Bovine herpesvirus type 1 glycoprotein H is essential for penetration and propagation in cell culture


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Bovine herpesvirus type 1 (BHV-1) glycoprotein H (gH) is a structural component of the virion which forms a complex with glycoprotein gL. To study the role of BHV-1 gH in the virus infectious cycle, a gH null mutant was constructed in which the gH coding sequences were deleted and replaced by the Escherichia coli lacZ cassette. The BHV-1 gH null mutant was propagated in trans-complementing MDBK cells, stably transfected with plasmid pMEP4 containing the BHV-1 gH gene under the control of the inducible mouse metallothionein promoter. Experiments with the BHV-1 gH null mutant showed that gH is essential in the infectious cycle of the virus and is specifically involved in virus entry and cell-to-cell spread. The lack of infectivity of virions devoid of gH is not due to a defect in attachment. Moreover, PEG-induced fusion of virions to target cells provides evidence that BHV-1 gH is required for virion penetration.

Bovine herpesvirus type 1 (BHV-1), a member of the subfamily Alphaherpesvirinae, causes infectious bovine rhinotracheitis (IBR), a disease of major economic concern (Wyler et al., 1989). The structure of BHV-1 is similar to that of other herpesviruses, consisting of a nucleocapsid containing the linear double-strand DNA genome which is surrounded by the tegument and a lipid bilayer envelope with the virus-encoded glycoproteins. These glycoproteins play an important role in the initiation of infection of target cells. Three major BHV-1 glycoproteins, gB, gC and gD, are involved in virus entry (Fitzpatrick et al., 1988; Liang et al., 1991; Okazaki et al., 1994; Thaker et al., 1994; Byrne et al., 1995). In addition, a fourth glycoprotein, gH, is necessary for entry of herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PrV) into target cells (Fuller et al., 1989; Babic et al., 1996). The homologous BHV-1 gH gene has been sequenced (Meyer et al., 1991) and BHV-1 gH has been identified as a 108 kDa structural component of the virus, expressed as a beta-gamma protein (Baranowski et al., 1995). BHV-1 gH forms a complex with another glycoprotein, gL, which is necessary for the proper processing and transport of gH but not gL (Khattar et al., 1996). In addition, the BHV-1 gH–gL complex is required for induction of a neutralizing antibody response and anchoring of gL to the plasma membrane (Khattar et al., 1996). Characterization of PrV and HSV-1 gH null mutants has indicated that gH of these viruses is an essential glycoprotein which is involved in both fusion between virion and cellular membranes during virus entry and in cell-to-cell spread of the virus (Forrester et al., 1992; Babic et al., 1996). For PrV, absence of gH prevents penetration and propagation of the virus in the nervous system of adult mice after intranasal inoculation, suggesting that transneuronal spread in vivo and direct cell-to-cell spread in cell culture are governed by similar mechanisms (Babic et al., 1996). The present study describes the construction and characterization of a BHV-1 gH null mutant and demonstrates that gH of BHV-1 is an essential glycoprotein necessary for both virus penetration and cell-to-cell spread.

Since previous data indicated that gH is required for virion infectivity in other alphaherpesviruses (Gompels & Minson, 1986; Forrester et al., 1992; Peeters et al., 1992) a cell line capable of supplying gH in trans was constructed by transfecting MDBK cells (ATCC CCL22) with the eukaryotic plasmid pMEP4 carrying the gH gene (PMEP4-gH) of the virulent BHV-1 Lam strain. To construct PMEP4-gH, BHV-1 Lam genomic DNA was firstly digested by Clal/Spel to generate a fragment of about 7 kb encompassing the gH gene. This fragment was inserted into Clal/Spel-cleaved pBluescript SK(+) to give plasmid SK7. After restriction analysis, plasmid SK7 was first linearized by BamHI, blunt-ended and cleaved by AAvI to generate a 2.7 kb fragment encompassing 40 bp of upstream sequences, the gH open reading frame (ORF) and the putative polyadenylation site of the BHV-1 gH transcript (sequence confirmation). This fragment was inserted into PnuI/NheI-cleaved eukaryotic expression plasmid pMEP4 under the control of the inducible mouse metallothionein promoter and containing the hygromycin-resistance gene. This promoter was chosen in order to avoid a potential cytotoxic activity due to constitutive expression of gH in MDBK cells. The mouse metallothionein promoter was induced by addition of ZnSO4 (80 µM) in cell culture medium. After transfection of PMEP4-gH into MDBK cells by lipo-
Infection (Felgner et al., 1987), transfectants were selected on the basis of their resistance to hygromycin (200 μg/ml).

After 3 weeks of selection, a sample of transfected cells was treated with ZnSO₄ (80 μM) for 8 h and analysed by radioimmunoprecipitation with BH8, a BHV-1 gH-specific monoclonal antibody, and a rabbit serum directed against a peptide of BHV-1 gH (Baranowski et al., 1995). In cells transfected with pMEP4-gH, a protein of 100 kDa was recognized by the rabbit gH antipeptide serum only. No signal was observed with BH8, which recognizes the 108 kDa mature glycosylated gH only (Baranowski et al., 1993). This 100 kDa protein corresponds to the BHV-1 gH protein expressed under tunicamycin treatment and is sensitive to Endo H treatment. All these results (data not shown) suggest that gH is expressed by the pMEP4-gH-transfected cells as a 100 kDa precursor and not as the mature form. Indeed, it was previously shown that gH expressed by BHV-1 infected cells is first detected as a 100 kDa precursor which is processed into the 108 kDa mature form (Baranowski et al., 1995). This 100 kDa gH precursor was sensitive to Endo H treatment, suggesting that it contains N-linked oligosaccharides exclusively of the high-mannose type (van Drunen Littel-van den Hurk & Babiuk, 1986). In contrast, the 108 kDa mature form of gH is resistant to Endo H treatment, indicating that it has entered the normal secretory pathway through the Golgi apparatus and contains complex-type oligosaccharides (Khattar et al., 1996). By using recombinant vaccinia viruses, Khattar et al. (1996) demonstrated that complex formation between gH and gL is necessary for proper maturation and folding on the cell surface of the gH mature form. Similarly, the absence of gL co-expression in pMEP4-gH-transfected cells could be responsible for the lack of maturation of gH.

Cells were then cloned by flow cytometry using a Becton-Dickinson fluorescence-activated cell sorter (Facstar Plus). One cell line, gH-PMEP4, was selected by radioimmunoprecipitation and grown in medium supplemented with 200 μg/ml hygromycin for ten additional passages. Finally, this gH-PMEP4 cell line was used for construction of the BHV-1 gH null mutant with the assumption that gL glycoprotein expressed by the mutant will be sufficient for the correct processing and maturation of gH expressed by the trans-complementing cell line.

To isolate a gH-deficient BHV-1 mutant, plasmid pCMV-gal was constructed by cloning a β-galactosidase cassette (MacGregor & Caskey, 1989) between the two flanking EcoI and HindIII sites and then inserted in the EcoRI/HindIII sites of pBluescript SK(+) to generate the plasmid SK-PCMV. The BHV-1 Aavr–Spel fragment (right flanking sequence of gH) was then cleaved from SK7 and inserted into the SpeI site of SK-PCMV plasmid in the opposite direction to the β-galactosidase cassette. The resulting plasmid was SK-PCMV-Spe. A second fragment of 1–1 kb [left flanking sequence of gH containing the thymidine kinase (TK) gene] was obtained by digestion of SK7 by HindIII and blunt-ended. This fragment was inserted into the blunt-ended KpnI site of SK-PCMV-Spe, in the same direction as the right flanking sequence, to generate the pCMV-gal.

MDBK cells were cotransfected using the lipofection method with 10 μg of BHV-1 Lam DNA and 5 μg of plasmid pCMV-gal and cotransfected viruses were isolated after 48–72 h. Selection of cells infected with a phenotypically complemented gH-deficient mutant (BHV1-gH+/−) was carried out by flow cytometry analysis using a FACS–β-galactosidase assay in which the β-galactosidase activity of infected cells was revealed by the procedure described by Nolan et al. (1988). Further cloning of BHV1-gH+/− was done by a limiting dilution method in conjunction with β-galactosidase staining (Schröder et al., 1997) to select a virus population with a 100% blue-plaque phenotype. To isolate a gH-rescue virus, genomic BHV1-gH+/− DNA was cotransfected into pMEP4-gH cells with SK7 plasmid containing the 7 kb Clal–Spel fragment encompassing the gH gene. Transfection progeny was selected for its ability to grow in normal MDBK cells and for its blue-plaque phenotype.

Southern blot hybridization confirmed the integration of the β-galactosidase cassette in place of the gH gene for BHV1-gH+/− virus and the restoration of the gH gene for gH-rescue virus (data not shown). The gH polyadenylation site is supposed to be the active one for the adjacent TK gene, the mRNA of TK/gH being bicistronic (Bello et al., 1992). In our construction, the gH polyadenylation site was deleted and an SV40 one was inserted after the TK gene. The presence of functional BHV-1 TK protein activity during BHV1-gH+/− infection was confirmed by [3H]thymidine autoradiographic assay (Tenser et al., 1983).

The BHV-1 Lam strain and the gH-rescue viruses were propagated in MDBK cells at an m.o.i. of 1 whereas BHV1-gH+/− mutant was grown in pCMV-gH trans-complemented cells induced with 20 μM ZnSO₄. Preliminary results showed that 20 μM ZnSO₄ is sufficient to induce gH expression without cell toxicity. When MDBK or pMEP4-gH cells were infected by BHV1-gH+/− (m.o.i. 1), only individual infected (blue) MDBK cells were detected by β-galactosidase staining, whereas blue plaques were apparent in the trans-complemented pMEP4-gH cells. The individual MDBK blue cells showed the rounding-up characteristic of infected cells, suggesting that infection still resulting in cell killing. These observations suggested that BHV1-gH+/− was unable to spread from infected to uninfected MDBK cells, indicating that gH is essential for direct cell-to-cell transmission of the virus.

To obtain virions devoid of gH (BHV1-gH−/−), MDBK cells were infected at a high m.o.i. (10–50) with BHV1-gH+/− and harvested after exhibiting pronounced cytopathic effect. The absence of gH from BHV1-gH−/− and the presence of gH expression in gH-rescue virus was verified by radioimmunoprecipitation assay using monoclonal antibody BH8.
Role of BHV-1 gH in cell penetration

Fig. 1. Attachment of BHV1-gH+/− to MDBK cells. Radiolabelled purified wild-type, gH-rescue BHV1-gH+/− and BHV1-gH+/− viruses were bound to cells for 2 h at 0 °C. The total binding fraction was determined as the amount of labelled virus which remained bound after washing with PBS supplemented by 1% BSA. The percentage of heparin-resistant binding was determined by three washings with PBS supplemented with heparin (250 UI/ml) after the attachment period. Addition of 250 UI/ml of heparin during the attachment process showed heparin-independent binding. The percentage of input radioactive material which remained bound to the cells after the respective treatments is indicated (Karger & Mettenleiter, 1993). Data were derived from three independent experiments and standard deviations are indicated.

Fig. 2. PEG-induced fusion of BHV1-gH+/− on gH-PMEP4 cells compared to wild-type and gH-rescue viruses. After the attachment period, viruses were treated with PEG or left untreated. Virus plaques were fixed and counted 48 h post-inoculation under microscopy after β-galactosidase staining. Data were derived from four independent experiments and standard deviations are indicated. The results were statistically evaluated by analysis of variance (ANOVA) determining the least significant difference.

(Baranowski et al., 1995). Glycoprotein gH was recognized by BH8 for all viruses except BHV1-gH−/− (data not shown). In gH-PMEP4 cells infected by BHV1-gH+/−, BH8 recognized the 108 kDa mature form of gH, indicating that the gH-PMEP4 cell line is effectively sufficient for the construction and replication of BHV1-gH+/−.

To analyse the ability of the BHV1-gH−/− mutant to bind to target cells, attachment assays on MDBK cells using radiolabelled virions as well as the differentiation of heparin-sensitive, heparin-resistant and heparin-independent binding were performed as described by Karger & Mettenleiter (1993). Radiolabelled virions were purified from supernatant medium by ultracentrifugation through a 10–25% Ficoll gradient (Lyaku et al., 1992) and incubated on MDBK cells for 2 h at 0 °C to allow attachment but not penetration. Addition of heparin (250 UI/ml) during the attachment process reduced the binding of all viruses by more than 85% (Fig. 1). After thorough washing with PBS supplemented with 1% BSA, the amount of labelled virus which remained bound to the monolayer was determined as the total binding fraction. The difference between the total binding fraction and heparin-resistant binding determined the heparin-sensitive primary adsorption of virions to target cells. The results showed that, in each of the three independent experiments, the heparin-sensitive fraction was consistently higher for BHV1-gH−/− than for wild-type, BHV1-gH+/− and gH-rescue viruses. BHV1 glycoprotein gC, and to a lesser extend gB, were shown to be important for this step of the infectious cycle (Liang et al., 1991). For BHV1-gH−/−, the absence of gH could enhance the capacity of these glycoproteins to bind heparan sulfates. Nevertheless, these results showed that BHV1-gH−/− attached to target cells as efficiently as wild-type and gH-rescue viruses, indicating that absence of gH from virions does not alter the attachment phenotype.

Since BHV-1 gH did not have any effect on virus attachment, we tested its role in the penetration process on MDBK target cells. A deficiency in penetration can usually be overcome by treating attached virions with polyethylene glycol (PEG). Therefore, we investigated the effect of PEG on the infectivity of BHV1-gH−/− compared to the parental strain and gH-rescue virus. Trans-complementing gH-PMEP4 cells were infected for 1 h at 37 °C with serial dilutions of the respective viruses. After this period the monolayer was washed and treated with PEG-8000 as described (Sarmiento et al., 1979; Rauh & Mettenleiter, 1991). After fusion, cells were incubated with MEM containing 10% foetal calf serum for 2 h after which the medium was replaced with MEM containing 10% of BHV-1-neutralizing newborn calf serum (titre 2048...
ED$_{50}$/ml). Two days after PEG treatment, plaques were counted after $\beta$-galactosidase staining. The results showed that the titre of BHV-1 Lam or rescue viruses did not change after PEG treatment (Fig. 2). In contrast, PEG treatment enhanced significantly ($P < 0.01$) the infectivity of BHV-1 gH$^{-/-}$ (17–45-fold increase; Fig. 2), demonstrating that attached virions lacking gH were blocked at the penetration level of infection. By using specific monoclonal antibodies, van Drunen Littel-van den Hurk et al. (1996) recently demonstrated that the BHV-1 gH–gL complex is essential for penetration of BHV-1 into susceptible cells. Glycoprotein gH is the second, after gB, most highly conserved herpesvirus glycoprotein (Baranowski et al., 1996). BHV-1 gH shares the fusogenic properties of this protein family, observed in the different herpesviruses studied so far. It cooperates with major glycoproteins to mediate virus penetration and cell-to-cell spread, but does not act as a receptor-binding protein, in contrast to glycoproteins gB and gD (Liang et al., 1995).

In conclusion, this study shows that BHV-1 gH is essential in the infectious cycle of the virus and is specifically involved in virus entry and cell-to-cell spread. The lack of infectivity of virions devoid of gH is not due to a defect in attachment. Moreover, PEG-induced fusion of virions to target cells provides evidence that BHV-1 gH is required for virion penetration in addition to gB and gD.

The authors thank Dr J. T. van Oirschot (ID-DLO Institute, Lelystad, Netherlands) for providing them with the BHV-1 Lam strain. The authors extend special thanks to Laurence Nols and Maria Loncar for their technical assistance. Purchase of the flow cytometer was supported in part by a grant from the ‘Loterie Nationale’ (no. 9.4505.92, Belgium). E. Hanon is a senior research assistant of the Fonds National Belge de la Recherche Scientifique (F.N.R.S.). This investigation was financially supported by the European Commission, Agro-Industrial Research grant AIR3-BM92-008 and the Fonds de la Recherche Scientifique et Medicale (FRSM) no. 3.4514.96 and 3.4612.95.

References


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Received 24 February 1998; Accepted 8 April 1998