Infected cell protein 0 encoded by bovine herpesvirus 1 can activate caspase 3 when overexpressed in transfected cells

Gail Henderson, Yange Zhang, Melissa Inman, Dallas Jones and Clinton Jones

Department of Veterinary and Biomedical Sciences, Nebraska Center for Virology, University of Nebraska, Lincoln, NE 68503, USA

Infection of cattle or bovine cells with bovine herpesvirus 1 (BHV-1) leads to increased apoptosis. Previous studies indicated that BHV-1 infected cell protein 0 (bICP0), the major transcriptional regulatory protein of BHV-1, is toxic in transiently transfected cells. Point mutations within the zinc RING finger of bICP0 reduced toxicity and eliminated the ability of bICP0 to activate viral gene expression. In mouse neuroblastoma cells (neuro-2A) and bovine turbinate cells, bICP0 activated caspase 3, a key regulatory protein in the apoptotic pathway. A pro-apoptotic gene (Bax), but not bICP0, induced caspase 3 cleavage and activation by 8 h after transfection of neuro-2A cells. Conversely, bICP0 or the N-terminal 356 aa of bICP0 did not induce caspase 3 cleavage in neuro-2A cells until 30 h after transfection, suggesting that bICP0 stimulates caspase 3 cleavage by an indirect mechanism. These studies indicate that the toxic functions of bICP0 correlate with caspase 3 cleavage and activation.

Bovine herpesvirus 1 (BHV-1) infection can cause conjunctivitis, pneumonia, genital disorders, abortions and an upper respiratory tract infection referred to as ‘shipping fever’ (Jones, 2003; Tikoo et al., 1995). Infection of cattle (Lovato et al., 2003; Winkler et al., 1999) or permissive bovine cells (Devireddy & Jones, 1999) with BHV-1 leads to rapid cell death, in part due to apoptosis. Viral gene expression is temporally regulated in three distinct phases: immediate-early (IE), early (E) and late (L). IE transcription unit 1 (IEtu1) encodes the BHV-1 infected cell protein 0 (bICP0) (Fig. 1a), which activates all three classes of viral genes (Wirth et al., 1991). bICP0 is expressed throughout productive infection because the gene has an IE promoter and a separate E promoter located at exon 2 (Wirth et al., 1992) (Fig. 1a).

Most alphaherpesviruses, including BHV-1, encode a bICP0-like transcriptional activator that contains a well-conserved C3HC4 zinc RING finger domain near the N terminus of these proteins (Everett et al., 1993, 1995; Everett, 1988; 2000; Lium & Silverstein, 1997). Mutational analysis has demonstrated the importance of the zinc RING finger domain in herpes simplex virus 1 (HSV-1) ICP0 (Everett et al., 1993, 1995; Everett, 1988, 2000; Lium & Silverstein, 1997), equine herpesvirus 1 ICP0-like protein (Bowles et al., 1997, 2000) and bICP0 (Inman et al., 2001). These ICP0 homologues transactivate all classes of viral genes (Bowles et al., 1997; Fraefel et al., 1994; Koppel et al., 1996, 1997; Lium et al., 1998). HSV-1 ICP0 (Everett et al., 1997, 1999a; b; Maul & Everett, 1994; Maul et al., 1993) and bICP0 (Inman et al., 2001; Parkinson & Everett, 2000) co-localize with and disrupt the proto-oncogene promyelocytic leukaemia protein-containing nuclear domains (ND10 or PODS). ICP0 can regulate steady-state levels of certain proteins (Everett et al., 1997, 1999a) because it has E3 ubiquitin ligase activity (Boutell et al., 2002; Van Sant et al., 2001). A recent study has demonstrated that ICP0 induces ubiquitination of the tumour suppressor protein p53 (Boutell & Everett, 2003). The E ubiquitin ligase activity of ICP0 results in perturbation of the cell cycle and altered cellular gene expression (for example, p21, gadd45 and mdm-2; Hobbs & DeLuca, 1999).

Our previous studies demonstrated that bICP0 induced cell death in transient transfection assays (Inman et al., 2001). However, these studies did not test how bICP0 killed these cells. There are at least three forms of programmed cell death – necrosis, apoptosis and paraptosis (Sperandio et al., 2000). Caspase 3 is induced when apoptosis occurs but not when necrosis or paraptosis occurs. Two major apoptotic pathways exist in mammals – the death receptor-mediated pathway and the mitochondrial pathway (Kruegger et al., 2001; Schmitz et al., 2000; Wang, 2001). Regardless of which pathway is activated, caspase 3 is cleaved and activated. Consequently, testing whether bICP0 induced caspase 3 cleavage would distinguish whether the toxic effects of bICP0 were caused by apoptosis, necrosis or paraptosis. For the studies presented below, three plasmids that expressed bICP0 or mutant forms of the protein (bICP0, ΔICP0 or 13G/51A; Fig. 1b) were utilized. The respective bICP0
plasmids expressed Flag-tagged proteins in transfected mouse neuroblastoma (neuro-2A) cells that migrated as expected (Fig. 1c). We consistently observed that slightly lower levels of wt bICP0 protein were expressed in neuro-2A cells compared with the ΔbICP0 and 13G/51A protein levels in transfected neuro-2A cells. Since expression of the respective bICP0 proteins was regulated by the human cytomegalovirus (CMV) promoter, subtle differences in protein expression were not related to differences in the promoters used in the respective plasmid constructs. Neuro-2A cells were used for these studies because they are derived from the peripheral nervous system (Olmsted et al., 1970) and thus may be a good model to examine the cytotoxicity of bICP0 in neurons.

To test whether bICP0 activated caspase 3, we co-transfected neuro-2A cells with one of the bICP0 expression plasmids and the pCaspase3-Sensor plasmid (Clontech). As controls, neuro-2A cultures were co-transfected with a CMV plasmid expressing the pro-apoptotic protein Bax (Upstate Biotechnology) or a blank CMV expression vector (pcDNA3.1 —) and the pCaspase3-Sensor plasmid. Plasmid pCaspase3-Sensor encodes the enhanced yellow-green variant of the green fluorescent protein (GFP) and has three copies of the SV40 large T antigen nuclear localization signal at the 3’ end of the GFP gene. At the 5’ terminus of the GFP gene, a sequence encoding the nuclear export signal (NES) of the mitogen-activated protein kinase is located. The NES is separated from GFP coding sequences by a 36 bp cassette containing the region of poly(ADP-ribose) polymerase cleaved by caspase 3. As expected, GFP was primarily localized to the cytoplasm of healthy cells (Fig. 2a). When neuro-2A cells were co-transfected with the CMV Bax expression plasmid and the pCaspase3-Sensor plasmid,
caspase 3 was activated, the NES was cleaved and GFP had localized to the nucleus of transfected cells by 30 h post-transfection (Fig. 2b). Bax was used as a positive control for apoptosis induction because it localizes to the mitochondria and induces cytochrome C and Smac/Diablo release, which results in caspase 3 cleavage and activation (Wang, 2001). Neuro-2A cells transfected with ΔbICP0 (Fig. 2c) or bICP0 (Fig. 2d) appeared to contain more cells with GFP localized to the nucleus relative to cells transfected with the blank expression vector (Fig. 2a).

The percentage of apoptotic cells in the respective samples was calculated from three independent studies by determining the percentage of GFP+ cells that contained nuclear localization. Neuro-2A cells (4 x 10^5) in a 35 mm dish were co-transfected with 0.5 μg pCaspase3-Sensor plasmid and 0.25 μg of the designated plasmids. Transfection was carried out using TransIT transfection reagents (Mirus) as described by the manufacturer. Five hours after the neuro-2A cultures had been transfected, cultures were rinsed with serum-free medium (Eagle’s MEM), and fresh medium containing 10% fetal calf serum and 2.5 mM sodium butyrate was added and cultures incubated overnight. The following morning, cultures were rinsed with medium containing 10% fetal calf serum and fresh medium was added. The CMV Bax expression vector expresses the pro-apoptotic protein Bax (b). Representative cells are shown 30 h post-transfection with the blank expression vector pcDNA3.1– (a), ΔbICP0 (c) or a bICP0 expression plasmid (d). GFP+ cells were viewed with a Leica fluorescence microscope. (e) Determination of apoptotic cells in transiently transfected neuro-2A cells. Neuro-2A cells (4 x 10^5) in 35 mm dishes were transfected with the designated plasmids as described above. The percentage of non-apoptotic cells (number of cells containing a GFP signal in the cytoplasm divided by the total number of GFP+ cells) was determined at 30 h post-transfection by counting the number of GFP+ cells in a given area. The values obtained are the mean of three independent experiments. At least 300 GFP+ cells were counted for each respective sample in each experiment.

**Fig. 2.** bICP0 induces caspase 3 activation. Neuro-2A cells (4 x 10^5) in a 35 mm dish were co-transfected with 0.5 μg pCaspase3-Sensor plasmid and 0.25 μg of the designated plasmids. Transfection was carried out using TransIT transfection reagents (Mirus) as described by the manufacturer. Five hours after the neuro-2A cultures had been transfected, cultures were rinsed with serum-free medium (Eagle’s MEM), and fresh medium containing 10% fetal calf serum and 2.5 mM sodium butyrate was added and cultures incubated overnight. The following morning, cultures were rinsed with medium containing 10% fetal calf serum and fresh medium was added. The CMV Bax expression vector expresses the pro-apoptotic protein Bax (b). Representative cells are shown 30 h post-transfection with the blank expression vector pcDNA3.1– (a), ΔbICP0 (c) or a bICP0 expression plasmid (d). GFP+ cells were viewed with a Leica fluorescence microscope. (e) Determination of apoptotic cells in transiently transfected neuro-2A cells. Neuro-2A cells (4 x 10^5) in 35 mm dishes were transfected with the designated plasmids as described above. The percentage of non-apoptotic cells (number of cells containing a GFP signal in the cytoplasm divided by the total number of GFP+ cells) was determined at 30 h post-transfection by counting the number of GFP+ cells in a given area. The values obtained are the mean of three independent experiments. At least 300 GFP+ cells were counted for each respective sample in each experiment.

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<tr>
<th>Plasmid</th>
<th>GFP+ cells in nucleus (%)</th>
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<tr>
<td>Blank</td>
<td>15±10</td>
</tr>
<tr>
<td>bICP0</td>
<td>60±7</td>
</tr>
<tr>
<td>ΔbICP0</td>
<td>78±9</td>
</tr>
<tr>
<td>13G/51A</td>
<td>33±12</td>
</tr>
<tr>
<td>Bax</td>
<td>86±6</td>
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GFP (Fig. 2e). When cells were transfected with bICP0 for 30 h, approximately 60% of the GFP+ cells contained the signal in the nucleus, whereas only 15% of the cells transfected with the blank expression vector contained GFP in the nucleus (Fig. 2e). The ΔbICP0 construct resulted in 78% of the GFP+ cells having nuclear localization by 30 h post-transfection. An intact zinc RING finger played a role in activating caspase 3, since neuro-2A cultures transfected with the 13G/51A construct had 33% of cells with nuclear-localized GFP. As expected, in cells transfected with Bax, more than 85% of the cells were apoptotic. A Tukey–Kramer multiple comparisons post-NOVA test revealed a significant difference between the 13G/51A construct and wt bICP0, the ΔbICP0 construct or Bax (P<0.001). Furthermore, the percentage of cells expressing activated caspase 3 following transfection with 13G/51A was significantly different compared with cells transfected with the blank expression vector (P<0.01).

The results in Fig. 2 suggested that caspase 3 was activated by bICP0 and that cells transfected with the ΔbICP0 construct exhibited a higher frequency of caspase 3 cleavage and activation. To confirm this observation, we examined caspase 3 cleavage in transiently transfected neuro-2A cells (Fig. 3a). Caspase 3 cleavage requires proteolytic processing at Asp-175 into a 19 kDa (pro-domain plus large fragment) and 17 kDa fragment (large fragment) (Nicholson & Thornberry, 1997; Thornberry et al., 1997; Wang, 2001; Wolf & Green, 1999). An antibody that specifically recognizes the 17 and 19 kDa fragments of cleaved caspase 3 (catalogue #9661; Cell Signalling) was used for this study. Relative to neuro-2A cultures transfected with pcDNA3.1−, cultures transfected with bICP0 or ΔbICP0 contained higher levels of cleaved caspase 3 at 30 h post-transfection (Fig. 3a). Cells transfected with ΔbICP0 or Bax contained similar levels of cleaved caspase 3. Increasing the concentration of ΔbICP0 plasmid did not increase the levels of cleaved caspase 3. We believe that increasing the concentration of ΔbICP0 led to increased cell lysis and, consequently, we were unable to observe higher levels of cleaved caspase 3 at 30 h post-transfection. The zinc RING finger mutant (13G/51A) contained lower levels of cleaved caspase 3 compared with

![Fig. 3. bICP0 induces caspase 3 cleavage. Neuro-2A cells (1 x 10⁶) in a 60 mm dish were transfected with the designated bICP0 plasmids (0.5 or 2 μg DNA) as described in Fig. 2 or a blank expression vector (pcDNA3.1−; lane B). As a positive control, cultures were also transfected with 1.5 μg CMV Bax. At 30 h post-transfection, total cell lysate was prepared as described by the supplier of the cleaved caspase 3 antibody using their cell lysis buffers. (a) Western blot analysis was performed with an antibody that recognizes cleaved caspase 3. (b) Western blot analysis was performed with an antibody that recognizes β-actin (Santa Cruz Biotechnology). The arrow denotes the position of β-actin. (c) Cells were transfected as described above using 2 μg DNA and cell lysate prepared at 8 h post-transfection. Western blot analysis was performed with an antibody that recognizes cleaved caspase 3. (d) BT cells were transfected with 2 μg of the designated plasmids. At 24 h post-transfection, cell lysate was prepared and Western blot analysis performed. The arrows denote the location of cleaved caspase 3 in (a), (c) and (d). The size of pro-caspase 3 is approximately 37 kDa and is denoted by an oval. For the Western blots in (a–d), approximately 100 μg protein was loaded in each lane. The results are representative of three different experiments.]
cultures transfected with bICP0, which was consistent with the results in Fig. 2. Neuro-2A cells transfected with 13G/51A contained higher levels of cleaved caspase 3 compared with cultures transfected with the blank expression vector. Regardless of the plasmid used for transfection, similar levels of β-actin were present in the cells at 30 h post-transfection (Fig. 3b). bICP0 protein levels were also similar at 30 h post-transfection because the respective bICP0 constructs were cloned into a human CMV IE expression vector (Inman et al., 2001) (for example, see Fig. 1c).

We analysed caspase 3 cleavage at 8 h post-transfection because we predicted that if bICP0 directly induced caspase 3 cleavage, higher levels of the cleaved forms of caspase 3 would be present relative to mock-transfected cells. bICP0, ΔbICP0, 13G/51A and cells transfected with the blank expression vector (Fig. 3, lane B) did not contain readily detectable levels of cleaved caspase 3 at 8 h post-transfection (Fig. 3c). In contrast, cleaved caspase 3 was detected in cells transfected with Bax at 8 h post-transfection (Fig. 3). These studies indicated that wt bICP0 or ΔbICP0 induced caspase 3 cleavage in neuro-2A cells much later than Bax.

A low-passage-number bovine cell line, bovine turbinate (BT) cells, was also transfected with the bICP0 constructs or Bax and caspase 3 cleavage was monitored by Western blot analysis (Fig. 3d). The bICP0 constructs induced higher levels of caspase 3 cleavage in BT cells at 24 h post-transfection relative to cells transfected with the blank expression vector. At 24 h post-transfection, similar levels of β-actin and the respective bICP0 proteins were present in the samples obtained from transfected BT cells (data not shown). In contrast to the results with neuro-2A cells, the 13G/51A construct appeared to induce similar levels of cleaved caspase 3 to wt bICP0 or the ΔbICP0 construct, suggesting that certain cells are more sensitive to the toxic effects of bICP0. When a human cell line (293) that expresses adenovirus E1A and E1B proteins was transfected with ΔbICP0, an increase in cleaved caspase 3 was detected (data not shown). However, wt bICP0 did not consistently have a dramatic effect on caspase 3 cleavage. Collectively, these results suggested that cell type-specific factors regulate the ability of bICP0 to induce caspase 3 cleavage and cell death.

Our results suggested that ΔbICP0 was more efficient at activating caspase 3 in neuro-2A and 293 cells because ΔbICP0 lacked certain functions that wt bICP0 possessed. A previous study demonstrated that ΔbICP0 was predominantly localized to the cytoplasm of transfected neuro-2A cells, whereas bICP0 was primarily localized to the nucleus (Inman et al., 2001), suggesting that interactions between cytoplasmic factors and bICP0 stimulated toxicity and apoptosis. The plasmid ΔbICP0 does not activate an HSV-1 thymidine kinase promoter (Inman et al., 2001; Zhang & Jones, 2000), which implies that the ability of bICP0 to activate transcription plays a role in cell survival. Finally, these studies indicated that the ability of bICP0 to activate caspase 3 cleavage occurred by an indirect mechanism, since caspase 3 cleavage was slow relative to Bax. Regardless of the mechanism by which bICP0 stimulates caspase 3 cleavage, these studies strongly suggest that the ability of bICP0 to promote caspase 3 cleavage plays an important role with respect to the toxic effects of bICP0.

During BHV-1 productive infection, caspase 3 is activated (Lovato et al., 2003), but induction of apoptosis is a relatively late event (Devireddy & Jones, 1999), which correlates well with the finding that bICP0 activates caspase 3 cleavage more slowly than Bax. Alphaherpesviruses encode several anti-apoptotic proteins during productive infection (Aubert & Blaho, 2001; Jones, 1998, 2003), suggesting that the ability of bICP0 to induce caspase 3 cleavage is delayed during productive infection. In general, it is believed that virally induced apoptosis and/or caspase activation is detrimental for virus replication and spread. However, there are examples of caspase activation promoting virus replication. For example, caspase cleavage of a non-structural protein stimulates replication of Aleutian mink disease parvovirus in cultured cells (Best et al., 2003). Caspase 3 can also enhance HSV-1 reactivation from latency when latently infected neurons are explanted (Hunsperger & Wilcox, 2003). Since bICP0 promotes reactivation from latency (Jones, 2003), the ability of bICP0 to activate caspase 3 may stimulate reactivation from latency and/or productive infection.

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References


