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Identification and Characterization of a Bovine Herpesvirus-1 (BHV-1) Glycoprotein gL Which Is Required for Proper Antigenicity, Processing, and Transport of BHV-1 Glycoprotein gH[1]

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DNA sequence analysis of the bovine herpesvirus-1 (BHV-1) genome revealed the presence of an open reading frame named UL1 which exhibited limited homology to glycoprotein gL of herpes simplex virus-1 (S. K. Khattar, S. van Drunen Littel-van den Hurk, L. A. Babiuk, and S. K. Tikoo, *Virology* 213, 28–37). To identify the BHV-1 UL1 protein, rabbit antisera were prepared against two synthetic peptides that were predicted by computer analysis to encompass antigenic epitopes. Sera against both peptides immunoprecipitated a 16- to 17-kDa protein from *in vitro* translated *in vitro* transcribed mRNA, BHV-1-infected MDBK cells, and purified virions. Enzymatic deglycosylation and lectin binding assays confirmed that the BHV-1 UL1 protein contains only O-linked oligosaccharides and was named glycoprotein gL. Sera against UL22 protein immunoprecipitated a protein of 108 kDa from BHV-1-infected MDBK cells and purified virions, which was modified only by N-linked oligosaccharides and was named glycoprotein gH. Glycoprotein gL expressed by recombinant vaccinia virus was properly processed and secreted into the medium. In contrast glycoprotein gH expressed by recombinant vaccinia virus was found to be retained in the rough endoplasmic reticulum. However, gH coexpressed with gL by recombinant vaccinia viruses was properly processed and transported to the cell surface, suggesting that complex formation between gH and gL is necessary for the proper processing and transport of gH but not gL. In addition gH–gL complex formation is also required for induction of neutralizing antibody response and anchoring of gL to the plasma membrane.

Bovine herpesvirus-1 (BHV-1), an alphaherpesvirus (Roizman *et al.,* 1982), is a predominant cause of respiratory disease, abortion, and genital infections in cattle (Yates, 1982). Like other herpesviruses, BHV-1 consists of a nucleocapsid containing the linear double-stranded DNA genome, which is surrounded by an electron-dense zone called the tegument and a lipid bilayer envelope. In this membrane are embedded the virus-encoded glycoproteins. These glycoproteins play an important role in the initial stages of virus–cell interactions and act as major antigenic determinants for the humoral and cellular immune responses of the host. Although 10 glycoprotein genes have been localized and sequenced in BHV-1 designated gB, gC, gD, gE, gG, gH, gI, gK, gL, and gM, only few have been characterized at the protein level (reviewed by Tikoo *et al.,* 1995; Khadr *et al.,* 1996; Khattar *et al.,* 1995; Vlcek *et al.,* 1995), all of which exhibit homology to respective glycoproteins of herpes simplex virus-1. While gC, gE, gG, and gI are nonessential for virus replication *in vitro* (Liang *et al.,* 1991; van Engelenburg *et al.,* 1994; Harrach, unpublished data), gD has been shown to be an essential component of the virion (Fehler *et al.,* 1992).

Glycoprotein gH, an essential component of the virion, is found in all herpesviruses and constitutes the second most highly conserved group of herpesviral glycoproteins. Homologs of gH have been found to be required for penetration and cell to cell spread (Gompels and Minson, 1986; Fuller *et al.,* 1989; Haddad and Hutt-Fletcher, 1989; Peeters *et al.,* 1992; Forrester *et al.,* 1992). In addition, antibodies against gH homologs possess potent complement-independent neutralizing activity (Buckmaster *et al.,* 1984; Gompels and Minson, 1986; Montalvo and Grose, 1986; Miller and Hutt-Fletcher, 1988; Fuller *et al.,* 1989; Forrester *et al.,* 1991; Gompels *et al.,* 1991). The expression of gH homologs in the absence of other virus proteins leads to the synthesis of the glycoprotein which is improperly transported and antigenically different from native gH, suggesting that gH homologs must interact with another viral protein in order to attain proper structure and function (Gompels and Minson, 1989; Foa-Tomasi *et al.,* 1991; Gompels *et al.,* 1991; Roberts *et al.,* 1991; Spaete *et al.,* 1993). Recently, an accessory molecule named glycoprotein gL (Hutchinson *et al.,* 1992; Kaye *et al.,* 1992; Roop *et al.,* 1993; Liu *et*

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al., 1993) was identified as the viral protein that forms a complex with gH, which is necessary for attaining proper antigenic conformation and transport of gH.

BHV-1 gII/108, a glycoprotein of 108 kDa (van Drunen littel-van den Hurk *et al.,* manuscript in preparation), is the target of complement-independent neutralizing monoclonal antibodies (MAbs) which inhibit penetration but do not prevent attachment of the virus to the cell (Baranowski *et al.,* 1993). gII/108 glycoprotein has been suggested to be involved in viral entry into the cell and in cell to cell spread of the virus (Baranowski *et al.,* 1993). Preliminary evidence suggests that gII/108 may be the homolog of glycoprotein H (Baranowski *et al.,* 1995; van Drunen littel-van den Hurk *et al.,* manuscript in preparation). However, in contrast to other herpesviruses, analysis of purified labeled BHV-1 virions by immunoprecipitation has suggested that gII/108 may not be a component of the virion (Baranowski *et al.,* 1993). In addition, no other protein has been found associated with gII/108 glycoprotein (Baranowski *et al.,* 1995). Since we recently described the sequence of the BHV-1 UL1 gene that has the potential to encode the homolog of HSV-1 glycoprotein gL (Khattar *et al.,* 1995), we sought to determine if BHV-1 encodes a functional gL and whether it is necessary for the correct transport and antigenicity of BHV-1 glycoprotein gH. In this communication, we describe the characterization of gH and gL glycoproteins and show that BHV-1 encodes a functional glycoprotein gL which forms a complex with gH and is required for proper post-translational modification and transport of gH.

MATERIALS AND METHODS

Reagents and media

Cell culture media and fetal bovine serum were purchased from GIBCO/BRL (Burlington, Ontario, Canada). Reagents for DNA manipulations and protein A–Sepharose were purchased from Pharmacia (Dorval, Quebec, Canada) and used as recommended by the manufacturer. The enzyme neuraminidase, 5′-bromo-2′-deoxyuridine, biotinylated lectins, and other reagents for protein analysis were obtained from Sigma Chemicals Co. (St. Louis, MO) and Pierce (Rockford, IL). The enzymes *O*-glycosidase, PNGase F, and endo H were purchased from Boehringer Mannheim Canada (Laval, Quebec, Canada). The Glycotrack kit was purchased from Oxford Biosystems Ltd. (Rosedale, NY). The reagents for immunofluorescence staining and other immunological assays were purchased from Zymed Laboratories Inc. (San Francisco, CA) and Bio-Rad (Mississauga, Ontario, Canada). Radioisotopically labeled compounds and reagents for fluorography were purchased from ICN, (Irvine, CA). Reagents for *in vitro* transcription and translation including plasmid pSP64polyA were purchased from Promega/Fisher, Scientific Ltd. (Nepean, Ontario, Canada). RIBI adjuvant was purchased from RIBI Immunochemicals (Hamilton, Ontario, Canada), while VSA3 is a proprietory adjuvant of VIDO/Biostar (Saskatoon, SK, Canada).

Cells and viruses

Madin–Darby bovine kidney (MDBK) cells, human thymidine kinase negative (TK^-) cells, and BSC-1 cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum. $LMTK^-$ cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% fetal bovine serum. Virus stocks of BHV-1 were prepared in MDBK cells (Babiuk and Rouse, 1975). Wild-type vaccinia virus (VV) was propagated in BSC-1 cells while recombinant VVs were cultivated in $LMTK^-$ cells (Mackett *et al.,* 1984).

Antipeptide sera and other antibodies

Two synthetic peptides, BUL1-1 (19 residues), representing amino acids 23 to 41, and BUL1-2 (16 residues), representing amino acids 143 to 158 of UL1 ORF (Khattar *et al.,* 1995), were synthesized onto a hydroxymethyl–phenyl resin using an Applied Biosystems 430 A solid-phase peptide synthesizer employing Fmoc chemistry. Rabbits were injected with 0.2 mg each of peptide conjugated to keyhole limpet hemocyanin in Freund's complete adjuvant. Subsequent booster immunizations were given with 0.2 mg peptide conjugated to egg white ovalbumin in RIBI adjuvant at 4-week intervals. MAbs (5C8, 1A9, and 3E2) specific for UL22 protein were generated essentially as described previously (van Drunen Littel-van den Hurk *et al.,* 1984) except that the antigen used for immunizing mice was envelope fraction of BHV-1 depleted of major glycoproteins gB, gC, and gD. Rabbit polyclonal serum specific for UL22 was prepared by injecting rabbits twice intramuscularly with 25 μg of affinity purified protein in VSA3.

Plasmid constructions

The full-length UL1-encoding gene was excised from a plasmid designated pSK2.2 [PTZ18R containing a 2.2-kb *Hin*dIII–*Nru*I fragment (Khattar *et al.,* 1995)] as a 600-bp *Nla*III–*Nhe*I fragment (blunt end repaired by T4 DNA polymerase) and inserted into *Nco*I (blunt end repaired by mung bean nuclease) and *Sma*I-digested pTZ18RgIV (Tikoo *et al.,* 1993a), generating plasmid pSKgL. A 0.6-kb *Bgl*II fragment derived from pSKgL was inserted into *Bam*HI-digested pSP64polyA, generating plasmid pSKiL, and to *Bgl*II-digested pVVSL (Tikoo *et al.,* 1993a), generating plasmid pSKvL.

The 2.9-kb *Avr*II fragment containing the UL22 gene (Meyer *et al.,* 1991) was excised from plasmid pSD106 (Mayfield *et al.,* 1983), blunt end repaired with T4 DNA polymerase, and ligated to *Bam*HI linkers. After *Bam*HI digestion of the ligation mixture, a 2.8-kb fragment was purified and ligated to *Bam*HI-digested pSP64polyA, gen-

erating plasmid pSKiH, and to *Bgl*II-digested pVVSL (Tikoo *et al.,* 1993a), generating plasmid pSKvH.

In vitro transcription and translation

The 0.5–5.0 μg of plasmid DNA (pSKiL or pSKiH) was *in vitro* transcribed with SP6 polymerase as described by the supplier (Promega). RNA was translated *in vitro* for 2 hr at 30° using a rabbit reticulocyte lysate containing 40 μCi of [^{35}S]cysteine. Canine pancreatic microsomal membranes were added to some reaction mixtures to process the *in vitro* translated proteins. After synthesis, the *in vitro* translated proteins were analyzed on sodium dodecyl sulphate (SDS)–polyacrylamide gels (PAGE) with or without immunoprecipitation.

Radiolabeling of cells and immunoprecipitation

For immunoprecipitation, LMTK$^-$ cells were infected with VVs at a multiplicity of infection of 5. After 90 min of adsorption, the cells were washed and incubated in cysteine-free DMEM for 3 hr before labeling with [^{35}S]-cysteine (100 μCi/ml). After 4–8 hr of labeling, the cells and/or media were harvested. BHV-1-infected MDBK cells were labeled as described previously (van Drunen Littel-van den Hurk *et al.,* 1984). In pulse chase experiments, cells were labeled at 6 hr postinfection with 100 μCi of [^{35}S]cysteine for 30 min. Depending on the specific experiment, either the cells were harvested immediately or the label was removed and the cells were incubated for different time periods in DMEM containing an excess of cold cysteine (chase). Proteins were immunoprecipitated from the medium or from infected cells lysed with modified radioimmunoprecipitation assay buffer and analyzed by SDS–PAGE as described previously (van Drunen Littel-van den Hurk *et al.,* 1984).

Isolation of recombinant vaccinia viruses

The desired recombinant VVs were made by homologous recombination as previously described (Mackett *et al.,* 1984), except that the linearized plasmid (pSKvL or pSKvH) DNA was electroporated into the wild-type VV-infected cells by using a Pharmacia Gene Pulser set at 200 V and 500 μF. The recombinant VVs were identified by screening TK$^-$ plaques for the expression of recombinant proteins by immunocytochemistry (Tikoo *et al.,* 1990) before plaque purification and making viral stocks. The recombinant VVs were named SKgL (expressing UL1 protein) and SKgH (expressing UL22 protein).

Western blotting

Affinity purified protein, potassium tartrate gradient purified BHV-1, and recombinant VV-infected LMTK$^-$ cells were suspended in modified lysis buffer and the proteins were separated by SDS–PAGE (van Drunen Littel-van den Hurk *et al.,* 1984). For Western blotting the proteins were subsequently transferred to nitrocellulose membranes and reacted with UL1- or UL22-specific antiserum. Bound antibody was visualized after incubation of membranes with biotinylated secondary antibody using the strepavidin/biotinylated alkaline phosphatase complex catalyzed chromogenic reaction of the Bio-Rad immunoblot assay kit. For carbohydrate detection, the membranes were treated with periodate followed by biotin–hydrazide (Glycotrack kit). For determining the type of carbohydrate attached, the membranes were incubated with a range of different biotinylated lectins. Finally, the biotinylated compounds were detected with streptavidin–alkaline phosphatase conjugate.

Immunofluorescence

MDBK or LMTK$^-$ cells were seeded and infected in four-well Lab-Tek chamber slides. The infected cells were fixed with either 2% paraformaldehyde for 15 min at 4° for surface staining or 100% methanol for 15 min at −20° for internal staining. The cells were incubated with rabbit antipeptide, rabbit antiprotein, or preimmune serum and goat anti-rabbit immunoglobulin G conjugated to FITC and analyzed.

Immunization of rabbit and antibody titer determination

Rabbits were immunized intraperitoneally with 0.2 ml of 10^6 PFU of wild-type VV or recombinant VVs (SKgL or SKgH) per milliliter at 0 and 21 days postinfection. In addition, one group of rabbits was immunized with 0.2 ml of a suspension containing equal PFUs of recombinant VVs SKgL and SKgH. Sera were obtained 2 weeks after each immunization. BHV-1-specific total antibody responses were measured by enzyme-linked immunosorbent assay (ELISA) (van Drunen Littel-van den Hurk *et al.,* 1984) with purified BHV-1 as an antigen. Serum neutralization antibody responses were determined as described previously (van Drunen Littel-van den Hurk *et al.,* 1984), using 100 PFU of BHV-1. Titers were expressed as the reciprocals of the highest antibody dilution that caused a 50% reduction in number of plaques relative to the control.

RESULTS

Production of UL1 protein-specific antiserum

In order to identify and characterize the protein encoded by the UL1 gene, we made antipeptide serum. Analysis of the UL1 gene product by an antigen program (Hopp and Woods, 1981) of the PC/GENE software package identified two highly immunogenic regions, one site close to the amino terminus of the predicted protein and one at the carboxy-terminal location. Peptide BUL1-1 of 19 amino acids corresponding to residues 23 to 41 and peptide BUL1-2 of 16 amino acids corresponding to resi-

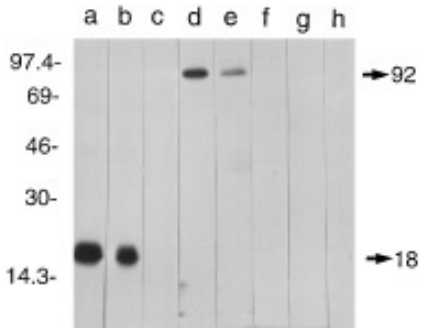

FIG. 1. Immunoprecipitation of proteins synthesized by *in vitro* transcription and translation of pSKiL and pSKiH plasmids. [^{35}S]Cysteine-labeled *in vitro* translated pSkiL products (lane a), immunoprecipitated with anti-BUL1-1 peptide serum (lane b) and preimmune serum (lane c); [^{35}S]cysteine-labeled pSkiH products (lane d), immunoprecipitated with anti-UL22 serum (lane e) and preimmune serum (lane f); and [^{35}S]cysteine-labeled pSP64polyA products, immunoprecipitated with anti-BUL1-1 peptide serum (lane g) and anti-UL22 serum (lane h), were separated on 12.5% SDS–PAGE under reducing conditions. The mol wt markers in kDa are indicated to the left.

dues 143 to 158 of the predicted UL1 primary translation product were synthesized and used for immunization of rabbits. Sera collected after the fourth boost were analyzed in detail. As shown below, sera against both peptides showed specific positive reactions. In contrast, none of the sera obtained after immunization of rabbits with an irrelevant peptide recognized any BHV-1-specific proteins (data not shown).

In vitro translation and immunoprecipitation of UL1 and UL22 proteins

In order to determine the identity of UL1 and UL22 ORFs and to check whether the antibodies against these gene products recognized their respective proteins, the two *in vitro* expression plasmids pSKiL and pSKiH were generated in which the coding sequence of UL1 and UL22, respectively, was placed downstream of the SP6 promoter. *In vitro* translation of pSKiL RNA resulted in the synthesis of a polypeptide of 18 kDa (Fig. 1, lane a). The same protein was also recognized by anti-BUL1-1 peptide serum (Fig. 1, lane b). Similarly, *in vitro* translation of pSKiH RNA resulted in the synthesis of a polypeptide of 92 kDa (Fig. 1, lane d) which was recognized by anti-UL22 serum (Fig. 1, lane e). However, these proteins were not immunoprecipitated with anti-UL1 peptide serum (Fig. 1, lane g) or anti-UL22 serum (Fig. 1, lane h) from reactions in which pSP64polyA RNA was translated *in vitro.* In addition, preimmune serum did not immunoprecipitate any protein from the reactions containing pSKiL (Fig. 1, lane c) or pSKiH (Fig. 1, lane f) RNAs.

In vivo expression of UL1 and UL22 proteins

To further characterize the proteins and to confirm the specificity of our antiserum, immunoprecipitation assays were performed. Radiolabeled proteins from mock-infected or BHV-1-infected cell lysates were immunoprecipitated and analyzed by SDS–PAGE. Compared to preimmune serum (Fig. 2A, lane b), both anti-BUL1-1 serum (Fig. 2A, lane c) and anti-BUL1-2 serum (Fig. 2A, lane f) recognized two protein species of 16 to 17 and 108 kDa. Preincubation of anti-BUL1-1 serum with BUL1-1 peptide inhibited detection (Fig. 2A, lane d), while addition of peptide BUL1-2 had no effect (Fig. 2A, lane e). Similarly, detection by anti-BUL1-2 peptide serum was inhibited by preincubation of serum with peptide BUL1-2 (Fig. 2A, lane g) but not with peptide BUL1-1 (Fig. 2A, lane h). These proteins were not detected in uninfected cells (Fig. 2A, lane a). In addition, anti-UL22 serum also recognized the two protein species of 108 and 16 to 17 kDa (Fig. 2A, lane i). To determine if the 16- to 17- and 108-kDa proteins are different and to examine if the anti-UL1 or anti-UL22 serum cross-react with UL22 or UL1 proteins, respectively, we constructed recombinant VVs expressing UL1 (SKgL) or UL22 (SKgH) proteins. The anti-BUL1-1 peptide serum (Fig. 2B, lane c) but not anti-UL22 serum (Fig. 2B, lane d) recognized a 16- to 17-kDa protein expressed in pSKgL VV-infected cells. The anti-UL22 serum (Fig. 2B, lane e) but not anti-BUL1-1 serum (Fig. 2B, lane f) recognized a protein of 100 kDa (precursor of UL22 protein) expressed in pSKgH VV-infected cells. These proteins were not detected in LMTK$^-$ cells infected with wild-type VV (Fig. 2B, lane b) or in uninfected cells (Fig. 2B, lane a). These results suggest that UL1 and UL22 proteins form a complex in BHV-1-infected cells which is recognized by both anti-BUL1-1 and anti-UL22 sera.

UL1 and UL22 proteins are not associated by disulfide bonds

To determine if the complex formation between UL1 and UL22 proteins depended on interchain disulfide bonds, the proteins from radiolabeled lysates of BHV-1-infected MDBK cells were immunoprecipitated with anti-BUL1-1 (Fig. 3, lanes a and b) or anti-UL22 serum (Fig. 3, lanes c and d) and analyzed by SDS–PAGE under reducing (Fig. 3, lanes a and c) and nonreducing (Fig. 3, lanes b and d) conditions. As seen in Fig. 3, both sera clearly recognized two proteins of 108 and 16 to 17 kDa from BHV-1-infected MDBK cells when the proteins were analyzed by either reducing or nonreducing conditions. These results suggest that UL1 and UL22 proteins are not associated by disulfide bonds.

The UL1 and UL22 proteins are glycosylated

Sequence analyses of UL1 protein homologs of most alphaherpesviruses except pseudorabies virus (PRV) show at least one N-linked glycosylation consensus sequence (Kornfeld and Kornfeld, 1985; Davison and Scott, 1986; McGeoch *et al.,* 1988; Telford *et al.,* 1992; Klupp *et al.,* 1994). However, PRV and the BHV-1 UL1 do not show

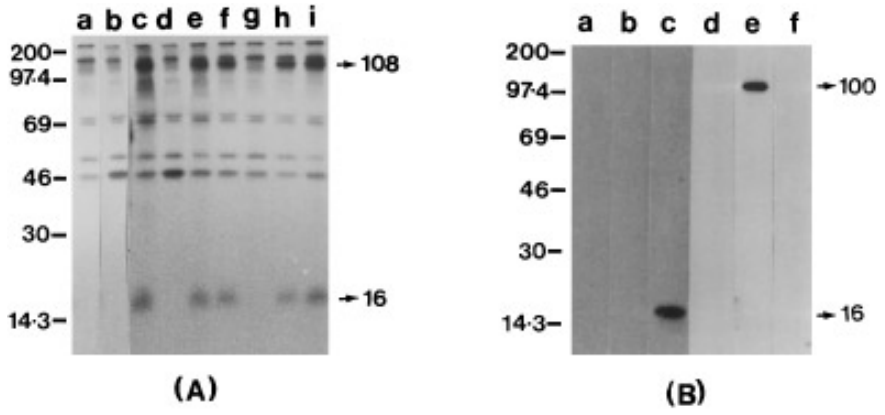

FIG. 2. *In vivo* expression of UL1 and UL22 proteins. (A) Proteins from lysates of [^{35}S]cysteine-labeled mock-infected (lane a) or BHV-1-infected (lanes b–i) MDBK cells were immunoprecipitated with preimmune serum (lane b), anti-BUL1-1 peptide serum (lanes a, c), anti-BUL1-1 peptide serum preincubated with BUL1-1 peptide (lane d), anti-BUL1-1 serum preincubated with BUL1-2 peptide (lane e), anti-BUL1-2 peptide serum (lane f), anti-BUL1-2 peptide serum preincubated with BUL1-2 peptide (lane g), anti-BUL1-2 peptide serum preincubated with BUL1-1 peptide (lane h), and anti-UL22 serum (lane i) and were separated on 15% SDS–PAGE under reducing conditions. (B) Proteins from lysates of [^{35}S]cysteine-labeled LMTK$^-$ cells mock-infected (lane a) or infected with wild-type vaccinia virus (lane b), recombinant VV SKgL (lanes c, d), and recombinant VV SKgH (lanes e, f) were immunoprecipitated with anti-BUL1-1 peptide serum (lanes a–c, f) or anti-UL22 serum (lanes d, e) and separated on 12.5% SDS–PAGE under reducing conditions. The mol wt markers in kDa are indicated to the left.

such a consensus sequence (Klupp *et al.,* 1994; Khattar *et al.,* 1995). *In vitro* translation of *in vitro* transcribed UL1 mRNA (Fig. 4A, lane a) in the presence of canine microsomal membranes did not show any decrease in the mobility of UL1 protein (Fig. 4A, lane b). In addition, treatment of UL1 protein with endo H (Fig. 4A, lane d) or PNGase F (Fig. 4A, lane c) did not show any increase in the mobility of the protein. Together these results suggest that UL1 protein is not modified by N-linked oligosaccharides. Moreover, an increase in the mobility of UL1 protein translated *in vitro* in the presence of canine microsomal membranes suggests that the signal sequence is cleaved (Fig. 4A, lane b). To determine whether the UL1 protein contained O-linked oligosaccharides we treated the UL1 protein with neuraminidase and *O*-glycosidase. No increase in the mobility of the protein was observed following such treatment (Fig. 4A, lane e). Second, attempts to label the protein with [^{3}H]glucosamine were unsuccessful (data not shown). However, periodate oxidation of UL1 protein detected carbohydrates (Fig. 4B, lane b). So we tested different lectins with specificities for different carbohydrate groups for their ability to bind to the protein. Only *Dolichus biflorus* lectin, which is specific for *N*-acetyl galactosamine [(GalNAc)-α-3GalNAc] bound to the UL1 protein (Fig. 4B, lane c). Treatment of the protein with neuraminidase and *O*-glycosidase (Fig. 4B, lane d) but not PNGase F (Fig. 4B, lane e) caused the loss of *D. biflorus* lectin binding due to removal of sugar moieties. Since these data indicated that O-linked oligosaccharides are attached to the UL1 protein, we named the UL1 translational product BHV-1 glycoprotein gL in accordance with the uniform nomenclature system of alphaherpesvirus glycoproteins as described earlier (Tikoo *et al.,* 1995).

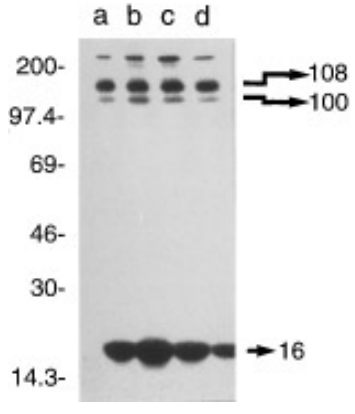

FIG. 3. UL1 and UL22 proteins are not associated by disulfide bonds. Proteins from lysates of [^{35}S]cysteine-labeled BHV-1-infected MDBK cells were immunoprecipitated with anti-BUL1-1 serum (a, b) or anti-UL22 serum (c, d) and separated on 10% SDS–PAGE under reducing (a, c) or nonreducing (b, d) conditions. The mol wt markers in kDa are indicated to the left.

Homologs of the herpesvirus UL22 proteins have been found to be glycosylated (Cranage *et al.,* 1988; Gompels and Minson, 1989; Klupp *et al.,* 1992). An *in vitro* translation of *in vitro* transcribed UL22 mRNA (Fig. 4C, lane a) in the presence of canine microsomal membranes showed a decrease in mobility of UL22 protein (Fig. 4C, lane b). UL22 protein synthesized in BHV-1-infected cells was labeled by [^{3}H]glucosamine (Fig. 4C, lane c). Treatment of UL22 protein (Fig. 4D, lane a) with PNGase F (Fig. 4D, lane b) but not with endo H (Fig. 4D, lane c), neuraminidase (Fig. 4D, lane d), or neuraminidase and *O*-glycosidase (Fig. 4D, lane e) showed an increase in the mobility of the protein. None of the lectins specific for O-linked oligosaccharides bound to the UL22 protein (data not shown). Taken together these results sug-

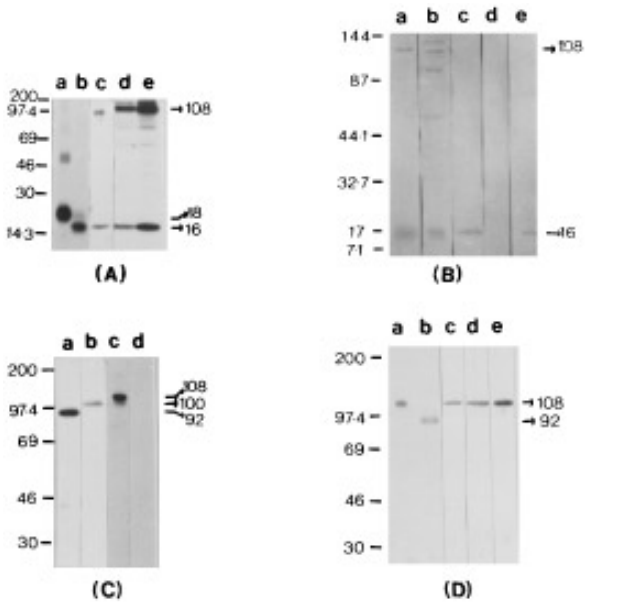

FIG. 4. Glycosylation of UL1 and UL22 proteins. (A) Immunoprecipitation of [^{35}S]cysteine-labeled *in vitro* translated pSKiL RNA products in the absence (lane a) or presence (lane b) of canine microsomal membranes with anti-BUL1-1 peptide serum. ^{35}S-labeled cytoplasmic extracts from MDBK cells infected with BHV-1 were immunoprecipitated with anti-BUL1-1 peptide serum and treated with PNGase F (lane c), endo H (lane d), or neuraminidase and *O*-glycosidase (lane e). All these proteins were separated on 15% SDS–PAGE. (B) For lectin binding, solublized purified BHV-1 virions (lanes a, b) or affinity purified UL1 protein (lanes c–e) were separated on 15% SDS–PAGE under reducing conditions. The separated proteins were probed in Western blots with anti-BUL1-1 peptide and anti-UL22 serum (lane a), oxidized with periodate and treated with biotin hydrazide (lane b) and probed before (lane c) or after digestion with neuraminidase and *O*-glycosidase (lane d) or PNGase F (lane e) with biotinylated *Dolicus biflorus* lectin. (C) Immunoprecipitation of proteins from [^{35}S]cysteine-labeled *in vitro* translated pSKiH RNA in the absence (lane a) or presence (lane b) of canine microsomal membranes, or from lysates of [^{3}H]glucosamine-labeled MDBK cells BHV-1 infected (lane c) or mock infected (lane d) by anti-UL22 serum. Immunoprecipitated proteins were separated on 10% SDS–PAGE under reducing conditions. (D) Proteins from [^{35}S]cysteine-labeled BHV-1-infected MDBK cells were immunoprecipitated with anti-UL22 serum. The immunoprecipitated proteins were mock digested (lane a) or treated with PNGase F (lane b), endo H (lane c), neuraminidase (lane d), or neuraminidase and *O*-glycosidase (lane e) and analyzed on 10% SDS–PAGE. The mol wt markers in kDa are indicated to the left.

gested that the UL22 protein is modified by N-linked oligosaccharides only and was named glycoprotein gH.

BHV-1 gL and gH are part of BHV-1 virions

In order to determine whether these two glycoproteins are components of the virion, BHV-1 virions were labeled with [^{35}S]cysteine and purified by sucrose gradients. Purified labeled virions were solubilized, and the proteins were immunoprecipitated by anti-BUL1-1 serum or anti-UL22 serum and analyzed by SDS–PAGE. As seen in Fig. 5, compared to preimmune serum (lane a), both anti-BUL1-1 (lane b) and anti-UL22 sera (lane c) specifically recognized 108- and 16- to 17-kDa virion proteins.

BHV-1 gL is required for proper processing and transport of BHV-1 gH

Two types of experiments were performed to investigate the processing of recombinant gH protein. (i) Pulse chase studies were performed to study the maturation of recombinant gH expressed by SKgH VV. (ii) Since endo H sensitivity indicates the retention of protein in the endoplasmic reticulum, endo H enzyme treatment of steady-state-labeled proteins was done to investigate whether the recombinant gH was retained in the endoplasmic reticulum (endo H sensitive) or transported to the Golgi apparatus (endo H resistant), where N-linked oligosaccharides are modified and O-linked oligosaccharides are added.

Wild-type gH expressed by BHV-1-infected cells was first detected as a 100-kDa precursor which was processed into a 108-kDa mature form of the protein (Fig. 6A, panel 1). The mature form of wild-type gH (Fig. 6B, lane a) was resistant to endo H enzyme treatment (Fig. 6B, lane b), indicating that it entered the normal secretory pathway through the Golgi apparatus, where N-linked oligosaccharides on gH were modified to the complex type prior to transport of gH to the cell surface. In contrast, gH protein expressed by recombinant VV SKgH was detected as a precursor (100 kDa) which was never processed into high-molecular-weight mature forms (Fig. 6A, panel 2). In addition, this recombinant protein (Fig. 6B, lane c) was sensitive to endo H treatment (Fig. 6B, lane d), indicating that this protein contained N-linked oligosaccharides of the high-mannose type exclusively (Kornfeld and Kornfeld, 1985). These findings suggest

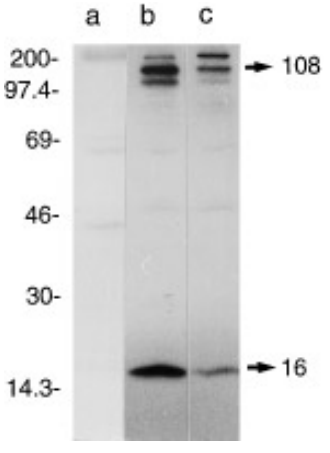

FIG. 5. Identification of the BHV-1 gL and gH proteins in purified virions. [^{35}S]cysteine-labeled sucrose gradient purified BHV-1 virions were solubilized and proteins were immunoprecipitated with preimmune serum (lane a), anti-BUL1-1 serum (lane b), or anti-UL22 serum (lane c) and analyzed on 12.5% SDS–PAGE. The mol wt markers in kDa are indicated to the left.

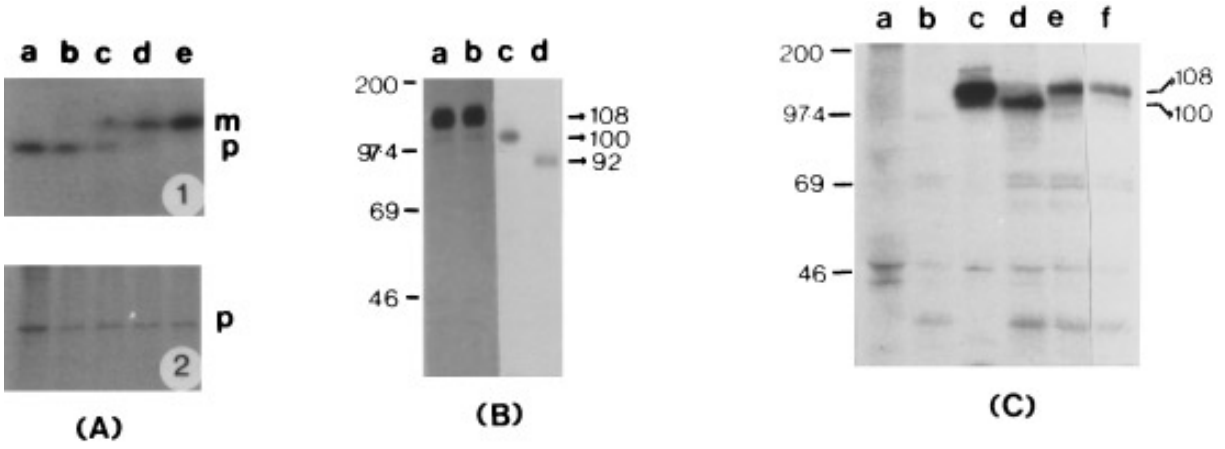

FIG. 6. Processing of glycoprotein gH. (A) BHV-1-infected MDBK cells (panel 1) or recombinant VV SKgH-infected LMTK$^-$ cells (panel 2) were pulse labeled with [^{35}S]cysteine for 15 min (lane a) and chased for 15 (lane b), 30 (lane c), 60 (lane d), or 120 (lane e) min in unlabeled medium. Cell extracts were immunoprecipitated with a UL22 MAb pool and analyzed by 12.5% SDS–PAGE under reducing conditions. (B) Proteins from [^{35}S]cysteine-labeled lysates of BHV-1-infected MDBK cells (lanes a, b) or recombinant VV SKgH-infected LMTK$^-$ cells (lanes c, d) were immunoprecipitated with anti-UL22 serum. Immunoprecipitated proteins were mock digested (lanes a, c) or treated with endo H (lanes b, d) and analyzed by 7.5% SDS–PAGE under reducing conditions. (C) Proteins from [^{35}S]cysteine-labeled lysates of mock-infected (lane a) or BHV-1-infected MDBK cells (lane c), wild-type vaccinia virus-infected (lane b), recombinant VV SKgH-infected (lane d), and recombinant VV SKgH plus recombinant VV SKgL-infected (lane e) LMTK$^-$ cells were immunoprecipitated with anti-UL22 serum. Immunoprecipitated proteins from recombinant VVs SKgH- and SKgL-infected LMTK$^-$ cells were treated with endo H (lane f). Proteins were analyzed by 7.5% SDS–PAGE under reducing conditions. The mol wt markers in kDa are indicated to the left.

that recombinant gH was not transported from the endoplasmic reticulum to the Golgi.

To determine if coexpression of gH and gL proteins would help in proper processing and transport of recombinant gH, LMTK$^-$ cells were infected with SKgH or SKgL individually or together. The radiolabeled proteins were immunoprecipitated with anti-UL22 serum and analyzed by SDS–PAGE. Compared to gH alone (Fig. 6C, lane d) coexpression of gL and gH results in the synthesis of a 108-kDa protein (mature form) (Fig. 6C, lane e) that is indistinguishable from gH synthesized in BHV-1-infected cells (Fig. 6C, lane c) and which is resistant to endo H treatment (Fig. 6C, lane f). This suggests that gH and gL form a complex which assists in the processing and transport of gH.

To examine if the gH–gL complex formation was necessary for the processing and transport of gL, radiolabeled proteins from infected cell lysates were immunoprecipitated with anti-BUL1-1 serum. As seen in Fig. 7A, the electrophoretic mobility of recombinant gL synthesized in SKgL-infected cells (lane d) was similar to authentic gL produced in BHV-1-infected cells (lane c) or gL produced in SKgL- and SKgH-infected cells (lane b). In addition, recombinant gL produced in SKgL-infected LMTK$^-$ cells contained O-linked oligosaccharides (Fig. 7B, lane c). This suggests that gH is not required for processing and transport of gL from the endoplasmic reticulum to the Golgi.

BHV-1 gL is not anchored independently to cell membranes

Recombinant gH when expressed alone, was detected intracellularly (Fig. 8e) but was not detected on the surface of the infected cells (Fig. 8f) or the culture media (Fig. 9, lane b). In contrast, coexpression of gH and gL proteins resulted in the detection of gH on the surface of the infected cells (Fig. 8h) but not in the culture media (Fig. 9, lane c), confirming that processing and transport of gH is dependent on gL. Similarly, recombinant gL, when expressed alone, was detected intracellularly (Fig. 8c) but was not detected on the surface of the infected

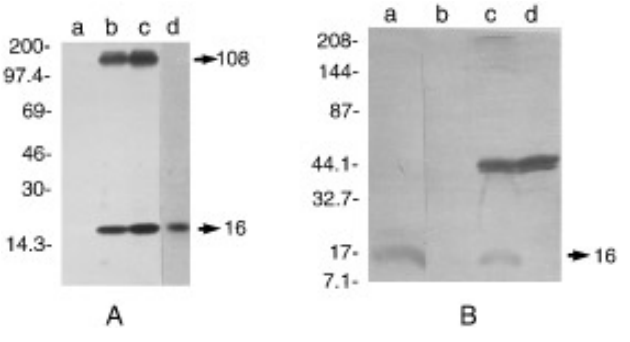

FIG. 7. Processing of glycoprotein gL. (A) Proteins from lysates of [^{35}S]cysteine-labeled wild-type VV (lane a)-, recombinant VV SKgL (lane d)-, and recombinant VV SKgL plus recombinant VV SKgH (lane b)-infected LMTK$^-$ cells or BHV-1-infected MDBK cells (lane c) were immunoprecipitated with anti-BUL1-1 peptide serum and analyzed on 15% SDS–PAGE under reducing conditions. (B) Proteins from lysates of mock-infected (lane d) cells, SKgL-infected LMTK$^-$ cells (lanes b, c), or BHV-1-infected MDBK cells with (lane a) were separated on SDS–PAGE under reducing conditions. After Western blotting, blots were probed with either anti-BUL1-1 serum (lane a) or biotinylated lectin (*Dolichos biflorus*) before (lanes c, d) or after neuraminidase and *O*-glycanase treatment (lane b). The mol wt markers in kDa are indicated to the left.

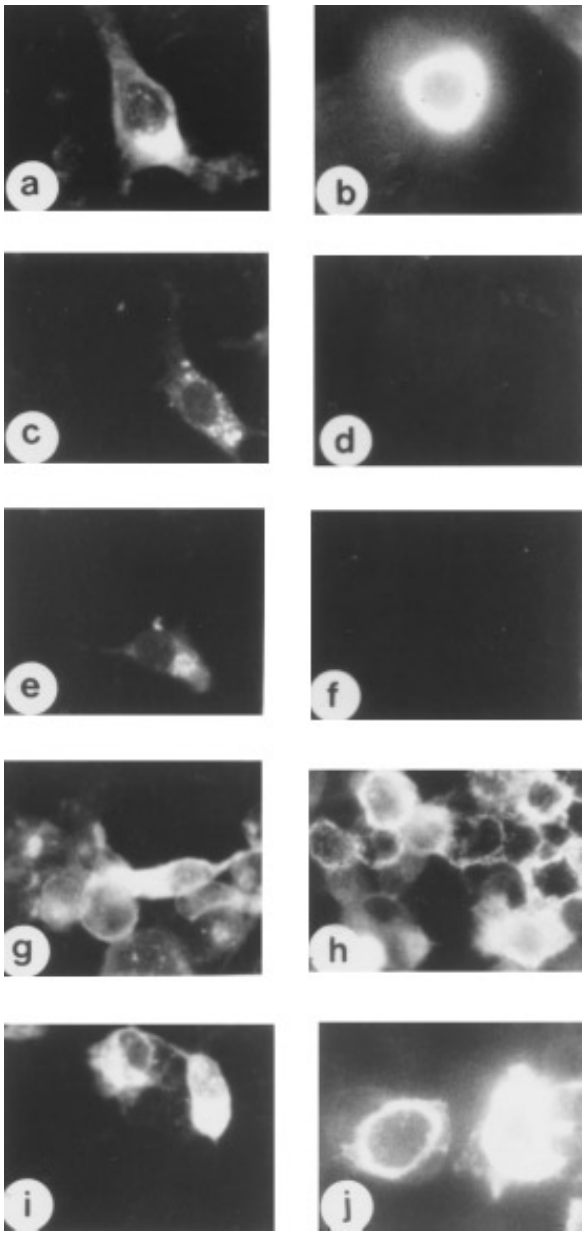

FIG. 8. Analysis of distribution of gH and gL glycoproteins. BHV-1-infected MDBK cells infected with BHV-1 (a, b) or LMTK$^-$ cells infected with recombinant VV SKgL (c, d), recombinant VV SKgh (e, f), or recombinant VVs SKgH plus SKgL (g, h, i, j) were fixed either with 2% paraformaldehyde to detect protein on the surface of the cells (b, d, f, h, j) or permeabilized with methanol to detect protein in the cells (a, c, e, g, i). The fixed cells were treated with either anti-UL22 serum (a, b, e–h) or anti-BUL1-1 peptide serum (c, d, i, j) followed by fluorescein-conjugated goat anti-rabbit IgG. Mock-infected BHV-1 or LMTK$^-$ cells, after fixing with paraformaldehyde or permeabilizing with methanol, looked similar to those in panel d.

cells (Fig. 8d). Coexpression of gL and gH resulted in the detection of gL on the surface of infected cells (Fig. 8j). However, gL was immunoprecipitated from the culture media of cells infected with recombinant VV SKgL alone (Fig. 9, lane a) but not from cells coinfected with the recombinant VVs SKgL and SKgH (Fig. 9, lane d).

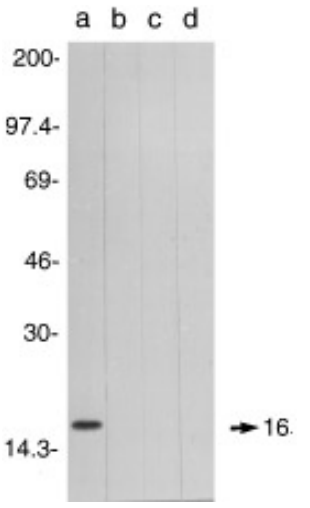

FIG. 9. Secretion of glycoprotein gH and gL. [^{35}S]Cysteine-labeled culture medium from LMTK$^-$ cells infected with recombinant SKgL (lane a), recombinant SKgH (lane b), or recombinant SKgL plus SKgH (lanes c, d) were immunoprecipitated with anti-BUL1-1 peptide serum (lanes a, d), anti-UL22 serum (lanes b, c), or anti-BUL1-1 peptide serum (lane a, d), and proteins were analyzed by 15% SDS–PAGE under reducing conditions. The mol wt markers in kDa are shown to the left.

These results suggest that gL is membrane associated as a result of its interaction with gH.

Immunogenicity of recombinant gH and gL proteins

In order to examine if complex formation between gH and gL was necessary for the development of neutralizing antibody responses, rabbits were immunized with the recombinant VVs SKgH and SKgL individually or in combination. As shown in Table 1, ELISA titers were detected in rabbits immunized with gH, gL, and gH–gL. However, complement-independent serum neutralizing antibody responses could only be detected in rabbits immunized with gH–gL.

TABLE 1

Serologic Response of Rabbits Immunized with Recombinant SKgL or SKgH[a]

Antigen	ELISA antibody titer[b]	Virus-neutralizing antibody titer[c]
Wild-type VV	<10	<2
Recombinant gL	160	<2
Recombinant gH	640	<2
Recombinant gH plus gL	2560	16

[a] Rabbits were immunized intraperitoneally with 0.2 ml of 10^6 PFU per milliliter at 0 and 21 days postinfection and sera were obtained after 2 weeks of immunization.

[b] ELISA titers versus 0.1 μg of purified BHV-1 per well (van Drunen Littel-van den Hurk *et al.,* 1984).

[c] Virus-neutralizing antibody titers versus 100 PFU of BHV-1 in the absence of complement (van Drunen Littel-van den Hurk *et al.,* 1984).

DISCUSSION

Several studies have shown that a novel glycoprotein designated gL is encoded by members of all herpesvirus subgroups, including alpha, beta, and gamma herpesviruses, which is required for the correct processing and transport of glycoprotein H (Hutchinson *et al.,* 1992; Kaye *et al.,* 1992; Liu *et al.,* 1993; Yaswen *et al.,* 1993; Spaete *et al.,* 1993, Forgani *et al.,* 1994). This glycoprotein complex (gH–gL) has been proposed to be essential for virus penetration and cell to cell spread. Although these two glycoproteins are structural components of all herpesviruses examined so far, the interaction between them differs among the different herpesviruses (Duus *et al.,* 1995), suggesting that this complex may be involved in virus-specific interactions and thus may determine the tropism of the virus. In this report we show that BHV-1 also encodes functional gL and gH glycoproteins, which are structural components of virion and form a complex which appears to be required for the proper processing, transport, and antigenicity of gH and anchoring of gL on the cell surface.

The BHV-1 UL1 gene has been suggested to encode a protein of 158 amino acids which is predicted to contain an N-terminal signal sequence with no potential site(s) for the addition of N-linked oligosaccharides (Khattar *et al.,* 1995). Two different antipeptide sera immunoprecipitated a protein of 16 to 17 kDa from BHV-1-infected cells and purified virions which we designated glycoprotein gL based on following observations: (i) the BHV-1 UL1 gene is a positional homolog of the other herpesvirus glycoprotein gL, (ii) the N-terminal hydrophobic region of the BHV-1 UL1 protein acts as a signal sequence as it is cleaved, (iii) the BHV-1 UL1 protein is glycosylated, and (iv) the BHV-1 UL1 protein is functionally equivalent to glycoprotein gL of other herpesviruses. BHV-1 gL does not need any other viral protein for its posttranslational processing, since cells infected with recombinant VV SKgL secrete gL which is indistinguishable from gL produced in BHV-1-infected cells or in cells transfected with gL (data not shown). In contrast, a recent report suggests that HSV-1 gL produced in transfected cells (Dubin and Jiang, 1995) is processed differently than gL produced in recombinant VV-infected cells (Hutchinson *et al.,* 1992).

However, BHV-1 gL does need a glycoprotein for its association with the plasma membrane. Three lines of evidence suggest that glycoprotein gH is required for the anchoring of gL to the plasma membrane: (i) there is no other hydrophobic domain in gL which might serve as putative transmembrane anchor except the N-terminal signal sequence, which is cleaved in the mature form; (ii) gL is secreted from cells infected with recombinant VV SKgL but not from cells coinfected with recombinant VV SKgL and SKgH; and (iii) cell surface expression of gL results from complex formation with gH, which has a well-defined carboxy-terminal hydrophobic transmembrane anchor domain. A similar observation was made earlier for human cytomegalovirus (CMV) gL (Spaete *et al.,* 1993) and herpes simplex virus-1 (HSV-1) gL (Dubin and Jiang, 1995).

Previous studies have suggested that BHV-1 gII and gp108 are identical proteins (van Drunen Littel-van den Hurk *et al.,* manuscript in preparation). Using cross-immunoprecipitation with monoclonal antibodies to BHV-1 gp108 and anti-UL22 peptide antiserum, it was suggested recently that gp108 is the product of the UL22 (gH) ORF (Baranowski *et al.,* 1995). Our results involving the expression of the UL22 gene *in vitro* and *in vivo* confirm and extend the previous observations and provide further evidence that gp108, gII, and the translation product of UL22, glycoprotein gH, are identical proteins and contain only N-linked oligosaccharides.

BHV-1 glycoproteins gB, gC, and gD expressed by recombinant VVs are transported and processed similar to the authentic glycoproteins produced in BHV-1-infected cells (van Drunen Littel-van den Hurk *et al.,* 1989; Tikoo *et al.,* 1993a,b). However, the characteristics of gH expressed by recombinant VV SKgH differed from those of authentic gH produced in BHV-1-infected cells. The recombinant gH was slightly smaller, possessed endo H-sensitive glycans, and was not transported to the cell surface. This is unlikely to be due to some anomaly in the vaccinia virus expression system since similar results were found with BHV-1 gH expressed by transfected MDBK cells (unpublished results). This defect in intracellular transport appears to be a conserved feature of herpesvirus gH homologs, including HSV-1 (Gompels and Minson, 1989).

The proper processing and transport of recombinant gH could be restored by coexpression of gH with gL. Further evidence that gH and gL form a complex comes from the observation that both glycoproteins were immunoprecipitated from BHV-1-infected cells with either anti-gH or anti-gL serum. This gH–gL interaction appears to be noncovalent since the same molecular weight proteins were detected by PAGE analysis under reducing and nonreducing conditions. Although similar gH–gL complex formation has been observed in other herpesviruses, the nature of the interactions differ. For example, the HSV-1 gH is associated noncovalently with gL (Hutchinson *et al.,* 1992; Kaye *et al.,* 1992) and CMV gH is associated with gL by disulfide linkages (Spaete *et al.,* 1993).

Like other herpesvirus gH proteins, BHV-1 gH has been suggested to be involved in virus penetration, cell to cell spread, and induction of neutralizing antibodies (Baranowski *et al.,* 1993). Using an affinity purified gH:gL complex, we demonstrated that preincubation of cells with the complex prevented virus penetration but did not prevent virus attachment (van Drunen Littel-van den Hurk *et al.,* manuscript in preparation). In addition, neutralizing antibodies could be detected in sera of rabbits immu-

nized with both recombinant VV SKgL and recombinant VV SKgH but not when the rabbits were immunized with individual recombinant VV. These results clearly suggested that formation of the gH–gL complex is necessary for the biological and immunological functions of gH. However, it is not clear what the contribution of gL is to these biological and immunological functions. It is possible that a functional domain composed of parts of both gH and gL is produced as a result of their interaction. Alternatively, gL may be helping gH in attaining the biologically active configuration and thus may not be directly involved in these functions. Experiments are in progress to prove or refute these alternatives.

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