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## Glycoprotein H (gH/gp108) and glycoprotein L form a functional complex which plays a role in penetration, but not in attachment, of bovine herpesvirus 1

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**The glycoproteins of bovine herpesvirus 1 (BHV-1) play important roles in the interactions between virions and target cells. A 108 kDa glycoprotein, designated gH or gp108, has been identified by two different panels of monoclonal antibodies. The gH- and gp108-specific monoclonal antibodies were shown to react with the same protein, which was identified by N-terminal sequencing as the homologue of herpes simplex virus type 1 (HSV-1) gH. When BHV-1 gH was purified by immunoadsorbent chromatography, gL was co-purified. The gH–gL complex induced the production of antibodies that neutralized virus infectivity and inhibited virus penetration. Affinity-purified gH–gL did prevent penetration, but not attachment of BHV-1, which suggests that the gH–gL complex is essential for penetration of BHV-1 into susceptible cells.**

Bovine herpesvirus 1 (BHV-1), a member of the subfamily *Alphaherpesvirinae*, is distributed world-wide. It causes a variety of diseases including respiratory and genital infections, conjunctivitis, encephalitis, abortions and fatal multisystemic infections of neonates. Four major glycoproteins, designated gI (130 kDa), gH (108 kDa), gIII (91 kDa) and gIV (71 kDa) (van Drunen Littel-van den Hurk & Babiuk, 1986), have been identified by BHV-1 specific monoclonal antibodies. Three additional glycoproteins, designated gp108, gp93 and gp42, have been defined by use of monoclonal antibodies (Baranowski *et al.*, 1993). The gI, gIII and gIV glycoproteins are homologous to herpes simplex virus type 1 (HSV-1) gB, gC and gD, respectively, and are all involved in virus entry (Liang *et al.*, 1991). However, the identities and functions of gH, gp108, gp93 and gp42 remain to be established. Monoclonal

antibodies specific for gH and gp108 precipitate glycoproteins with the same apparent molecular mass, suggesting that gH is gp108.

A fourth glycoprotein, gL, is necessary for entry of HSV-1 (McGeoch & Davidson, 1986; Fuller *et al.*, 1989). Genes homologous to that encoding HSV-1 gL have been found in the genomes of other herpesviruses, including varicella-zoster virus and Epstein–Barr virus (EBV) (McGeoch & Davidson, 1986), human cytomegalovirus (HCMV; Cranage *et al.*, 1988), equine herpesvirus (EHV; Nicolson, 1990), pseudorabies virus (PRV; Klupp & Mettenleiter, 1991) and human herpesvirus-6 (HHV-6; Liu *et al.*, 1993). The BHV-1 gene homologous to gL of HSV-1 has been sequenced (Meyer *et al.*, 1991). This sequence contains an open reading frame of 842 amino acids and six potential N-linked glycosylation sites. Glycoprotein II contains N-linked carbohydrates of the complex and high-mannose types. The apparent molecular mass of the unglycosylated precursor of gH is 90 kDa (van Drunen Littel-van den Hurk & Babiuk, 1986), suggesting that gH may be the gL homologue of HSV-1. The purpose of the present study was to determine the identity and biological functions of the gH and gp108 glycoproteins.

BHV-1 strain Cooper was propagated in Madin–Darby bovine kidney (MDBK) cells in minimum essential medium (MEM) (Gibco) supplemented with 2% fetal bovine serum (FBS) (Gibco). A 108 kDa protein was purified from BHV-1 infected MDBK cells by affinity chromatography as described by van Drunen Littel-van den Hurk & Babiuk (1985). Briefly, the BHV-1 infected MDBK cells were lysed in 0·01 M-Tris–HCl, 0·5 M-NaCl, 1% NP40, 1% sodium deoxycholate, pH 7·5 and cycled three times through a monoclonal antibody immunoadsorbent column specific for gp108 (Baranowski *et al.*, 1993). After washing the column, the bound protein was eluted with 0·05 M-diethylamine, pH 11·5, neutralized with 1 M-Tris–HCl, pH 7·0, and after concentration on an Amicon P30 membrane, dialysed against PBS. An average of 5 µg of gp108 was purified from  $2 \times 10^7$  cells (one T150 flask). Analysis of the purified preparation by PAGE demonstrated

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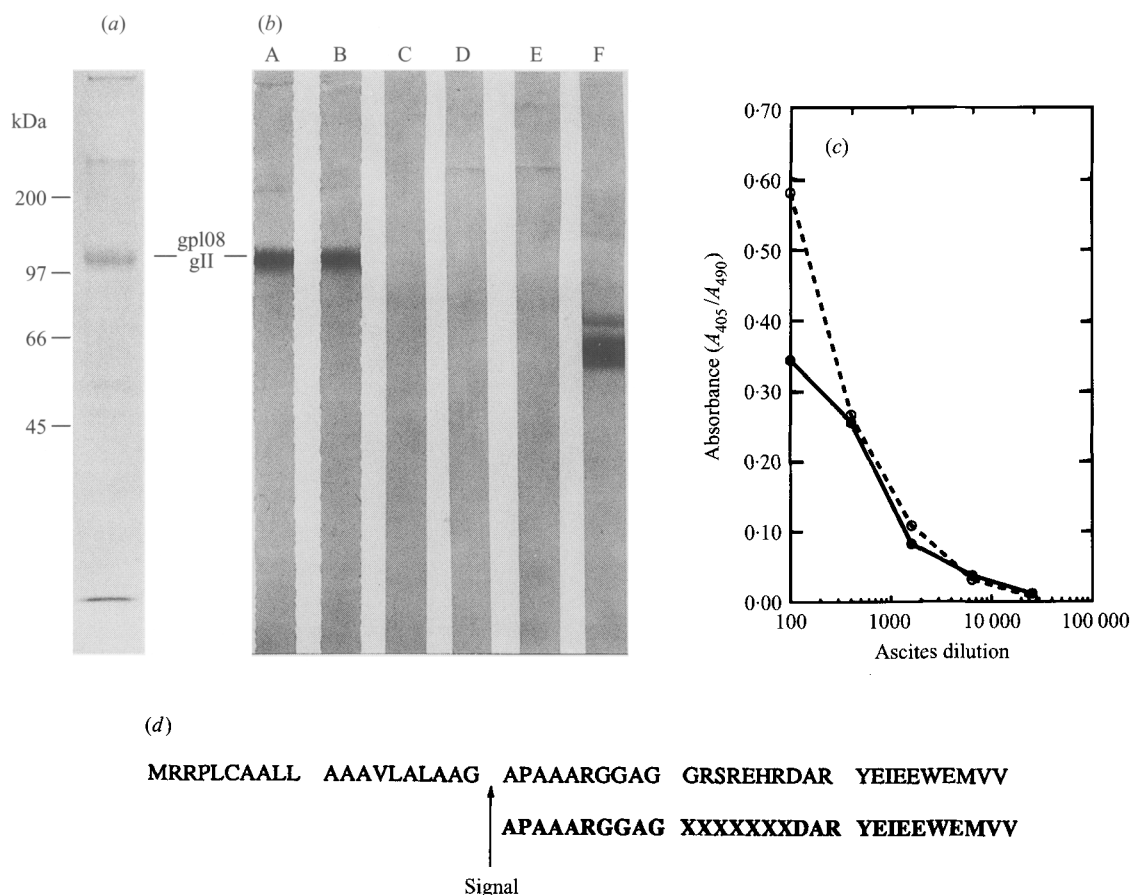
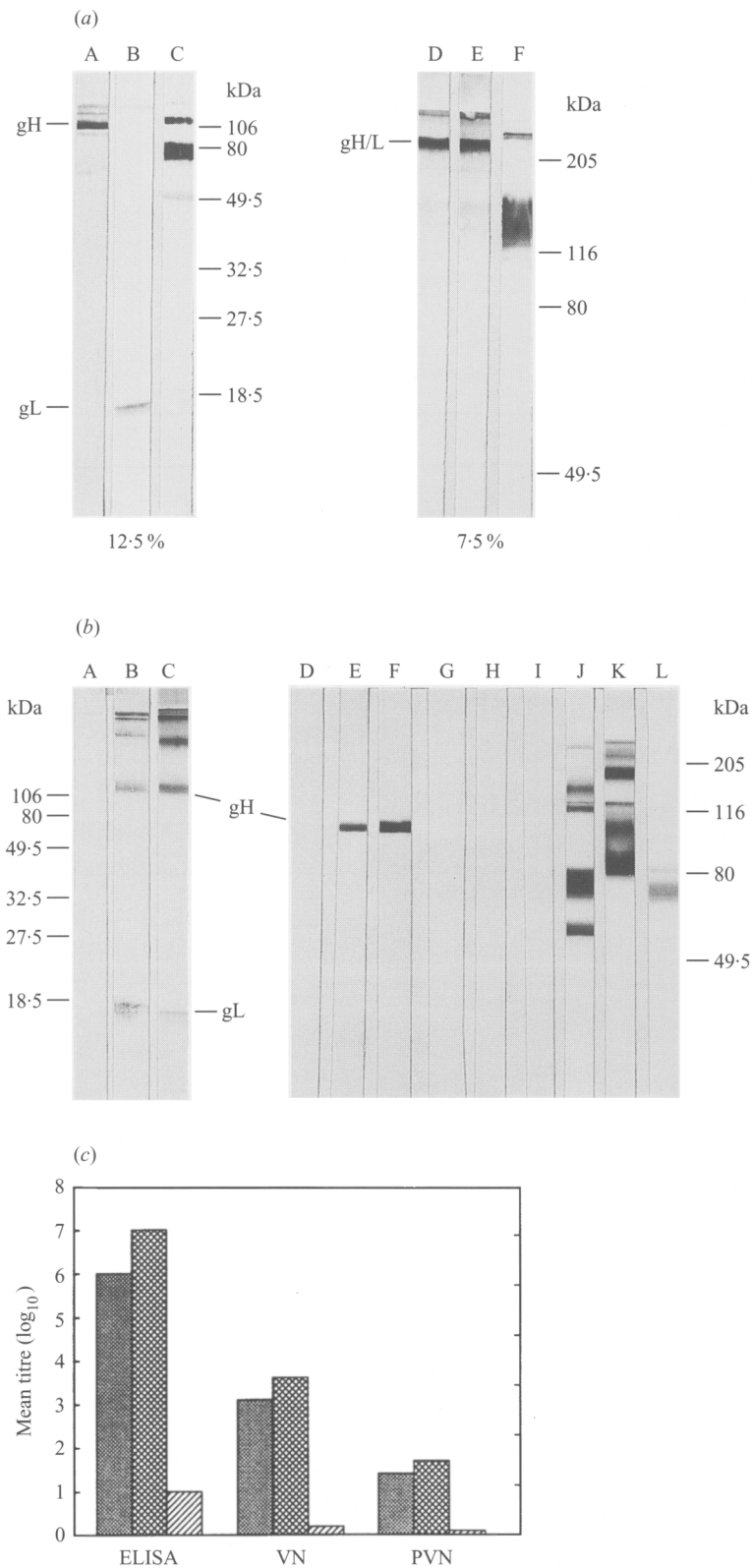


Fig. 1. Analysis of gII/gp108, purified on a gp108-specific monoclonal antibody column. (a) Affinity-purified 108 kDa glycoprotein was run on an 8.5% polyacrylamide gel under reducing conditions and stained with CBB. The position of the 108 kDa glycoprotein is indicated on the right. Molecular mass markers are shown on the left. (b) Affinity-purified 108 kDa glycoprotein (lanes A, B and C) and gD (lanes D, E and F) were run on an 8.5% semi-denaturing polyacrylamide gel, transferred to nitrocellulose and probed with gII-specific monoclonal antibody 3D9T (lanes A and D), gp108-specific monoclonal antibody BH23 (lane B and E) or gD-specific monoclonal antibody 9D6 (lanes C and F). The position of the 108 kDa glycoprotein is indicated on the left. (c) Reaction of 3D9T (●) and BH23 (○) with the 108 kDa glycoprotein (0.1 µg per well) in an indirect ELISA. (d) N-terminal amino acid sequence of purified gII/gp108. The N-terminal sequence determined by Edman degradation analysis is displayed in bold below the predicted sequence of BHV-1 gH. X represents degradation cycles which resulted in undetectable derivatives. The signal cleavage site is indicated with an arrow.

the presence of a Coomassie Brilliant Blue (CBB) stained 108 kDa protein (Fig. 1a). The purified protein was applied onto an 8.5% semi-denaturing polyacrylamide gel, transferred to nitrocellulose and probed with the gII-specific monoclonal antibody 3D9T (van Drunen Littel-van den Hurk & Babiuk, 1986) or the gp108-specific monoclonal antibody BH23 (Baranowski *et al.*, 1993). The reaction was visualized with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Boehringer Mannheim) and 4-chloronaphthol (Bio-Rad). The gII-specific monoclonal antibody reacted with the protein purified on the gp108-specific monoclonal antibody column (Fig. 1b). A gD-specific monoclonal antibody did not recognize gp108, nor was purified gD recognized by 3D9T or BH23 (Fig. 1b). In addition, monoclonal antibodies 3D9T and BH23 showed very similar reactions with purified gp108 in an indirect ELISA (Fig. 1c), which confirms that gII and gp108 are the same glycoprotein.

To determine whether gII/gp108 is indeed the gH homologue of HSV-1, N-terminal sequencing was performed. Affinity-purified gII/gp108 was applied onto an 8.5% reducing polyacrylamide gel, transferred to PVDF (Millipore) and stained with CBB. The glycoprotein band was cut from the PVDF membrane and subjected to N-terminal sequencing by Edman degradation using an Applied Biosystems protein sequencer A477 coupled to a phenylthiodantoin acid analyser A120. Sequence was obtained from two regions. The first 10 amino acids of gII/gp108 corresponded to amino acids 21–30 of the predicted polypeptide sequence of the BHV-1 gH homologue (Meyer *et al.*, 1991). Another 13 amino acids of gII/gp108 were also identifiable and corresponded to amino acids 38–50 of the predicted sequence of gH. The N-terminal residue of this glycoprotein was Ala-21 (Fig. 1d), which demonstrates that the signal sequence is cleaved. This analysis showed that gII/gp108 is the gH homologue of HSV-1



**Fig. 2.** Analysis of affinity-purified gH/gL. (a) Affinity-purified gH/gL (lanes A, B, D and E) or gB (lanes C and F) were separated on a 12.5% reducing or 7.5% non-reducing polyacrylamide gel, transferred to nitrocellulose and probed with gH-specific (lanes A and D), gL-specific (lanes B and E) or gB-specific antibody (lanes C and F). (b) Specificity of gH/gL-specific rabbit sera. BHV-1 (lanes A, B, D and E), gH/gL (lanes C and F), gB (lanes G and J), gC (lanes H and K) and gD (lanes I and L) were run on a 12.5% (lanes A, B and C) or 8.5% (lanes D to L) polyacrylamide gel, transferred to nitrocellulose and probed with preimmune rabbit serum (lanes A and D), gH/gL hyperimmune serum (lanes B, C, E to I), gB-specific monoclonal antibody cocktail (lane J), gC-specific monoclonal antibody cocktail (lane K) or gD-specific monoclonal antibody cocktail (lane L). The positions of gH and gL are indicated in the middle. The positions of molecular mass markers are shown (left and right). (c) Rabbits received two intramuscular immunizations of 25 µg gH/gL (■) or PBS (▨) in VSA3. A gD-specific antibody (▤), 9D6 (Hughes *et al.*, 1988) is included as control. ELISA titres were determined against gH/gL, or gD in the case of 9D6, and expressed as the reciprocal of the highest dilution resulting in a reading 2SDs above the control value. Virus neutralizing (VN) titres were expressed as a 50% endpoint using 100 p.f.u. of virus. Post-adsorption virus neutralizing (PVN) titres were determined after allowing the virus to adsorb to the cells at 4 °C. Values are expressed as log<sub>10</sub>.

and thus may be named gH, in accordance with the nomenclature for herpesvirus glycoproteins proposed at the XVIIIth International Herpesvirus Workshop.

In HSV-1, a glycoprotein designated gL has been identified that forms a complex with gH (Hutchinson *et al.*, 1992). Homologues for gL that form heterodimers with gH have also been described for several herpesviruses including HCMV (Kaye *et al.*, 1992), HHV-6 (Liu *et al.*, 1993), EBV (Yaswen, 1993), and PRV (Klupp *et al.*, 1994). Recently, the BHV-1 gL gene was sequenced (Khattar *et al.*, 1995). Anti-gL peptide sera were raised, which precipitated a 16–17 kDa glycoprotein (Khattar *et al.*, 1995). To determine whether gL was co-purified on the gH-specific immunoabsorbent column, the purified glycoprotein was applied onto a 12.5% polyacrylamide gel and either stained with CBB or transferred to nitrocellulose and probed with gH-specific or gL-specific rabbit serum (Fig. 2*a*, lanes A and B). In addition to gH, a 16–17 kDa polypeptide visualized on the stained gel (data not shown) reacted with the gL-specific rabbit serum on the Western blot (Fig. 2*a*), suggesting that gH and gL are co-purified as a complex. When the gH–gL complex was analysed by electrophoresis under non-reducing conditions, the gH- and gL-specific antibodies both reacted with a polypeptide with an apparent molecular mass of approximately 250 kDa (Fig. 2*a*, lanes D and E), suggesting that the affinity-purified gH–gL complex may exist as a tetramer, consisting of two gH and two gL molecules. Alternatively, this high-molecular mass complex may be formed following purification. This is similar to BHV-1 gB, which under reducing conditions has apparent molecular masses of 130 kDa (uncleaved form), 74 kDa and 55 kDa (cleaved forms) (Fig. 2*a*, lane C). Under non-reducing conditions gB exhibits a monomeric form of 130 kDa and a dimeric form of 260 kDa (Fig. 2*a*, lane F) (van Drunen Littel-van den Hurk & Babiuk, 1986).

When HSV-1 gH is expressed in vaccinia virus, it is aberrantly processed and not transported to the cell surface, unless co-expressed with gL, so gL appears to be essential for proper folding and surface expression of gH (Hutchinson *et al.*, 1992). Vaccinia virus expressed gH failed to induce neutralizing antibodies or induce protection in mice, whereas gH/gL co-expressed in vaccinia virus induced moderate levels of protection (Browne *et al.*, 1993). To identify the functional properties of BHV-1 gH/gL, rabbits were immunized twice intramuscularly with 25 µg gH/gL in VSA3, a proprietary adjuvant (VIDO/Biostar). On a Western blot, the rabbit sera recognized gH and gL (Fig. 2*b*, lanes B, C, E and F). No reaction was observed between the gH/gL-specific antibodies and gB, gC or gD (Fig. 2*b*, lanes G, H and I). ELISA and neutralization assays were performed as described by van Drunen Littel-van den Hurk *et al.* (1985). The sera of the immunized animals contained high levels of gH/gL-specific antibodies that neutralized virus infectivity (Fig. 2*c*). To determine whether gH/gL is involved in virus penetration, a post-adsorption neutralization assay was performed as described by Hughes *et*

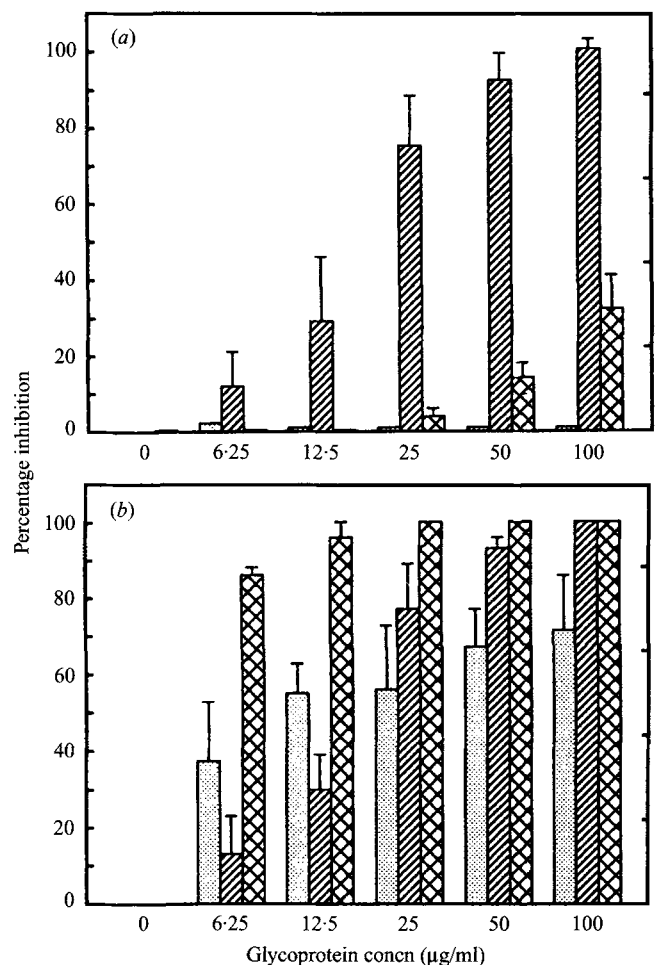


Fig. 3. Inhibition of virus attachment (*a*) or penetration (*b*) by purified glycoproteins gH/gL (■), gC (▨) or gD (▩). Glycoproteins were diluted in cold MEM to specified concentrations and 50 µl of each glycoprotein was added to pre-cooled MDBK cells. After a 1 h incubation on ice, 50 µl, containing 200 p.f.u. of BHV-1 was added to the wells and incubated on ice for a further 1 h. Subsequently, the cells were washed and overlaid with MEM containing 2% FBS and methylcellulose. Plaques were counted 3 days later. Percentage inhibition was calculated relative to a medium control. The mean number of plaques formed  $\pm$  s.d. for three experiments was  $85 \pm 10$ . The number of plaques formed in the absence of glycoprotein was defined as 100% plaque formation. The values represent the average of three experiments and the bars indicate s.d. values.

*al.* (1988) for gD-specific antibodies. This assay relies on the adsorption of virus at low temperature (4 °C), so that the virus envelope and the cell membrane cannot fuse and penetration cannot occur. Penetration is then initiated by a temperature shift to 37 °C. Antibodies are tested for effects on penetration by treatment of adsorbed virus with antibody prior to the temperature shift. The rabbit sera neutralized the virus after adsorption, which suggests that BHV-1 gH/gL is involved in virus penetration (Fig. 2*c*). To determine whether gH/gL also has a function in virus adsorption, it was tested for its inhibitory effect on virus adsorption by plaque assay as described by Liang *et al.* (1991). Briefly, confluent MDBK cell monolayers in 24-well plates (approximately  $1.5 \times 10^5$  cells

per well) were cooled on ice for 1 h. Viral glycoproteins were diluted in cold MEM to specified concentrations and added to the cells in volumes of 50 µl per well. The plates were incubated for 1 h on ice with purified glycoproteins gH/gL, gC or gD, before addition of 200 p.f.u. per well of BHV-1 in a 50 µl volume of MEM. After a further 1 h on ice, the monolayers were washed three times with MEM containing 2% FBS and overlaid with MEM containing 2% FBS and methylcellulose. Fig. 3(a) shows that in contrast to glycoprotein gC, which plays a major role in attachment of BHV-1, gH/gL did not have any effect on virus attachment. Glycoprotein D partially inhibited attachment at high concentrations, which confirms our previous observations (Liang *et al.*, 1991). To confirm that gH/gL plays a role in penetration, a penetration inhibition assay was performed as described by Highlander *et al.* (1987). Again, confluent MDBK cells were incubated for 1 h on ice with different concentrations of purified glycoproteins gH/gL, gC or gD and then virus was added for 1 h on ice. In this assay, this step was followed by incubation at 37 °C for 30 min and a citrate wash (40 mM-citric acid, 10 mM-KCl, 135 mM-NaCl, pH 3), before overlay. Fig. 3(b) demonstrates that gH/gL inhibited penetration, although not as effectively as gD, which has a very strong inhibitory effect. In contrast, gC had no inhibitory effect on virus penetration. These data show that BHV-1 gH/gL plays a role in penetration, in addition to cell fusion, as determined by Baranowski *et al.* (1993), which is in agreement with the functions ascribed to HSV-1 gH/gL. Indeed, when gH/gL, gC and gD were left on the BHV-1 infected MDBK cells during plaque formation as described by Johnson *et al.* (1990), the blocking observed for these three glycoproteins was almost identical to that in the penetration assay (data not shown).

In conclusion, this work has shown that the previously identified BHV-1 glycoproteins gII and gp108 are the same protein and are encoded by the homologue of the gH gene of HSV-1. Like HSV-1 gH/gL (Fuller *et al.*, 1989), BHV-1 gH and gL form a functional complex, which plays a role in virus penetration.

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