The Effect of Bovine Herpesvirus Type 1 Glycoproteins gI and gIII on Herpesvirus Infections

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SUMMARY

We expressed the bovine herpesvirus type 1 (BHV-1) glycoproteins, gI and gIII, in bovine cells using a bovine papillomavirus vector. The proteins expressed by these cells had the same Mr as the native BHV-1 proteins and monoclonal antibodies detected no differences in their antigenic structure. Cells expressing gI were infected with either BHV-1 or herpes simplex virus type 1 (HSV-1). The number of plaques in gI-expressing cells was similar to that seen with normal fibroblasts infected with BHV-1 or HSV-1. However, BHV-1 or HSV-1 plaques produced in gI-expressing cells were smaller and darker than those seen in normal fibroblasts indicating an interference with cell-to-cell transmission or cellular lysis. Virus growth curves and [35S]methionine labelling of BHV-1-infected gI-expressing cells showed no difference in virus production, virus protein synthesis or cellular protein shutdown when compared to BHV-1-infected normal cells. This led us to conclude that the gI protein may interfere with a cellular protein(s) responsible for the cytopathic effects of BHV-1 infection. Cells expressing gIII were fully susceptible to BHV-1 infection.

Bovine herpesvirus type 1 (BHV-1), an alphaherpesvirus, is responsible for infectious bovine rhinotracheitis and infectious pustular vulvovaginitis in cattle (McKercher, 1978; Thomson, 1980; Yates, 1982). Work on BHV-1 envelope glycoproteins has focused on determining their function in cell–virus interaction and their importance in the bovine immune response to BHV-1. Three BHV-1 glycoproteins, gI, gIII and gIV, have been isolated (Marshall et al., 1986; van Drunen Littel-van den Hurk et al., 1984, 1985), molecularly cloned and mapped (Zamb, 1987). The specific functions of these BHV-1 glycoproteins in viral infection and virus–cell interactions have not been well defined. Murine cells expressing gI fuse and form polykaryons (Fitzpatrick et al., 1988b). Work by Okazaki et al. (1987) suggested that gIII may be the viral attachment protein. Bovine antigen-presenting cells contain a 45K to 60K protein that cross-reacts with antibodies directed against gIII. The possible role of this molecular mimicry of gIII in the pathogenesis of BHV-1 infection is under investigation (Fitzpatrick et al., 1988a).

The herpes simplex virus type 1 (HSV-1) glycoproteins gB and gC have been shown to be homologous to BHV-1 gI and gIII, respectively (Misra et al., 1988; Zamb, 1987). The role of gB and gC in infection and cell–virus interactions has been thoroughly investigated (Spear, 1985). HSV-1 gB is essential for penetration of virus into cells and plays a role in cell fusion (Spear, 1985). HSV-1 gC is not essential for productive HSV-1 infection but has a role in formation of C3b receptors on infected cells (Spear, 1985).

The BHV-1 glycoproteins elicit antibodies that neutralize the virus and lyse infected cells in the presence of complement (Gerber et al., 1978; Lupton & Reed, 1980; Marshall et al., 1988). Neither active (Israel et al., 1988) nor passive (Marshall & Letchworth, 1988) immunization with these glycoproteins protects calves against BHV-1 infection but calves are protected...
against death from bovine respiratory disease on challenge with a combined infection of BHV-1 and Pasteurella haemolytica (Babiuk et al., 1987).

We constructed an inducible expression system to produce gI and gIII in bovine cells for use in glycoprotein functional assays and as immunogens. Our system used a bovine papillomavirus type 1 (BPV-1) vector containing the heavy metal-inducible mouse metallothionein (MMT) promoter. In this communication we report the derivation and characterization of these expressed proteins and the effect they have on infection with BHV-1 and HSV-1.

Recombinant DNA plasmids were constructed using standard cloning methods (Maniatis et al., 1982). The plasmid pBPV-B#1 was constructed by ligating the gI gene (Zamb, 1987) into p341 (Eiden et al., 1985) between the MMT promoter and the simian virus 40 (SV40) polyadenylation site. This plasmid was ligated to the BPV fragment of BamHI-digested pNeoBPV100 (Lusky & Botchan, 1984). This ligation resulted in the plasmid pBPV-B#1 containing the gI gene, the MMT promoter, the SV40 polyadenylation site and the entire genome of BPV (Fig. 1a). Plasmid pBPV-C#1 was constructed by ligating the gIII gene (Zamb, 1987) into p341 (Eiden et al., 1985). The resultant plasmid, pMC10B, was digested, and the fragment containing the MMT promoter, gIII gene and SV40 polyadenylation site was isolated and ligated into the BamHI site of pCGBPVgAB5 (Matthias et al., 1983). Plasmid pCGBPVgAB5 contains the entire genome of BPV-1 and a neomycin phosphotransferase gene providing G418 resistance in eukaryotic cells. Ligation of the gIII gene, MMT promoter and SV40 polyadenylation site into this BPV plasmid resulted in the plasmid pBPV-C#1 (Fig. 1b).

Bovine fibroblasts were used as the eukaryotic host for selection and expression of the recombinant plasmids. Fibroblasts harvested from bovine skin (Freshney, 1983) and grown in MEM (Gibco) containing 5% foetal calf serum were plated at a density of 2.5 x 10^6 cells/25 cm² flask. These cells were transfected with 18 μg of pBPV-B#1 and 2 μg of pSV2neo (Southern & Berg, 1982) or 1 μg of pBPV-C#1 using the calcium phosphate precipitation method (Graham & van der Eb, 1973) except that the cells were osmotically shocked with 15% glycerol for 2 min, 3.5 h after the addition of the precipitate. Cells were grown in medium containing 500 μg/ml of active G418 for 2 to 3 weeks. G418-resistant foci of fibroblasts were isolated and cloned using an agarose overlay (Anonymous, 1980). These resistant clones were screened in an indirect immunofluorescence assay using anti-BHV-1 monoclonal antibodies, 5106 (gI) or 1808 (gIII) (Marshall et al., 1986). Cells expressing gI had fluorescence in the cytoplasm and on the cell membrane (Fig. 2b). The intensity of staining was less than in BHV-1-infected cells stained with a gI antibody (Fig. 2a). Fluorescence in gIII-expressing cells was also visible on cell surface membranes and in the cytoplasm (Fig. 2d) at levels lower than gIII-stained BHV-1-infected cells (Fig. 2c). Mock-infected cells did not fluoresce after reacting with either the gI or gIII antibodies (results not shown).

In order to compare the characteristics of the recombinant gI to those of the native protein, three gI-expressing cell lines, LB70-B#1, LB70-B#3 and LB64, were labelled with [35S]methionine, immunoprecipitated, analysed by SDS-PAGE, and visualized by autoradiography (Marshall et al., 1986). Three proteins with Mr values of 54K, 74K and 130K were present in all three gI-transfected lines (Fig. 3a, lanes 3 to 8) but the LB64 line had faint bands visible only on the original autoradiograph. These proteins had the same Mr as the native gI protein produced by BHV-1-infected bovine fibroblasts (Fig. 3a, lane 2). The recombinant gI protein from the LB70-B#3 cell line was immunoprecipitated by monoclonal antibodies against four epitopes (Marshall et al., 1988) suggesting that it had the same immunoreactive domains as the native viral glycoprotein (results not shown). Stimulation with cadmium increased recombinant gI protein production to levels greater than in BHV-1-infected cells (Fig. 3a, lanes 4 and 6). Immunoprecipitation of 54K, 74K and 130K proteins from 125I surface-labelled LB70-B#3 cells (Markwell & Fox, 1978) confirmed that the recombinant gI protein was being expressed on the cell membrane (results not shown).

To characterize the recombinant gIII protein, one gIII-expressing cell line, LBI-d, was surface-labelled with Na125I, immunoprecipitated, analysed by SDS-PAGE, and visualized by autoradiography (Marshall et al., 1986). A protein of 94K to 97K was present in the gIII-transfected cells (Fig. 3b, lanes 3 and 4). This protein had the same Mr as the native protein
Fig. 1. Construction of pBPV-B #1 and pBPV-C #1. (a) Procedure for construction of pBPV-B #1. The 3.8 kb EcoRI-SalI fragment of gI was isolated, repaired and ligated with BamHI linkers. This fragment was ligated into the BglII site of p341 between the mouse MMT promoter and the SV40 polyadenylation site (SV40). The resultant plasmid, pMB7-H, was digested with BamHI. The 7.9 kb BPV fragment was isolated and ligated with BamHI-linearized pMB7-H resulting in the plasmid pBPV-B #1. (b) Procedure used for construction of pBPV-C #1. The 2.6 kb EcoRI-BamHI fragment of gIII was isolated, repaired and ligated with BamHI linkers. This fragment was ligated into the BglII site of p341 as described above. The resultant plasmid, pMC10-B, was digested with BamHI and HindIII. The 5.3 kb fragment was isolated, single-stranded ends were filled in, and after ligation with BamHI linkers was inserted at the BamHI site of pCGBPVΔAB, resulting in pBPV-C #1. Arrows indicate the direction of transcription. Restriction enzyme abbreviations are: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; and S, SalI.
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Fig. 2. Immunofluorescence analysis of gl- and glII-expressing cells. (a) BHV-1-infected bovine fibroblasts reacted with 5106 (gl) antibody; (b) pBPV-B#1-transfected cells reacted with 5106 antibody; (c) BHV-1-infected bovine fibroblasts reacted with 1808 (gII) antibody; (d) pBPV-C#1-transfected cells reacted with 1808 antibody. Normal bovine fibroblasts were infected with the Cooper strain of BHV-1 for 18 h prior to fixation. pBPV-B#1- or pBPV-C#1-transfected cells were treated with 1 μM-CdCl₂. The cells were fixed with methanol, incubated with either 5106 or 1808 antibody, and then incubated with a fluorescein-conjugated goat anti-mouse antibody and photographed.

Immunoprecipitated from BHV-1-infected cells (Fig. 3b, lane 2). The recombinant protein was immunoprecipitated from the LB1-d cell line by monoclonal antibodies directed against five epitopes (Marshall et al., 1988) suggesting that it had the same immunoreactive domains as the native gIII protein (results not shown). Recombinant gIII expression was increased in gIII-transfected cells by the addition of cadmium chloride (Fig. 3b, lane 3). Lysates from bovine fibroblasts containing BPV plasmids alone did not have bands at 54K, 74K or 130K following immunoprecipitation with a gl monoclonal antibody or at 97K following immunoprecipitation with a gIII monoclonal antibody (results not shown). Southern blot analysis (Southern, 1975) of LB70-B #1 and LB70-B#3 showed the presence of 25 to 50 copies/cell of pBPV-B #1. The LB1-d line had only one to five copies/cell of pBPV-C#1 (results not shown).

The influence of gl expression on transfected cells was also investigated. Exposure of gl-expressing cells for 30 s to media that ranged in pH from 4.8 to 6.35 did not result in polykaryon formation unlike HSV gB-expressing cells that fuse at these pH values (Ali et al., 1987). Induction of gl expression with 1 μM-CdCl₂ for 3 to 4 days resulted in cell death but not in polykaryon formation. Normal fibroblasts did not die when treated with the same amount of CdCl₂ (results not shown).

Viral plaque assays were used to determine the effect of gl and gIII expression on BHV-1 infection. Confluent monolayers of normal, gl-expressing (LB70-B#3) or gIII-expressing (LB1-d) fibroblasts were infected with 100 p.f.u. of BHV-1, and overlaid with 0.5% agarose.
Fig. 3. Comparison of native and recombinant gl and gIII. (a) Autoradiograph of immunoprecipitated gl from cells transfected with pBPV-B#1. Non-transfected cells were mock-infected (lane 1) or infected with the Cooper strain of BHV-1 (lane 2) for 18 h before labelling. Transfected cells (LB70-B#1, lanes 3, 4; LB70-B#3, lanes 5, 6; LB64-B, lanes 7, 8) were untreated (lanes 3, 5 and 7) or treated with 1 µM-CdCl₂ for 18 h prior to labelling (lanes 4, 6 and 8). The 25 cm² flasks of cells were incubated for 1 h with methionine-free medium and then labelled for 5 h with 50 µCi/ml of [3⁵S]methionine. The cell lysate was prepared and immunoprecipitations were done as described (Marshall et al., 1986) using the 5106 (anti-gI) antibody (b) Autoradiograph of immunoprecipitated gIII from cells transfected with pBPV-C#1. The cells were treated before labelling as described above. The cells were surface-labelled with 200 µCi/ml Na¹²⁵I using a modification of the method previously described (Marshall et al., 1986). A coverslip coated with 20 µg of Iodogen (Pierce Chemicals) was floated on top of a 30 × 15 cm well of confluent cells for 15 min at 25 °C. The coverslip was then removed and cell lysate and immunoprecipitates were prepared (Marshall et al., 1986) using the 1808 (anti-gIII) antibody. The positions of M₉ markers are indicated. Lane 1, control; lane 2, BHV-1-infected; lane 3, transfected and treated with CdCl₂; lane 4, transfected but not CdCl₂-treated.

After 3 days, the monolayers were fixed, stained and the plaques were counted, measured and photographed. There was no difference between the number of plaques in BHV-1-infected normal fibroblasts, BHV-1-infected gl-expressing cells or BHV-1-infected gIII-expressing cells (Table 1). The appearance of BHV-1 plaques in the gl-expressing bovine fibroblasts (Fig. 4c and d) was quite different from the normal bovine fibroblasts (Fig. 4a) or gIII-expressing bovine fibroblasts (Fig. 4b). The gl-expressing fibroblasts had much smaller plaques and many were not clear (Table 1). The gIII-expressing fibroblasts had plaques of similar morphology to those seen in normal fibroblasts (Table 1).

To determine the specificity of the plaque changes, the plaque assay was repeated with 200 p.f.u. of HSV-1 (MacIntire strain) in normal, gl-expressing LB70-B#1 or LB70-B#3
Fig. 4. Comparison of BHV-1 plaque morphology in normal, gl- and gIII-expressing cells. Normal bovine fibroblasts (a), gIII-expressing cells (b) and gl-expressing cells (c, d) were infected with 100 p.f.u. of BHV-1 (Cooper), overlaid with 0.5% agarose and incubated for 3 days. The cells were fixed with formalin, stained with crystal violet, and photographed. Bar marker represents 0.1 mm.

Table 1. Effect of gl or gIII expression on BHV-1 plaque formation in bovine fibroblasts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment</th>
<th>No. plaques*</th>
<th>&gt;500 μm Clear</th>
<th>301-500 μm Clear</th>
<th>100-300 μm Clear</th>
<th>100-150 μm Clear</th>
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<tr>
<td>Normal bovine fibroblasts</td>
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<td>45, 51</td>
<td>40</td>
<td>45</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>69, 68</td>
<td>25</td>
<td>50</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>gl-expressing cells</td>
<td>1</td>
<td>50, 42</td>
<td>0</td>
<td>6</td>
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<td>63</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>61, 57</td>
<td>0</td>
<td>8</td>
<td>23</td>
<td>69</td>
</tr>
<tr>
<td>gIII-expressing cells</td>
<td>1</td>
<td>50, 40</td>
<td>37</td>
<td>47</td>
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<td>0</td>
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<td></td>
<td>2</td>
<td>42, 38</td>
<td>9</td>
<td>55</td>
<td>36</td>
<td>0</td>
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</table>

* Duplicate cultures.

fibroblasts. There was no statistical difference in the number of plaques in the three cell lines either with or without Cd²⁺ (Table 2). The plaques formed by HSV-1 in gl-expressing cells were smaller and more opaque than those seen in normal fibroblasts (Table 2). The HSV-1 plaques in the gl-expressing cells were larger and more opaque than the BHV-1 plaques (Tables 1 and 2). The HSV-1 plaque morphology was very similar to that seen for BHV-1 in Fig. 4. In order to compare the specificity of this gl effect for herpesvirus infections, normal, gl- or gIII-expressing fibroblasts were infected with 1000 p.f.u. of vesicular stomatitis virus. There was no difference in either plaque number or morphology between any of the three groups of cells (results not shown).

To investigate the impact of gl on BHV-1 virus production, 25 cm² flasks containing confluent monolayers of normal and gl-expressing fibroblasts were infected with BHV-1 at an m.o.i. of 10. Supernatant and cell lysate from individual flasks were harvested at intervals of 6 h over a 24 h period. Virus titres were determined as described by Rovozzo & Burke (1973) for
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Table 2. Effect of gi expression on HSV-1 plaque formation in bovine fibroblasts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Experiment</th>
<th>No. plaques*</th>
<th>&gt;500 μm Clear</th>
<th>301-500 μm Clear</th>
<th>151-500 μm Dark</th>
<th>100-150 μm Dark</th>
</tr>
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<tbody>
<tr>
<td>Normal bovine</td>
<td>1</td>
<td>44, 61</td>
<td>60</td>
<td>40</td>
<td>0</td>
<td>0</td>
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<tr>
<td>fibroblasts</td>
<td>2†</td>
<td>70, 73</td>
<td>74</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LB70-B #1</td>
<td>1</td>
<td>53, 56</td>
<td>0</td>
<td>5</td>
<td>85</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>2†</td>
<td>61, 76</td>
<td>0</td>
<td>0</td>
<td>74</td>
<td>26</td>
</tr>
<tr>
<td>LB70-B #3</td>
<td>1</td>
<td>61, 65</td>
<td>0</td>
<td>8</td>
<td>77</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2†</td>
<td>86, 76</td>
<td>0</td>
<td>11</td>
<td>81</td>
<td>8</td>
</tr>
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</table>

* Duplicate cultures.
† 1 μM-CdCl₂.

each time point. No differences in virus titre were seen between the normal cells and gi-expressing cells (results not shown).

The influence of gi on viral and cell protein synthesis during infection with BHV-1 was analysed by radiolabelling. Normal and gi-expressing fibroblasts were infected with BHV-1 at an m.o.i. of 10. At 6 h intervals over a 24 h period, the replicate cultures were radiolabelled with 50 μCi/ml of [35S]methionine for 5 h and harvested as described by Marshall et al. (1986). Autoradiographs of this whole cell lysate indicated no difference in the shutdown of cellular proteins nor in viral protein synthesis in normal or gi-expressing cells infected with BHV-1 (results not shown).

Although BPV vectors have been used to express the HSV-1 thymidine kinase gene (Lusky et al., 1983) and other surface proteins such as the envelope glycoprotein of human T cell leukaemia virus (Eiden et al., 1985), hepatitis B surface protein (Wang et al., 1983), and the influenza virus haemagglutinin (Sambrook et al., 1985), this represents the first expression of a herpesvirus glycoprotein gene using a BPV vector. The gi and gIII proteins expressed by our transfected bovine cells were indistinguishable from native BHV-1 gi and gIII by comparison of Mr and antigenic areas defined by all monoclonal antibodies used to date. Membrane expression of the gi- and gIII-transfected proteins was evident from the surface-labelled immunoprecipitation and membrane immunofluorescence.

A useful aspect of this system was the inducibility of gene expression. Maintenance of the cells at a low level of expression was made necessary by the cytopathic effect exerted by higher levels of gi. Addition of the heavy metal ion, cadmium, increased expression of gi and gIII to levels near those of infected cells for use in functional studies and for harvesting proteins for use as immunogens.

The function of BHV-1 gi and gIII in viral infection has not been established. Our plaque assay indicated that the cellular expression of gi does not inhibit entrance of BHV-1 or HSV-1 into cells as seen by the similar number of plaques in the infected normal and gi-expressing cells. The effect of gi expression on plaques does not appear to involve decreased virus production or release, viral protein synthesis or cellular protein shutdown. However, the gi-expressing fibroblasts had smaller and darker plaques than infected normal fibroblasts. Expression of gi therefore appears to delay lysis and death in cells infected with either BHV-1 or HSV-1. We hypothesize that gi interferes with a cellular protein responsible for fusion or other cytopathic effects to the cell. Interference with a cellular protein necessary for fusion has been hypothesized for another HSV-1 fusion protein, gD (Campadelli-Fiume et al., 1988). Cell lines expressing gIII were fully susceptible to BHV-1 infection as shown by normal plaque size, number and morphology.

The similarity of the HSV gB to BHV-1 gi at an amino acid, antigenic and structural level is very high (Misra et al., 1988; Whitbeck et al., 1988). Therefore the comparable effect on plaque formation seen in HSV-1- and BHV-1-infected gi-expressing cells is not surprising. Although these molecules are closely related, gi does not form polykaryons at low pH in bovine cells.
whereas HSV-1 gB-expressing simian cells do (Ali et al., 1987). The fusion function of gI may be species-specific as the gI gene used in our studies was the same as that used by Fitzpatrick et al., 1988b) when they observed polykaryon formation in gI-expressing murine cells.

The molecular investigation of the biological functions of gI and gII in BHV-1 infection needs to be pursued further. These recombinant proteins could be used to isolate their cellular target proteins in order to further define cell–virus interaction. If these cellular proteins were identified, their role as viral attachment proteins or cell fusion proteins could be assessed.

Since the submission of our manuscript, Conway et al. (1989) have reported on the use of a BPV vector to express gp340/220 of Epstein–Barr virus in murine cells with an MMT promoter. Unlike our system, they did not increase expression of this protein following induction with heavy metals.

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