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Analysis of the biochemical properties of, and complex formation between, glycoproteins H and L of the γ_2 herpesvirus bovine herpesvirus-4

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Genes encoding glycoprotein gH and gL homologues were localized in the genome of the gammaherpesvirus bovine herpesvirus-4 (BHV-4). Both genes were sequenced and glutathione *S*-transferase fusion proteins were produced and used to immunize rabbits against the translation products of the two genes. The anti-gH serum recognized a protein with an apparent molecular mass (MM) of 110 kDa both in infected cells and in virions. This protein was sensitive to endo-*β*-*N*-acetylglucosaminase-H (endoH) and endoglycosidase F-*N*-glycosidase F (endoF-PNGaseF) digestion. A protein with the same relative mobility was immunoprecipitated from infected cells radiolabelled with [^{3}H]glucosamine which confirmed that this product (gp110), now designated BHV-4 gH, was glycosylated. Western blotting with the anti-gL serum detected in infected cells a product with an apparent MM ranging from 31–35 kDa and diffusely migrating protein species ranging from 45–65 kDa. Tunicamycin, monensin, endoH or endoF-PNGaseF treatments showed that both the 31–35 kDa and the 45–65 kDa proteins were glycosylated, gp31–35 being a precursor of the 45–65 kDa glycoprotein species. In radioimmunoprecipitation assays, the anti-gL serum immunoprecipitated from infected cells two glycosylated proteins with apparent MMs of 31–35 kDa (gp31–35) and 45–55 kDa (gp45–55). However a third glycoprotein, gp110, was also immunoprecipitated together with gp31–35 and gp45–55. gp110 and gp45–55 were subsequently confirmed to be virion glycoproteins corresponding to mature forms of BHV-4 gH and gL respectively. In addition, the present study clearly demonstrated complex formation between BHV-4 gH and gL both in virions and in infected cells.

Introduction

Genes encoding glycoproteins gH and gL have been found in nearly all members of the family *Herpesviridae* (Davison & Scott, 1986; Gompels & Minson, 1986; McGeoch & Davison, 1986; Cranage *et al.*, 1988; Gompels *et al.*, 1988; Josephs *et al.*, 1991; Meyer *et al.*, 1991; Kaye *et al.*, 1992; Klupp *et al.*, 1992, 1994; Telford *et al.*, 1992, 1993, 1995; Xu *et al.*, 1994*a*, *b*; Liu *et al.*, 1993; Scott *et al.*, 1993; Yoshida *et al.*, 1994; Khattar *et al.*, 1995; Moore *et al.*, 1996). The only exception is channel catfish virus (CCV), which shares few common genomic characteristics with the other herpesviruses (Davison, 1992).

Glycoproteins gH and gL are essential for virion infectivity. Glycoprotein H has been shown to take part in fusion of the viral envelope and the plasma membrane of host cells during the infection process (Gompels & Minson, 1986; Haddad & Hutt-Fletcher, 1989; Foà-Tomasi *et al.*, 1991; Peeters *et al.*, 1992) as well as in cell-to-cell spread of virions by fusion of plasma membranes (Foà-Tomasi *et al.*, 1991; Gompels *et al.*, 1991; Peeters *et al.*, 1992; Wilson *et al.*, 1994). However, except for a few observations (Roop *et al.*, 1993; Novotny *et al.*, 1996; Duus & Grose, 1996) the precise functions of gL are still poorly understood. The two glycoproteins have been shown to be inevitably associated on the surface of infected cells forming a complex of either two (Hutchinson *et al.*, 1992; Kaye *et al.*, 1992; Liu *et al.*, 1993; Forghani *et al.*, 1994; Stokes *et al.*, 1996; van Drunen Littel-van den Hurk, 1996) or three

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The nucleotide sequences of BHV-4 gH and gL have been submitted to the EMBL database and assigned accession numbers Z79633 and Z79634, respectively.

proteins (Yaswen *et al.*, 1993; Li *et al.*, 1995). Indeed, in the γ_1 herpesvirus Epstein–Barr virus (EBV), a third glycoprotein, gp42/38, which is the translation product of the BZLF2 open reading frame (ORF), is associated with the gH and gL homologues and seems to be directly involved in the penetration of EBV into B lymphocytes (Li *et al.*, 1995).

Since no other virus belonging to this subfamily has been studied to date with regard to the properties of the gH and gL homologues, the purpose of this study was to investigate the characteristics and properties of these two glycoproteins in the gammaherpesvirus bovine herpesvirus-4 (BHV-4), which also belongs to the *Gammaherpesvirinae*. Although the whole BHV-4 genome has not yet been sequenced, its overall genomic organization has recently been elucidated and shown to be similar to that of EBV (Bublot *et al.*, 1992; Lomonte *et al.*, 1995). However, the gene arrangement in BHV-4 is more closely related to that of the γ_2 herpesviruses herpesvirus saimiri (HVS) and equine herpesviruses-2 and -5 (EHV-2, -5) (Albrecht *et al.*, 1992; Bublot *et al.*, 1992; Telford *et al.*, 1992, 1995; Lomonte *et al.*, 1995). On the basis of these data, the BHV-4 gH and gL homologous genes were localized in the genome and identified as positional homologues of the EBV BXLF2 (gH) and BKRF2 (gL) genes respectively (Bublot *et al.*, 1992; Lomonte *et al.*, 1992). In the present study, both genes were sequenced and the biochemical properties of their translation products analysed. This study also demonstrates the formation of a gH–gL complex both in virions and in infected cells.

Methods

■ **Viruses and cells.** Madin–Darby bovine kidney (MDBK) cells (ATCC-CCL22) were grown at 37 °C in minimal essential medium (MEM) (Gibco-BRL) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 IU of penicillin and 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO_2. The BHV-4 V.Test strain (Thiry *et al.*, 1981) used in this study was prepared from subconfluent MDBK cells infected at an m.o.i. of 0·5 p.f.u. per cell and maintained in MEM with 2% horse serum (HS) at 37 °C. After virus attachment for 2 h, the medium was removed and replaced by fresh MEM containing 5% HS. When about 90% of the cell monolayer exhibited cytopathic effect (CPE), at about 72 h post-infection (p.i.), the virus was purified from the culture supernatant. Cells and cell debris were removed by centrifugation at 3000 ***g*** for 30 min and virus particles in the supernatant were pelleted at 100 000 ***g*** for 1 h. The virus pellet was then resuspended in PBS and purified on a preformed discontinuous gradient of 10–25% Ficoll 400 (Sigma) as described by Lyaku *et al.* (1992).

■ **DNA sequencing and computer analysis.** Nucleotide sequences were determined on both strands of cloned dsDNA fragments containing the gH and gL homologous genes by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using the T7 sequencing kit (Pharmacia) and appropriate primers recognizing sequences inside each gene. Sequence data were compiled and analysed using the PHYLIP software package, version 8.1 (UNIX), of the University of Wisconsin Genetics Computer Group (GCG) (Devereux *et al.*, 1984). Translated ORFs were compared to protein sequences present in the SWISS-PROT database (release 32.0 and 32.+) using the FASTA program (K-tuple of 2; Pearson & Lipman, 1988).

■ **Plasmid constructs and fusion proteins**

Construction of plasmids expressing GST–gH and GST–gL. Two plasmids were constructed, one with part of the gL gene and the other with part of the gH gene cloned in-frame with the *Schistosoma japonicum* glutathione *S*-transferase (GST) gene, following the protocol described below.

Glycoprotein gL or gH homologous ORFs cloned into pBluescript SK(+) (Stratagene) were cleaved by restriction endonucleases *Apa*LI and *Eco*RI or *Xho*II (Eurogenetec), respectively. Fragments containing the gL ORF (nt 93–420) or the gH ORF (nt 69–2121) were blunt ended with bacteriophage T4 DNA polymerase (Boehringer Mannheim) and inserted respectively into plasmids pGEX-5X-1 and pGEX-3X (Pharmacia), both cleaved by *Sma*I. The constructs, named pGEX-gL and pGEX-gH, were analysed for correct orientation and in-frame insertion of the fragments and transferred into *Escherichia coli* strain strain DH5α. One bacterial clone containing each plasmid was selected to produce the GST–gL and GST–gH fusion proteins.

Synthesis and purification of fusion proteins. The fusion proteins GST–gL and GST–gH were synthesized and purified according to the protocol described below. One litre of NZY medium (Gibco-BRL) containing 50 μg/ml of ampicillin was inoculated with 10 ml of an overnight bacterial culture and shaken moderately at 37 °C for 7 h. Expression of the fusion proteins was induced by addition of IPTG (0·5 μM final concentration) to the medium with shaking for 1 h. After induction of protein synthesis, the fusion products were purified according to the manufacturer's protocol for the Redipack GST purification kit (Pharmacia). Elution buffer (10 mM reduced glutathione, 50 mM Tris–HCl, pH 8) containing the GST fusion proteins was aliquoted and stored at −80 °C until use. The purity and quantity of each fusion protein were checked by migration in an SDS–polyacrylamide (PA) gel and staining with Coomassie blue R-250 (LKB).

■ **Production of fusion protein antisera.** The affinity-purified fusion proteins GST–gL and GST–gH were used to immunize four 8-week-old New Zealand White rabbits. Twenty μg of GST–gL and GST–gH, each contained in 500 μl of Tris–HCl (50 mM, pH 8), were mixed with an equal volume of complete Freund's adjuvant (Sigma) until a stable emulsion was obtained. One ml of each emulsion was used to immunize one rabbit (two rabbits per fusion protein). After 4 weeks, rabbits were boosted three times at 3 week intervals with 1 ml of protein in incomplete Freund's adjuvant (Sigma). Fourteen days after the final boost, serum from the four rabbits was collected, aliquoted and stored at −20 °C. The anti-GST–gH and anti-GST–gL sera were termed anti-gH and anti-gL, respectively.

■ **Radiolabelling and immunoprecipitation.** Immunoprecipitation was done as previously described (Dubuisson *et al.*, 1989). Briefly, MDBK cells grown to 70% confluence were infected with BHV-4 at a m.o.i. of 2–4 p.f.u. per cell and incubated at 37 °C in MEM containing 2% HS. After virus attachment for 2 h, monolayers were washed thoroughly and incubated in medium containing one-tenth the normal concentration of methionine or glucose together with 50 μCi/ml [^{35}S]methionine or 50 μCi/ml [^{3}H]glucosamine, respectively. Forty-eight hours p.i., cells were lysed with radioimmunoprecipitation assay buffer (150 mM NaCl; 50 mM Tris–HCl, pH 7·2; 1% Triton X-100; 0·1% SDS; 1% sodium deoxycholate; 0·1% NaN_3; 200 mM PMSF). Cell lysates were clarified by centrifugation at 100 000 ***g*** for 1 h at 4 °C. Immunoprecipitated labelled proteins were separated in SDS–PA gels under reducing conditions. Molecular mass markers (Amersham; ^{14}C-labelled) were electrophoresed in parallel tracks. After electrophoresis, gels were treated with sodium salicylate, dried, and exposed to Kodak X-OMAT AR 5 films.

Radiolabelled BHV-4 was prepared by infecting subconfluent MDBK cells at a m.o.i. of 0·5 p.f.u. per cell in the presence of [^{35}S]methionine (50 μCi/ml) until all cells showed CPE. Labelled virus was purified from the culture supernatant and immunoprecipitated following the protocols described above.

■ **Western blotting (immunoblotting).** Western blot reactions were carried out on either infected cells or unlabelled immunoprecipitated proteins. Seventy percent confluent MDBK cell monolayers were infected with BHV-4 at an m.o.i. of 2–4 p.f.u. per cell and incubated at 37 °C in MEM containing 2% HS. After virus attachment for 2 h, medium was removed, replaced by fresh MEM containing 5% HS and incubated for 48 h. When at least 30% of the cell monolayer showed CPE, the cells were washed with PBS and lysed with Laemmli buffer (Laemmli, 1970). Samples were boiled and aliquots run in an SDS–PA gel. After electrophoresis, proteins were electrotransferred onto PVDF membranes (Immobilon-P transfer membranes, 0·45 μm pore size; Millipore) and detected with a chemiluminescence blotting substrate (POD) kit according to the manufacturer's protocol (Boehringer Mannheim).

Unlabelled immunoprecipitated proteins were dissociated by boiling in Laemmli buffer, electrophoresed in SDS–PA gels and analysed by Western blotting using the same protocol.

■ **Tunicamycin and monensin treatment.** Seventy percent confluent MDBK cell monolayers infected with BHV-4 at an m.o.i. of 2–4 p.f.u. per cell as described above were treated concurrently with 10 μg/ml tunicamycin or 10^{-6} M monensin (Sigma). Twenty-four hours p.i. cells were lysed with Laemmli buffer and used for immunoblotting.

■ **Endoglycosidase digestion.** BHV-4-infected cells (about 5×10^5, m.o.i. 2–4 p.f.u. per cell) were resuspended in 1 ml of endo-*β*-*N*-acetylglucosaminidase-H [endoglycosidase H (endoH); Boehringer Mannheim] buffer (100 mM sodium citrate, pH 5·5; 0·02% SDS; 100 mM 2-mercaptoethanol) or endoglycosidase F-*N*-glycosidase F (endoF-PNGaseF; Boehringer Mannheim) buffer (100 mM Na_2HPO_4–NaH_2PO_4, pH 7·2; 20 mM EDTA; 0·1% SDS). Samples were incubated for 5 min at 95 °C and then 0·2% Triton X-100 (Sigma) was added. After cooling at 37 °C, PMSF (0·5 mM final concentration) and endoH (10–50 mU/mg protein) or endoF-PNGaseF (0·2–2 U/mg of protein) were added to sample aliquots and incubated at 37 °C for 18 h prior to gel electrophoresis and immunoblotting.

Results

BHV-4 gH and gL ORF translation products

The gene encoding gH is 2121 nt long and the predicted protein contains 707 aa with a molecular mass (MM) of 81·5 kDa. Sixteen potential *N*-linked glycosylation sites are present in the sequence which shows a hydrophobicity profile characteristic of a type I glycoprotein, i.e. presence of hydrophobic regions in the N-terminal and C-terminal parts of the protein corresponding to potential signal and anchor sequences respectively (data not shown). The gL ORF is 420 nt long and is predicted to encode a 140 aa protein with an MM of 15·8 kDa. Six potential *N*-linked glycosylation sites are present in the sequence. The hydrophobicity profile is similar to that of the EBV BKRF2 (gL) gene product with a potential signal sequence but apparently without any hydrophobic sequence long enough to be considered as membrane spanning (data not shown). This characteristic is not unusual since it has already been described for gL of several other herpesviruses (Kaye *et al.*, 1992; Liu *et al.*, 1993; Dubin & Jiang, 1995; Li *et al.*, 1995).

Detection of BHV-4 proteins with the anti-gH and anti-gL sera

The anti-gH serum recognized a specific BHV-4 protein with an apparent MM of 110 kDa both in infected cells and in virions (Fig. 1*a*, lanes 2 and 3). A protein with an apparent MM of 65 kDa was also detected (Fig. 1*a*, lanes 1 and 2) but was clearly a cellular protein which cross-reacted with the anti-gH serum. In infected cells, the anti-gL serum specifically detected proteins with apparent MMs ranging between 31–35 kDa and 45–65 kDa (Fig. 1*b*, lanes 2). A unique but still diffuse band with an apparent MM ranging between 50 and 65 kDa was detected in virions (Fig. 1*b*, lane 3). No such proteins were detected using either the preimmune or the anti-GST sera (data not shown). The 110 kDa and the 50–65 kDa proteins, both present in virions, have higher MMs than the calculated MMs of the gH and gL homologous ORF translation products respectively. These proteins had therefore to undergo co- or post-translational modifications, probably by addition of *N*- and/or *O*-linked oligosaccharides. Indeed, the presence of potential *N*-linked glycosylation sites in both protein sequences is in agreement with this hypothesis.

Analysis of the glycosylation of the 110 kDa, 31–35 kDa and 45–65 kDa products

Tunicamycin and monensin are two inhibitors of glycosylation. Tunicamycin inhibits the transfer of the oligosaccharide part [(*N*-acetylglucosamine)$_2$(mannose)$_9$(glucose)$_3$] of the dolichyldiphosphate complex onto the asparagine residue of a *N*-glycosylation consensus site, Asn-X-Ser/Thr (with X being any amino acid except Pro or Asp) (Kornfeld & Kornfeld, 1985). This reaction occurs in the rough endoplasmic reticulum (RER) during translation of the protein. Monensin acts as a membrane ionophore which reversibly blocks the activity of the proteins present in the Golgi apparatus (Ledger *et al.*, 1984). Maturation of high mannose moieties as hybrid or complex structures and addition of *O*-linked oligosaccharide structures are two of the Golgi activities which are inhibited by addition of monensin to the cell culture medium.

Tunicamycin treatment of BHV-4-infected cells resulted in the disappearance of the 110 kDa protein detected by the anti-gH serum (Fig. 1*a*, lane 4). Surprisingly, no unglycosylated precursor was detected in tunicamycin-treated infected cells, either by immunoblotting or by immunoprecipitation (results not shown), most likely due to an instability of the non-glycosylated protein in such treated infected cells. Monensin treatment led to the detection of a 104 kDa protein (Fig. 1*a*, lane 5). These results showed the 110 kDa protein (gp110) to be glycosylated, as predicted. Moreover, these findings suggest that gp110 results from the maturation of a 104 kDa product which most likely possesses high mannose sugars

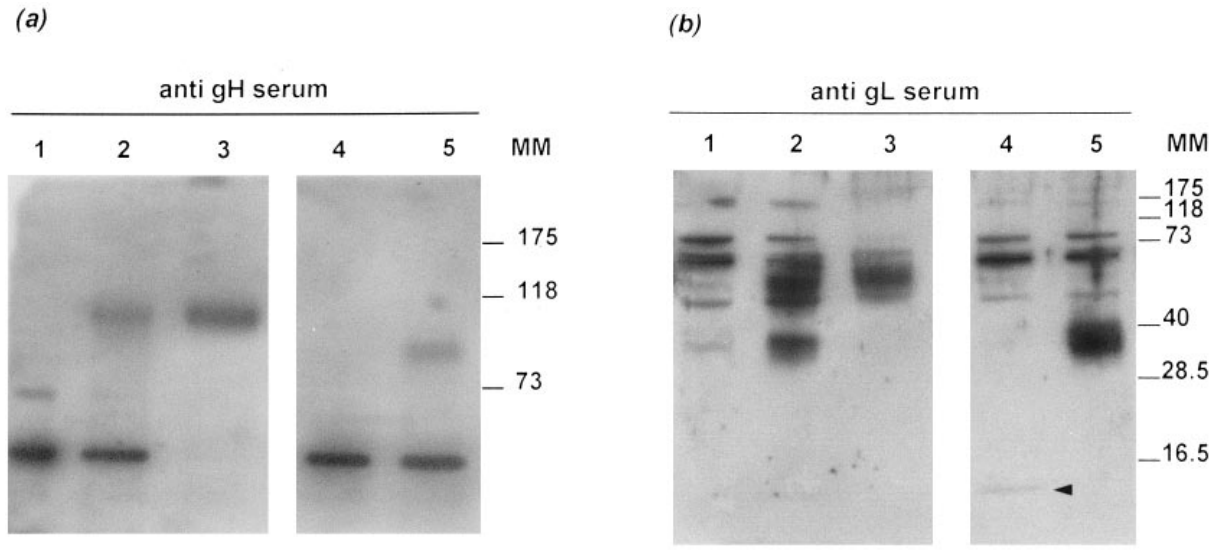

Fig. 1. Effect of tunicamycin and monensin treatment of infected MDBK cells on the detection of either the 110 kDa protein (*a*) or the 31–35 kDa and 45–65 kDa proteins (*b*). Western blotting (immunoblotting) was performed with either the anti-gH or anti-gL sera on proteins electrophoresed in SDS–7·5% (*a*) or –12% (*b*) PA gels before being electrotransferred onto PVDF membranes. Uninfected-cell proteins (lane 1), infected-cell proteins (lane 2), BHV-4 proteins (lane 3), tunicamycin-treated infected-cell proteins (lane 4), and monensin-treated infected-cell proteins (lane 5) are shown. The positions of MM markers (kDa) are shown. The arrowhead (lane 4*b*) points out the 14 kDa product detected with the anti-gL serum after tunicamycin treatment of infected cells. Anti-gH and anti-gL sera were diluted 100-fold in PBS.

processed in the Golgi apparatus, which results in the formation of the mature gp110 (gH) incorporated in the virus.

Tunicamycin treatment of infected cells led to the disappearance of both the 31–35 kDa and the 45–65 kDa protein species and to the concomitant detection of a faint but unique 14 kDa protein (Fig. 1*b*, lane 4). Treatment of infected cells with monensin led to the disappearance of the large upper 45–65 kDa signal but the 31–35 kDa protein remained detectable (Fig. 1*b*, lane 5). These results suggest that the 31–35 kDa (gp31–35) and 45–65 kDa (gp45–65) proteins are glycosylated and that gp31–35 is most likely an RER glycosylated form resulting from the addition of high mannose structures to the 14 kDa unglycosylated precursor of gL detected after tunicamycin treatment of infected cells. By inference, gp45–65 would then result from a further processing of gp31–35 in the Golgi apparatus. The width of the band signals obtained for both gp31–35 and especially gp45–65 could be due to: (i) heavy glycosylation of these two forms of BHV-4 gL and/or post-translational modifications of the proteins such as the addition of *O*-linked oligosaccharide structures, both of which may cause a disturbance in the migration of the glycoprotein in SDS–PA gels, or (ii) detection of several processed forms of gL.

Analysis of oligosaccharide structures present on gp110, gp31–35 and gp45–65

EndoH and endoF-PNGaseF glycosidases differ in their specificity towards oligosaccharide digestion. EndoH specifically cleaves high mannose and certain types of hybrid sugars processed only in the Golgi. EndoF-PNGaseF can cleave any type of *N*-linked sugar including complex oligosaccharide structures which result from the maturation of high mannose moieties during transport of the glycoprotein through the Golgi apparatus.

The susceptibility of gp110 to endoH and endoF-PNGaseF treatment was analysed. The results were the same for proteins from infected cells (Fig. 2*a*) and from virions (data not shown). The relative mobility of gp110 shifted to 104 kDa after treatment with endoH (Fig. 2*a*, lanes 2) whereas endoF-PNGaseF digestion led to the detection of a 77·5 kDa band (Fig. 2*a*, lanes 3), which is approximately the MM of gH calculated on the basis of its amino acid sequence and without considering the putative signal sequence. No such variations were observed when either infected-cell or viral proteins were incubated with endoH or endoF-PNGaseF buffers only (data not shown). These results suggest that gp110 oligosaccharide structures are mainly complex-type moieties with some hybrid sugars sensitive to endoH which probably remain linked to the mature protein.

The susceptibility of gp31–35 and gp45–65 to endoH and endoF-PNGaseF digestion was determined only for proteins from infected cells (as gp31–35 was not detectable in virions). After endoH treatment gp31–35 could not be detected and the intensity of the gp45–65 signal was reduced (Fig. 2*b*, lane 1). The predominant double bands observed for gp45–65 under these experimental conditions should represent proteins with various degrees of glycosylation and processing. A faint band

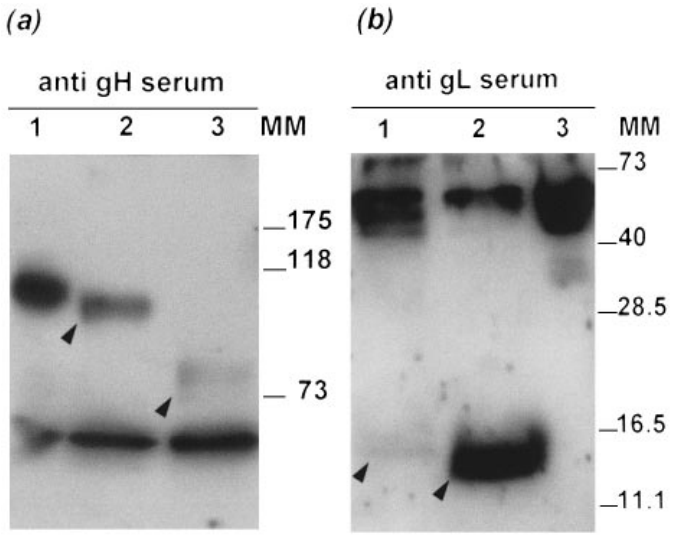

Fig. 2. Analysis of either gp110 (*a*) or gp31–35 and gp45–65 (*b*) oligosaccharide moieties by endoglycosidase digestion of infected-cell proteins. Western blotting was performed using either the anti-gH (*a*) or anti-gL (*b*) sera. Endoglycosidase-treated or -untreated proteins were electrophoresed in SDS–7·5% (*a*) or –15% (*b*) PA gels before Western blot detection. Untreated proteins (lanes 1*a* and 3*b*); endoH-digested proteins (lanes 2*a* and 1*b*) and endoF-PNGaseF-digested proteins (lanes 3*a* and 2*b*) are shown. The positions of MM markers (kDa) are shown. Arrowheads point out products obtained either after endoH digestion (lanes 2*a* and 1*b*) or after endoF-PNGaseF digestion (lanes 3*a* and 2*b*). Anti-gH and anti-gL sera were diluted 100-fold in PBS.

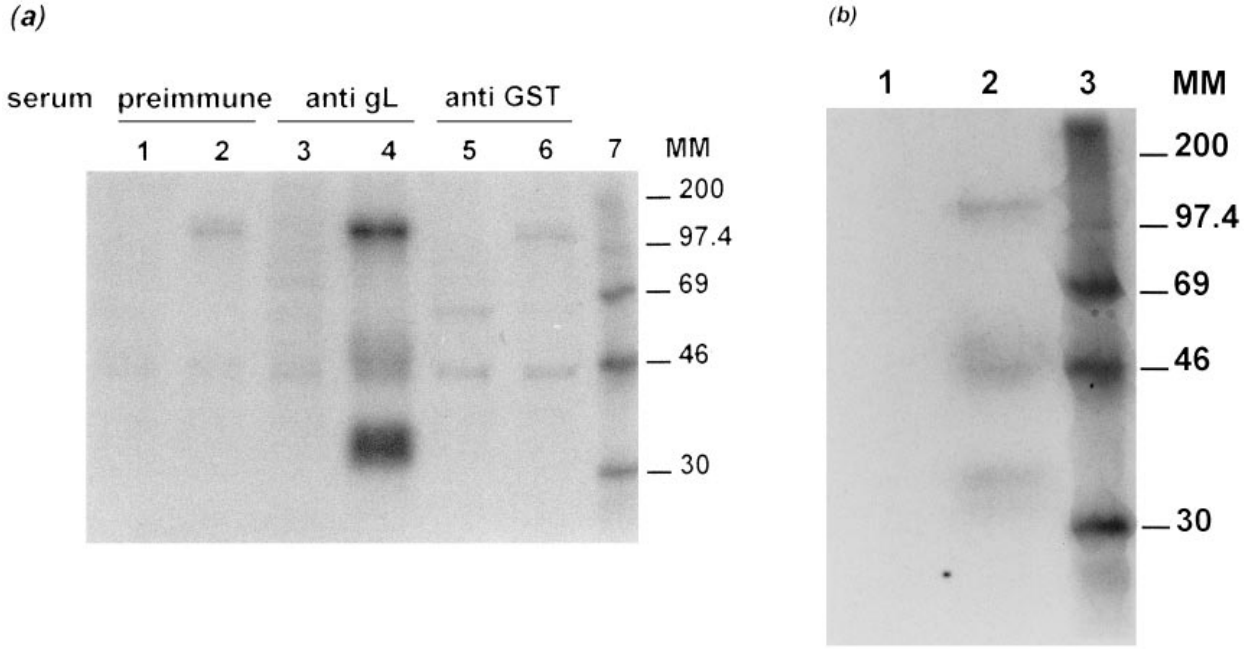

Fig. 3. Immunoprecipitation with the anti-gL serum of gp31–35, gp45–55 and gp110 from infected cells labelled with either [^{35}S]methionine (*a*) or [^{3}H]glucosamine (*b*). Labelled immunoprecipitated proteins were separated on an SDS–10% PA gel under reducing conditions. Labelled uninfected-cell proteins (lanes 1*a*, 1*b*, 3*a*, 5*a*), labelled infected-cell proteins (lanes 2*a*, 2*b*, 4*a*, 6*a*), and MM standards (kDa) labelled with ^{14}C (lanes 7*a* and 3*b*) are shown.

detected at 15 kDa (Fig. 2*b,* lane 1) correlated with the disappearance of gp31–35. This 15 kDa band almost corresponds to the MM of gL calculated on the basis of its amino acid sequence without considering its putative signal sequence. EndoF-PNGaseF digestion led to the detection of an intense band at 14–15 kDa which correlated with the absence of gp45–65 and gp31–35 (Fig. 2*b,* lane 2). No such modifications could be observed when proteins from infected cells were incubated with endoH or endo F-PNGaseF buffers only (data not shown). These results suggest that gp31–35 oligosaccharide structures are composed of high mannose moieties fully sensitive to endoH whereas gp45–65 sugars are mainly complex-type oligosaccharides. These findings are in agreement with the assumption that gp31–35 is a glycosylated precursor with gp45–65 representing Golgi-processed protein species of gL.

Analysis of BHV-4 gH–gL complex formation

Immunoprecipitation of BHV-4 gH and gL was done on infected-cell proteins labelled with either [^{35}S]methionine or

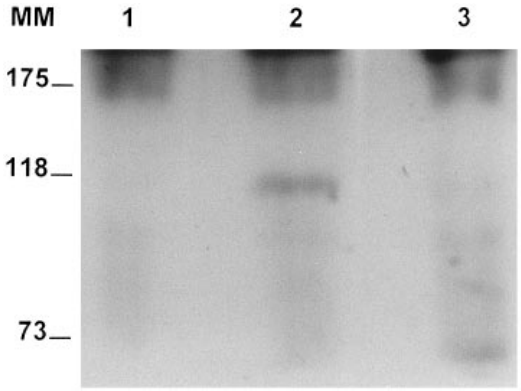

Fig. 4. Detection of BHV-4 gH (gp110) among the three proteins immunoprecipitated with the anti-gL serum. After migration on an SDS–7·5% PA gel, unlabelled proteins immunoprecipitated with the anti-gL serum were electrotransferred onto a PVDF membrane. BHV-4 gH (gp110) was detected by Western blotting using the anti-gH serum diluted 100-fold in PBS (lane 2). Preimmune serum (lane 1) and an anti-GST–BORFD1 serum (lane 3), raised against another GST fusion protein, were used as controls. The positions of MM markers (kDa) are shown.

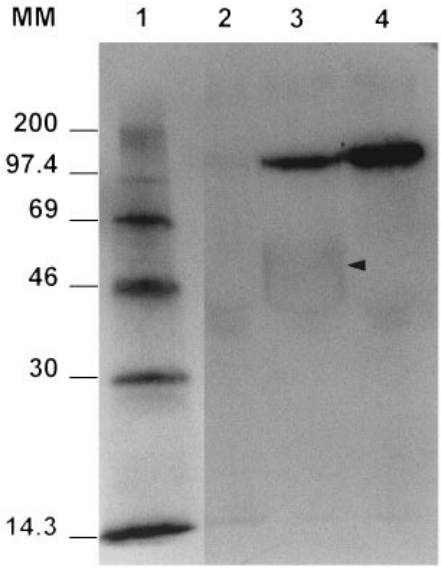

Fig. 5. Immunoprecipitation using anti-gL serum of mature BHV-4 gL (gp45–55) and gH (gp110) from purified virus labelled with [^{35}S]methionine. Immunoprecipitated proteins were separated on an SDS–12% PA gel under reducing conditions. MM standards (kDa) labelled with ^{14}C (lane 1), proteins immunoprecipitated with preimmune serum (lane 2), proteins immunoprecipitated with the anti-gL serum (lane 3), and gp110 immunoprecipitated with the anti-gH serum (lane 4) are shown. The signals for the MM standards resulted from an exposure time different to that for the rest of the gel.

[^{3}H]glucosamine. The anti-gH serum specifically immunoprecipitated a 110 kDa (gp110) protein both from [^{35}S]methionine- and [^{3}H]glucosamine-labelled infected cells (data not shown). Neither the preimmune nor the anti-GST sera immunoprecipitated any protein under the same experimental conditions. These results correspond to data obtained by immunoblot detection of proteins from BHV-4-infected cells with the anti-gH serum. The anti-gL serum specifically immunoprecipitated three glycoproteins. These glycoproteins, with apparent MMs of 31–35 kDa (gp31–35), 45–55 kDa (gp45–55) and 110 kDa (gp110), were detected both in [^{35}S]methionine- (Fig. 3*a*, lane 4) and in [^{3}H]glucosamine-labelled cells (Fig. 3*b*, lane 2); gp31–35 and gp45–55 most likely corresponded to glycoproteins with similar MMs detected by immunoblotting. Differences in apparent MM could in this case be attributed to the method used to detect these glycoproteins. Furthermore, the SDS–PA gel migration of protein species corresponding to gL could have been different following their immunoprecipitation with the anti-gL serum or when the whole infected-cell or virus-protein pool was run on the gel before immunoblotting.

Previous work by other investigators has shown the formation of a gH–gL complex in different herpesviruses. From our results, it was assumed that the gp110 immunoprecipitated together with gp31–35 and gp45–55 was gH. To verify this hypothesis, unlabelled proteins immunoprecipitated with the anti-gL serum were electrophoresed in an SDS–PA gel and then blotted on a PVDF membrane where detection was carried out using the anti-gH serum. Specific signal corresponding to gp110 was detected confirming complex formation between BHV-4 gH and gL in infected cells (Fig. 4, lane 2).

To show complex formation in virions, an immunoprecipitation assay using anti-gL as well as anti-gH sera was carried out on purified radiolabelled virus (Fig. 5). The anti-gL serum immunoprecipitated gp110 and gp45–55 (Fig. 5, lane 3) whereas the anti-gH serum only immunoprecipitated gp110 (Fig. 5, lane 4). These results show that in virions gH (gp110) is complexed with the gp45–55 form of gL.

Discussion

This study reports an analysis of the translation products of two BHV-4 ORFs which have been described as positional homologues of the EBV BXLF2 (gH) and BKRF2 (gL) genes. Western blot (immunoblot) and radioimmunoprecipitation assays using anti-gH serum specifically detected a 110 kDa glycoprotein (gp110) both in virus particles and in infected cells. This glycoprotein was then designated BHV-4 gH. Anti-gL serum detected several specific viral glycoproteins both by Western blotting and by immunoprecipitation assay. Products of 31–35 kDa (gp31–35) and 45–65 kDa (gp45–65) were detected by immunoblotting proteins from infected cells but the former was not detected in virions. These glycoprotein species were suggested to correspond to different glycosylated forms of BHV-4 gL. The width of the band signals obtained both for gp31–35 and especially for gp45–65 could be due to: (i) heavy glycosylation of the two forms of BHV-4 gL, (ii) detection of several processed forms of both gp31–35 and/or gp45–65 and/or (iii) post-translational modifications of the proteins such as addition of *O*-linked oligosaccharide structures which most likely resulted in the disturbance of migration of

the glycoprotein in SDS–PA gels. The latter suggestion is, of course, only valid for gp45–65 which results, unlike gp31–35, from processing by Golgi enzymes.

During glycoprotein biosynthesis the oligosaccharide moieties undergo extensive processing in specific intracellular locations (Kornfeld & Kornfeld, 1985). The acquisition of resistance to endoH corresponds to the intracellular transport time required for the high mannose glycosylated peptides to move from the RER to elements of the Golgi, where processing reactions convert them to endoH-resistant complex chains (Kornfeld & Kornfeld, 1985; Tarentino *et al.*, 1989). The complete sensitivity of gp31–35 to endoH digestion together with the resistance of gp45–65 to endoH and its sensitivity to endoF-PNGaseF digestion suggest that gp31–35 is a high mannose glycosylated product resulting from the glycosylation of a 14 kDa unglycosylated precursor; gp31–35 is then further processed to a gp45–65 glycoprotein species which carries mainly complex *N*-linked oligosaccharide structures.

Three glycoproteins, gp31–35, gp45–55 and gp110, were immunoprecipitated from infected cells using the anti-gL serum, gp31–35 and gp45–55 being most likely identical to those detected by Western blotting. We found that gp45–55 and gp110 were the mature associated forms of gL and gH respectively as evidenced by their presence in purified virions. The weak signal intensity of gp45–55 when labelled with [^{35}S]methionine as compared to the gp31–35 and gp110 bands suggested that gp45–55 possesses few methionine residues. Indeed, the BHV-4 gL homologous ORF encodes a protein containing only five methionine residues, two of which are located at positions 1 and 11 in the amino acid sequence, i.e. in the putative signal sequence. They would thus be eliminated by signal sequence cleavage during protein translocation through the RER membrane. This finding would explain the weak signal obtained for gp45–55 compared to gp110 (gH), which contains 16 methionine residues after cleavage of its putative signal sequence. The stronger signal observed for gp31–35 compared to gp45–55 could be explained by the independent immunoprecipitation of gp31–35 with regard to the gp45–55/gp110 complex. Indeed, the accumulation of free gp31–35 in the RER of infected cells together with the probable higher affinity of this form towards the anti-gL antibodies could explain the strength of the signal observed for gp31–35 as compared to that of gp45–55.

The association between HSV-1 gH and gL to form a protein heterodimer at the surface of infected cells and virion envelopes was described a few years ago (Hutchinson *et al.*, 1992; Roop *et al.*, 1993). Glycoprotein gL was shown to promote the correct folding and transport to the cell surface of gH in infected cells. This association has also been described for other herpesviruses (Kaye *et al.*, 1992; Liu *et al.*, 1993; Forghani *et al.*, 1994; Stokes *et al.*, 1996; van Drunen Littel-van den Hurk, 1996). In the present study, the biochemical properties of gH (gp110) and gL (gp45–55) of BHV-4 were determined and complex formation between the two glycoproteins was demonstrated both in infected cells and in virions. Detailed studies on the function of the BHV-4 gH–gL complex would further enhance our knowledge and understanding of these two glycoproteins in the *Gammaherpesvirinae*.

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