted by bICP0 for ubiquitination, they did demonstrate that bICP0 increases the level of protein polyubiquitination. These studies also demonstrated that an intact zinc RING finger domain is important for regulating bICP0 protein stability, potentially by catalyzing its own ubiquitination. Other sequences toward the C-terminus of bICP0 also appear to play a role in stability of the bICP0 protein. These studies demonstrated that deletion of sequences at the C-terminus of bICP0 alters bICP0 localization, levels, and cell morphology (Gaudreault and Jones, 2011).

PML protein levels were dramatically reduced in a proteasome-dependent manner in bovine cells following infection with BHV-1 or expression of bICP0 in the absence of other viral genes (Gaudreault and Jones, 2011). Because the underlying function of PML is still unclear, it is difficult to exactly define what the reduction of PML does for the virus. However, PML is known to promote apoptosis (Bernardi and Pandolfi, 2003;(Giorgi et al., 2010) and antiviral responses (Everett and Chelbi-Alix, 2007; Geoffroy and Chelbi-Alix, 2011; Tavalai and Stamminger, 2008), and these studies demonstrated that over-expression of PML appears to correlate with reduced BHV-1

replication. Although the zinc RING finger domain was found to be important for efficient PML degradation, altering the localization of bICP0 did not appear to affect its ability to reduce PML levels. This suggests that either bICP0 induces degradation of PML by an indirect mechanism or that bICP0 interacts with PML in the cytoplasm. Cytoplasmic PML has been shown to be important for regulating apoptosis (Giorgi et al., 2010) and in the resistance to viral infections (McNally et al., 2008), giving precedence for bICP0 to interact with PML in the cytoplasm.

Furthermore, bICP0 was found to be present in the cytoplasm of primary low-passage bovine cells at later times of BHV-1 productive infection (Gaudreault and Jones, 2011). This implicates a role for cytoplasmic bICP0 during infection, and suggests late viral proteins or induced factors may sequester bICP0 to the cytoplasm of productively infected cells. Recently, other work from our lab has shown cytoplasmic bICP0 can inhibit IFN-β promoter activity and induce degradation of IRF3 as efficiently as wt bICP0 (Da Silva and Gaudreault, unpublished). Thus, this data suggests a role for cytoplasmic bICP0 in repressing the innate immune response and altering cell fate after infection.

Attempts were also made to determine if the impaired replication potential of the bICP0 zinc RING finger mutant virus (51g) could be rescued in cells with defects in their IFN signaling. Although the reversion of the 51g mutation to wild type was an impediment to the progress of these experiments, the instability of the mutation within this highly conserved functional motif clearly indicates that the preservation of this cysteine residue is essential for the integrity of the zinc RING finger domain. Moreover,

the phenotype of the 51g virus further underscores the significant role bICP0 plays in the success of viral infection and the requirement of this particular motif for the critical functions attributed to bICP0.

In conclusion, the studies presented in this dissertation provide further evidence that will contribute to a better understanding of the role of BHV-1 encoded bICP0 during productive infection and the mechanisms underlying its critical functions.

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