Cleavage of the bovine herpesvirus glycoprotein B is not essential for its function

Earl Linwood Blewett and Vikram Misra*

Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

Herpes simplex virus glycoprotein B (HSVgB) and its bovine herpesvirus homologue (BHVgB) share similar primary structures. These glycoproteins are present in the envelope of the virion and are believed to initiate infection by fusing the virus envelope with a host cell membrane. BHVgB, like the membrane-fusing glycoproteins of most enveloped viruses, is normally cleaved and is present as a disulphide-linked complex in the virus envelope and host cell membranes. HSVgB, however, remains uncleaved, presumably because it lacks a similar protease recognition sequence. To determine whether the cleavage of BHVgB is essential for its role in initiating infection, we altered the coding sequence of this glycoprotein by removing the protease cleavage site and making this region similar to that of HSVgB. The mutant BHVgB gene was expressed by an HSV recombinant virus in mouse L cells and produced an uncleaved BHVgB. The uncleaved BHVgB could complement the function of HSVgB which had been neutralized by monoclonal antibody H233. When expressed in mouse L cells, the uncleaved mutant BHVgB retained its ability to fuse membranes.

Introduction

The virion envelope of herpesviruses contains a number of virus-encoded glycoproteins (Roizman & Sears, 1990). The primary structure of one of these, glycoprotein B (gB), is strongly conserved in every herpesvirus studied. The sequences of many of these gB homologues have been published: herpes simplex virus type 1 (HSV-1; Bzik et al., 1984), HSV-2 (Stuve et al., 1987), bovine herpesvirus type 2 (BHV-2; Hammerschmidt et al., 1988), varicella-zoster virus (VZV; Keller et al., 1986), pseudorabies virus (PRV; Robbins et al., 1987), BHV-1 (Misra et al., 1988), equine herpesvirus 1 (EHV-1; Whalley et al., 1989), EHV-4 (Riggio et al., 1989), Marek's disease virus (MDV; Ross et al., 1989), human cytomegalovirus (HCMV; Cranage et al., 1986), Epstein–Barr virus (EBV; Pellett et al., 1985) and herpes virus saimiri (HVS; Albrecht & Fleckenstein, 1990).

In HSV, gB is essential for virus infectivity (Little et al., 1981). Membranes containing HSVgB fuse with other membranes and this is believed to play a role in the initiation of infection (Ali et al., 1987; Cai et al., 1988a, b; DeLuca et al., 1982). The virus may gain entry to the host cell when the virion envelope fuses with a host cell membrane. Cell-to-cell virus spread may also be accomplished by gB-promoted membrane fusion. When HSVgB is expressed in mammalian cells it causes considerable cell membrane fusion resulting in syncytium formation (Ali et al., 1987). BHVgB expressed in mammalian cells also displays similar membrane-fusing abilities (Fitzpatrick et al., 1988). In recombinant pseudodiploid HSV which expresses both glycoproteins, BHVgB can complement an essential function of HSVgB which has been blocked by a neutralizing monoclonal antibody [Mab] H233 (Misra & Blewett, 1991).

Despite structural and functional homology, HSVgB and BHVgB differ in one major feature. BHVgB, like many of the other gB homologues, is cleaved but HSVgB is not (Pereira et al., 1981, 1982; van Drunen Littel-van Hurk & Babiuk, 1986).

Enveloped animal viruses have a fusion glycoprotein that enables them to enter the host cell (Spear, 1985; White et al., 1983). Entry is effected by fusion of the viral envelope with the host cell plasma membrane or by fusion with a host endosomal membrane. A common characteristic of these fusion proteins is cleavage, which is thought to expose an internal hydrophobic domain. With the exception of rhabdoviruses, the majority of viral fusion proteins must be cleaved to be functional (White et al., 1983). This has been demonstrated for influenza virus haemagglutinin (Bosch et al., 1979; Ohuchi et al., 1989), Sendai virus and measles virus F proteins (Alkhatib et al., 1990; Tashiro et al., 1989), Semliki Forest virus E protein (Lobigs & Garoff, 1990) and human immunodeficiency virus type 1 (HIV-1) gp160 (Bosch & Pawlita, 1990; Guo et al., 1990; McCune...
et al., 1988). The gB homologues of the herpesviruses HSV-1, HSV-2, BHV-2, EBV and HVS are not cleaved whereas those of VZV, PRV, BHV-1, EHV-1, EHV-4, MDV and HCMV are cleaved.

To determine whether the cleavage of BHVgB is essential for its ability to fuse membranes and initiate infection, we altered the gene for BHVgB by deleting several codons that specify amino acids at the cleavage site. The uncleaved BHVgB, produced by the mutant gene, was still able to complement the HSVgB function required for initiating infection and, when expressed in mammalian cells, retained the ability to fuse membranes.

**Methods**

**Virus.** The P8-2 strain of BHV-1 was isolated at the University of Wisconsin by J. R. Saunders. Its cultivation in Madin–Darby bovine kidney (MDBK) cells and its purification in potassium tartrate gradients have been described (Misra et al., 1981; Nelson et al., 1989). The antimitator strain of HSV-1 (PAA'5) was obtained from D. Coen (Harvard University, Cambridge, Mass., U.S.A.), and was grown and purified as described for BHV-1. The construction and characterization of pseudodiploid virus PsBHVGbB2 has been described (Misra & Blewett, 1991).

**Cells.** MDBK cells were obtained from the American Type Culture Collection. Murine thymidine kinase-negative (TK-) L cells (LTK-) were obtained originally from T. J. Kwoh, La Jolla Biological Laboratory, San Diego, Ca., U.S.A. Vero green monkey cells were obtained from B. Ziola, University of Saskatchewan.

**Plasmids and bacterial strains.** Escherichia coli JM101 (supE, thi, (lac-proAB), (F', traD36, pAB, lacIq, ZM15) (Yanisch-Perron et al., 1985) and E. coli RZ1032 [HfrKL16 PO/45 (lysA61-62), dutl, ung1, thi1, relA1] (Kunkel, 1985) were the host strains used for all recombinant plasmids and were grown in 2YT broth.

The plasmid pBHVGbB has a 3.6 kb HpaI–KpnI fragment of BHV-1 DNA cloned into the HincII–Kpnl site of pEMBL1+9+ (Dente et al., 1983). The HpaI–Kpnl fragment contains the entire coding sequence of BHVgB as well as 378 bp of its 5' and 416 bp of its 3' flanking sequences. The nucleotide sequence of the HpaI–Kpnl fragment has been described (Misra et al., 1988). The plasmid pHSV106, which has a 3.6 kb BamHI fragment containing the TK gene of HSV-1 cloned into pBR322, was obtained from Dr S. McKnight (Carnegie Institute, Baltimore, Md., U.S.A.). The sequence of the TK coding sequences has been published (McKnight, 1980). The mammalian expression vector pMSG was obtained from Pharmacia.

**DNA purification.** Plasmid DNA was prepared as described in Maniatis et al. (1982). Single-stranded DNA for sequencing was purified as instructed in Dente et al. (1983). HSV DNA for transfection was prepared as described earlier (Misra & Blewett, 1991).

**DNA analysis.** All restriction enzyme analysis, agarose gel electrophoresis and Southern blot procedures were essentially as described by Maniatis et al. (1982). Dideoxynucleotide sequencing was performed as described in Misra et al. (1988). The labelling of DNA with digoxigenin and its use in hybridization was as recommended by Boehringer Mannheim.

**Protein analysis.** Protein labelling, radioimmunoprecipitations, PAGE and autoradiography were performed as described in Misra et al. (1981) and Nelson et al. (1989).

Oligonucleotide-directed mutagenesis. A 40 base mutagenic oligonucleotide E10 (5' CGAACGCACGCTCGAGGGGC- CGCGCCTCTGCCC 3') was synthesized on an Applied Biosystems model 381A DNA synthesizer. This oligonucleotide contained two 20 base BHVGbB sequences flanking the DNA coding for the cleavage site. When used to prime an in vitro mutagenesis reaction this resulted in a BHVGbB coding sequence which lacked 45 bases (BHVGbA) as shown in Fig. 1(b). This oligonucleotide was used as described (Kunkel, 1985; Zoller & Smith, 1987). Single-stranded plasmid DNA was purified from putative clones and sequenced to determine whether the desired 45 base deletion had occurred in the correct location. The plasmid with the 45 base BHVGbB cleavage site deleted was designated pBHVGbA.

**Plasmid construction.** To construct vector pTKgB2 a DNA fragment containing the BHVGbB coding region was excised from pBHVGb and inserted into pHSV106 as described earlier (Misra & Blewett, 1991). A similar fragment was isolated from pBHVGbA and used to construct pTKgB2A. The vectors pTKgB2 and pTKgB2A were used in the construction of pseudodiploid viruses PsBHVGbB2 and PsBHVGbB2A, respectively.

To construct expression vectors pMSGgB1 and pMSGgB1A, Hpal–EcoRI fragments containing the BHVGbB coding region were purified from pBHVGb and pBHVGbA. The ends of these fragments were made blunt and cloned into the Smal site of the pMSG vector by standard techniques (Maniatis et al., 1982). The plasmid with the BHVGbB coding sequence inserted in the sense orientation behind the mouse mammary tumour virus promoter of pMSG was designated pMSGgB1. The plasmid with the corresponding mutant BHVGb was designated pMSGgB1A. These plasmids were used in the generation of the mouse cell lines LTKgB and LTKgB2.

**Generation of pseudodiploid HSV.** The construction and characterization of pseudodiploid HSV carrying the BHVGbB gene has been described (Misra & Blewett, 1991).

**Generation of mouse L cell lines expressing BHVGbB and BHVGbB2.** Plasmid DNA was obtained from standard mini-preparations and was sterilized prior to the experiment by ethanol precipitation and resuspended in H2O. DNA was added to the cells as a calcium phosphate precipitate using a CellPfect transfection kit (Pharmacia). 0.5 mM-sodium butyrate was obtained from Sigma, prepared and used according to Gorman & Howard (1983). The selective medium, designated GPT, was made and used as described (Gorman, 1985). Resistant cells were selected and individual clones were isolated by limiting dilution cloning.

**Neutralization of infection by MAb H233.** Experimental details are described in Misra & Blewett (1991). Briefly, 100 to 200 p.f.u. of virus and serially diluted MAb H233 (obtained from L. Pereira, University of California, San Francisco, U.S.A.) were incubated together then added to a confluent monolayer of MDBK cells which were overlaid with agarose. After 3 days incubation an agarose overlay containing neutral red was added. The plaques were counted 4 to 12 h later. The number of plaques after neutralization was calculated as a percentage of the plaques obtained without MAb and plotted against the inverse of the antibody dilution.

**Characterization of the syncytium-forming ability of the LTK–transfected cell lines.** The parental LTK– cells and the two transfected cell lines, LTKgB and LTKgB2A, were each seeded into four 15 × 60 mm Petri dishes (Falcon), such that they were confluent on the following day. Each cell line was treated with 50 mM-sodium butyrate and 5 × 10−6 M-dexamethasone to induce BHVGbB production. After 24 h of induction, the Petri dishes were separated into two groups, each having two Petri dishes of each line. The labels of these plates were removed and lids were coded by another worker. One group of plates
was examined for the presence of syncytia. Ten fields, 1.8 mm in diameter, on each plate were examined for the presence of syncytia. A syncytium was defined as a cell with multiple nuclei, which was at least the size of six or more normal cells. The second group of plates was treated with a low pH buffer, 100 mM-HEPES, 100 mM-MES pH 5.7, for 15 min at room temperature. These plates were examined for syncytia in the same manner. The average number of syncytia per field, before and after low pH treatment, was plotted on a bar graph.

Results

Oligonucleotide-directed mutagenesis

BHVgB is cleaved after the sequence RRARR (amino acid 505) (Fitzpatrick, 1989; Misra et al., 1988). The amino acid sequence RXRR is conserved in those gB homologues which are cleaved. This consensus sequence is absent in HSVgB. The amino acids of BHVgB and HSVgB are well conserved upstream and downstream of this sequence, as shown in Fig. 1 (a). The mutagenic oligonucleotide E10 was designed to introduce a 45 bp deletion in the BHVgB coding sequence at the cleavage site. This deletion removed the DNA coding for the protease cleavage site, making BHVgBA resemble HSVgB in this region (Fig. 1 b).

The oligonucleotide E10 was used as described to generate a number of putative clones. DNA of the clones coding for the cleavage region was sequenced, and a clone with the desired 45 base deletion was designated pBHVGBA (data not shown).

Construction of pseudodiploid viruses

The pseudodiploid viruses were constructed by homologous recombination of pTKgB2 and pTKgB2 into the PAA5 strain of HSV-1. Several bromovinyl deoxy-uridine-resistant virus clones were selected and grown. These clones were screened by immunoprecipitation for the production of BHVgB or BHVgBA. Viruses producing the BHVgB and BHVgBA proteins were designated PsBHVGAB2 and PsBHVGAB2, respectively and are shown in Fig. 3.

Restriction analysis and Southern blot of pseudodiploid viruses

To determine whether the HSV-TK/BHVgB gene hybrid had recombined into the correct position in the HSV genome, restriction enzyme analysis and a Southern blot were performed. Purified DNA from the HSV PAA5 virus, the PsBHVGAb2 virus and the PsBHVGAB2 virus was digested with BamHI and electrophoresed on a 0.7% agarose gel. DNA from pTKgB2 was also digested with BamHI and electrophoresed as a positive control (Fig. 2). In the HSV PAA5 DNA digestion (lane 3), a 3-5 kb band containing the TK gene is indicated (Wagner et al., 1981). In the digests of the pseudodiploid virus DNAs (lanes 4 and 5) this band was replaced by a larger 6-2 kb band. This band, designated TKgB, is indicated by an arrow (lane 6).

DNA fragments in the gel shown in Fig. 2 were transferred to a nylon membrane and probed with digoxigenin-labelled DNA containing the BHVgB coding sequence. Three bands hybridized to the probe. They were the TKgB fragment of PsBHVGa2, the TKgBA fragment of PsBHVGAB2 and the TKgB fragment of the positive control pTKgB (lanes 4, 5 and 6, respectively). The probe did not hybridize to any bands in the HSV PAA5 DNA digest.

Immunoprecipitation of BHVgB and BHVgBA

This experiment was performed to determine whether the pseudodiploid viruses synthesized BHVgB and BHVgBA. Cells infected with the pseudodiploid viruses were immunoprecipitated with a pool of BHVG-

![Fig. 1. Strategy for blocking cleavage by oligonucleotide-directed mutagenesis. The amino acid sequences of BHVgB and HSVgB in the region of the BHVgB cleavage site are aligned in (a). Identical amino acids shared by the two sequences are denoted by (:). Those amino acids which represent conservative substitutions are indicated by (.). The amino acids specifying the BHVgB cleavage site are in bold on a grey background. Spaces (−) have been introduced in the sequences to allow optimal alignment. The amino acids removed to eliminate the cleavage site are indicated with a bar. The alignment of the cleavage site deletion mutant BHVgBA and HSVgB is shown in (b).](image-url)
Fig. 2. Restriction digest and Southern blot of HSV-1 PAAr5 and pseudodiploid virus DNA. (a) Size markers (kb) are lambda DNA digested with HindIII (lanes 1 and 8), or lambda DNA digested with BstEII (lanes 2 and 7). The different HSV-1 DNAs, from PAAr5 (lane 3), PsBHVgB2 (lane 4) and PsBHVgBA2 (lane 5), were digested with BamHI. The plasmid pTKgB (lane 6) was also digested with BamHI as a positive control. Open arrows indicate the DNA fragment containing the HSV-1 TK gene. Closed arrows indicate TKgB, the DNA fragment containing BHVgB cloned into the HSV-I TK gene. (b) Southern blot of the gel probed with digoxigenin-labelled BHVgB.

specific MAbs. Fig. 3 shows BHVgB and BHVgBA as expressed in cells infected with the pseudodiploid viruses. No BHVgB was precipitated from cells infected with the HSV PAAr5 virus, or from the mock-infected MDBK cells (lanes 1 and 5, respectively).

The lane in which BHV-1-infected cell lysates were electrophoresed shows intensely labelled bands. The uncleaved BHVgB (approx. Mr, 130000) was present. The cleavage products with Mr's of 75000 and 55000 were also present. A faint band, representing a fast migrating precursor to the uncleaved gB, was also present (van Drunen Littel-van den Hurk & Babiuk, 1986).

The PsBHVgB2 virus expressed BHVgB. Immunoprecipitates from infected cells contained the uncleaved form of BHVgB as well as the cleavage products. The PsBHVgBA2 mutant expressed only the uncleaved BHVgB and its precursor. The removal of 15 amino acids from the putative cleavage region appeared to block BHVgB cleavage.

Neutralization of infection by MAb H233

We have previously shown that, unlike HSV-1, PsBHVgB2 is resistant to neutralization by MAb H233 which is specific for HSVgB (Pereira et al., 1981, 1989; Misra & Blewett, 1991). This MAb neutralizes the infectivity of HSV but does not neutralize BHV-1. We interpret this to mean that BHVgB can complement a function of HSVgB necessary for infection which is blocked by MAb H233.

To determine whether uncleaved BHVgB could functionally complement HSVgB, we assayed the ability of MAb H233 to neutralize PsBHVgBA2. Fig. 4 shows the results of the MAb neutralization experiments. Except at high concentrations of H233, both PsBHVgB2 and PsBHVgBA2 were resistant to neutralization. This suggests that the lack of cleavage and the deletion of the BHVgB cleavage sequence does not affect the ability of the protein to complement HSVgB.

Generation of mouse L cell lines expressing BHVgB and BHVgBA

To determine whether uncleaved BHVgB retained its ability to fuse membranes, the wild-type protein and the uncleaved mutant were expressed in mouse L cells. Syncytia were evident both in cell lines expressing the wild-type protein and those expressing the mutant
BHVGgB cleavage and function

BHVGgB protein. The different cell lines generated by cloning were screened by immunoprecipitation to detect BHVGgB or BHVGgBA production. Positive clones were identified, and those producing the most BHVGgB or BHVGgBA were designated LTKgB and LTKgBA, respectively. Cells expressing BHVGgB or BHVGgBA turned out to be slow-growing lines with evident syncytia. The clones that grew well and showed no syncytia also produced no detectable BHVGgB. This was especially apparent in the LTKgBA cell lines. Induction of BHVGgB and BHVGgBA synthesis by dexamethasone caused an increase in syncytium formation. Production of BHVGgB and BHVGgBA correlated with syncytium formation in cell culture.

Micrographs of the mouse L cell lines, induced by dexamethasone, are shown in Fig. 5. A few small syncytia are visible among the LTK- cells, whereas larger syncytia in greater numbers are evident in the LTKgB and LTKgBA cell lines.

Immunoprecipitation of BHVGgB and BHVGgBA from the LTKgB and LTKgBA cell lines

To determine whether BHVGgB or BHVGgBA was being expressed, lysates of the LTKgB and LTKgBA cell lines were immunoprecipitated with a pool of MAbs specific for BHVGgB (Fig. 6). The uncleaved BHVGgB and its cleavage products were visible in the positive control cells infected with BHV-1. The LTKgB line produced only the cleaved forms of BHVGgB. The LTKgBA cell line produced only the uncleaved glycoprotein.

Cell fusion assay

The syncytium-forming ability of cells expressing HSVgB or BHVGgB has been shown to be enhanced by a low pH treatment (Ali et al., 1987; Fitzpatrick et al., 1988). Fig. 7 shows the fusion abilities of three different cell lines with and without low pH treatment.

The parental mouse cells, the LTK- cell line, did not form many syncytia and the number did not appreciably increase with low pH treatment. The LTKgB cell line formed significantly more syncytia without low pH treatment than the parental LTK- cells and three times the number after low pH treatment. This confirms both

![Image of BHVGgB cleavage and function](https://www.microbiologyresearch.org)
E. L. Blewett and V. Misra

than the parental LTK- cell line, even without low pH treatment. In fact, the number of syncytia was the same as that of the potentiated wild-type cell line. The low pH treatment did not appreciably increase the number of syncytia.

Discussion

The primary and probably the tertiary structure of gB is strongly conserved among the herpesviruses. The exception to this is the presence or absence of a cleavage site. The gB proteins of HSV and BHV-1 have both been shown to possess membrane-fusing abilities (Ali et al., 1987; Fitzpatrick et al., 1988). In most cases a viral fusion protein must be cleaved to be functional (White et al., 1983). To determine whether cleavage of BHVgB is essential for its function, we have altered the coding sequence in order to remove the DNA coding for the cleavage site, and have expressed and characterized the uncleaved mutant protein.

When the DNA coding for 15 amino acids at the cleavage region of BHVgB was removed and the mutant gene expressed in mammalian cells, a protein of similar size to uncleaved BHVgB was produced. This mutant protein had the same apparent M, as the wild-type uncleaved BHVgB and could be immunoprecipitated by three different conformation-dependent MAbs which were specific for BHVgB. This suggests that the mutation did not drastically alter the conformation of the glycoprotein.

The functional abilities of the uncleaved BHVgB were tested. The pseudodiploid virus expressing uncleaved BHVgB was assayed for its ability to complement HSVgB. There was no difference between the ability of wild-type BHVgB and the uncleaved mutant to complement HSVgB which had been neutralized by MAb H233. The parental HSV was neutralized by this MAb. These results demonstrate that the mutant uncleaved BHVgB expressed by pseudodiploid virus could perform the function of HSVgB blocked by the MAb.

When BHVgBΔ was expressed in the LTKgBΔ cell line, significant numbers of syncytia were formed. Unlike cleaved BHVgB, a low pH treatment did not appreciably increase the number of syncytia produced by BHVgBΔ-expressing cells. This suggests that the 15 amino acid deletion may have changed the conformation of BHVgB, possibly mimicking the change induced by low pH. These conformational changes may be relatively minor and may not have affected the ability of MAbs to bind to the uncleaved BHVgBΔ. This indicates that the uncleaved mutant BHVgB has a functional membrane-fusing ability.
The membrane-fusing potential of HSVgB and BHVgB is enhanced by low pH treatment (Ali et al., 1987; Fitzpatrick et al., 1988). This is often the case when the virus enters host cells by an endosomal route (White et al., 1983). However, HSV is believed to enter cells by fusing with the host cell plasma membrane (Roizman & Sears, 1990), a process not usually associated with low pH enhancement. Other viruses such as HIV-1 may enter by fusing with host endosomal or plasma membranes (Marsh & Dalgleish, 1987) and it is possible that HSV and BHV-1 may also enter certain cell types by the endosomal route. Low pH enhancement of fusion may reflect this alternative route of entry. Another explanation could be that the low pH enhancement of membrane fusion by gB is an artefact and has no relevance to virus entry.

The cleavage of the membrane-fusing glycoprotein of an enveloped virus is essential for its function in many virus families. The distribution of proteases in host cells and organs can determine the tissue tropism and pathogenicity of a virus infection (Tashiro et al., 1989). In BHV-1, the importance of BHVgB cleavage in viral pathogenesis is not understood. Our results suggest that the cleavage of the glycoprotein may not play a role in its function and pathogenicity, at least not in the same manner as in other enveloped viruses. However, our results do not entirely rule out a role for BHVgB cleavage in BHV-1 pathogenesis. We have shown that the cleavage of BHVgB is not essential to enable the glycoprotein to fuse to mouse fibroblast cell membranes. This cleavage may become important for glycoprotein function in other cell types. We also tested the ability of BHVgB to fuse membranes and to complement HSVgB which had been blocked by an MAb. These are not necessarily the only functions of gB. Perhaps cleavage is essential in performing other untested functions or in other cell types in vivo.

We thank Pearse Ward for synthesizing the oligonucleotides used in this project, and David Fitzpatrick and Sireesh Tikoo for advice and kindly providing much needed reagents. This project was supported by an operating grant from the Natural Sciences and Engineering Research Council of Canada.

References


E. L. Blewett and V. Misra


(Received 22 March 1991; Accepted 31 May 1991)