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Structural, Functional, and Immunological Characterization of Bovine Herpesvirus-1 Glycoprotein gl Expressed by Recombinant Baculovirus

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Received April 2, 1992; accepted June 1, 1992

The major glycoprotein complex gl of bovine herpesvirus-1 was expressed at high levels (36 μg per 1 x 10⁶ cells) in insect cells using a recombinant baculovirus. The recombinant gl had an apparent molecular weight of 116 kDa and was partially cleaved to yield 63-kDa (glb) and 52-kDa (glc) subunits. This processing step was significantly less efficient in insect cells than the analogous step in mammalian cells, even though the cleavage sites of authentic and recombinant gl were shown to be identical. The oligosaccharide linkages were mostly endoglycosidase-H-sensitive, in contrast to those of authentic gl, which has mostly endoglycosidase-H-resistant linkages and an apparent molecular weight of 130/74/55 kDa. Despite the reduced cleavage and altered glycosylation, the recombinant glycoprotein was transported and expressed on the surface of infected insect cells. These surface molecules were biologically active as demonstrated by their ability to induce cell-cell fusion. Fusion was inhibited by three monoclonal antibodies specific for antigenic domains I and IV on gl. Domain I maps to the extracellular region of the carboxy terminal fragment glb and domain IV to the very amino terminus of the glb fragment, indicating that domains mapping in two distinct regions of gl function in cell fusion. Monoclonal antibodies specific for eight different epitopes recognized recombinant gl, indicating that the antigenic characteristics of the recombinant and authentic glycoproteins are similar. In addition, the recombinant gl was as immunogenic as the authentic gl, resulting in the induction of gl-specific antibodies in cattle.

Academic Press, Inc.

INTRODUCTION

Bovine herpesvirus-1 (BHV-1) gl belongs to a group of homologous glycoproteins that have been detected in all herpesviruses analyzed to date. The prototype for this family of glycoproteins is gB of herpes simplex virus (HSV) (Pellett et al., 1985b; Bzik et al., 1986). Glycoproteins with homology to gB have been described for human cytomegalovirus (HCMV) (Cranage et al., 1986), varicella zoster virus (VZV) (Keller et al., 1986), Epstein–Barr virus (EBV) (Pellett et al., 1985a), pseudorabies virus (PRV) (Robbins et al., 1987), BHV-1 (Whitbeck et al., 1988; Mira et al., 1988), BHV-2 (Hammerschmidt et al., 1988), equine herpesvirus-1 (EHV-1) (Whalley et al., 1989), EHV-4 (Higgin et al., 1989), Marek’s disease virus (Ross et al., 1989), herpesvirus of turkeys (Buckmaster et al., 1988), and herpesvirus saimiri (Albrecht and Fleckenstein, 1990). These glycoproteins show a high degree of homology at the DNA, protein, and structural level, which suggests that they play a central role in the biology of herpesviruses. This is underscored by the observation that HSV gB and PRV gll are essential for viral replication (Cai et al., 1987; Rauh et al., 1991). On the basis of the high degree of homology between the gB homologs, it is very likely that BHV-1 gl is an indispensible glycoprotein. A number of functions have been ascribed to this glycoprotein, among which are a role in attachment and penetration into susceptible cells (Liang et al., 1991), induction of cell-cell fusion (Fitzpatrick et al., 1988, 1990), and the induction of neutralizing antibodies (van Drunen Littel-van den Hurk and Babiuk, 1985). It is also a major target for the immune response of the host during BHV-1 infection (Collins et al., 1985; van Drunen Littel-van den Hurk and Babiuk, 1986a) and it confers protection from BHV-1 challenge in cattle (Babiuk et al., 1987; van Drunen Littel-van den Hurk et al., 1990a), which makes it a suitable subunit vaccine candidate.

Mature gl consists of three related polypeptides that are derived from a common 105-kDa primary translation product. After glycosylation the precursor is cleaved by a cellular protease into two smaller glycoproteins of 74 and 55 kDa that are covalently linked by disulfide bonds (Marshall et al., 1986; van Drunen Litt-
have proven to be valuable for foreign gene expression both in terms of the yield of the expressed protein and in the conservation of the biological properties of the derived protein (Luckow and Summers, 1988). Unfortunately, insect cells do not always authentically glycosylate and process the heterologous protein, resulting in aberrant forms with altered immunogenicity. Expression of the gl gene of BHV-1 in baculovirus resulted in the production of very high levels of gl. Although the recombinant gl was neither glycosylated nor cleaved as efficiently as the authentic gl, several of its functional, antigenic, and immunogenic properties were similar to those of authentic gl. It was transported to the cell surface and it mediated cell fusion. Affinity-purified recombinant gl induced the same level of antibodies in cattle as authentic gl. The availability of large amounts of gl will facilitate further studies on its function and potential as a subunit vaccine to BHV-1.

MATERIALS AND METHODS

Cells, viruses, and antibodies

Madin Darby bovine kidney (MDBK) cells were cultured in Eagle’s minimal essential medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO). Virus

Fig. 1. SDS-PAGE analysis of Bac-gl-infected Sf9 cells at 0 (B), 24 (C), 48 (D), and 72 (E) hr after infection, and of AcNPV-infected Sf9 cells at 48 hr (A) postinfection. Approximately 1.5 x 10^6 cells were applied per well. The polypeptides were separated on 10% polyacrylamide gels and stained with Coomassie brilliant blue. Molecular weight markers x 10^3 are indicated in the left margin. Recombinant glycoproteins are indicated as bgla, bglb, and bglc.
Fig. 3. Processing of recombinant and authentic gl. (A) Tunicamycin treatment. BHV-1-infected MDBK cells (lanes A and B) and Bac-gl-infected Sf9 cells (lanes C and D) were labeled with L-[35S]methionine and treated with tunicamycin (lanes B and D) or left untreated (lanes A and C). Cell lysates were precipitated with a gl-specific monoclonal antibody cocktail and analyzed on 7.5% polyacrylamide gels. (B) Endoglycosidase treatment. Bat-gl-infected Sf9 cells (lanes A, B, and C) and BHV-1-infected MDBK cells (lanes D, E, and F) were treated with endo H (lanes B and E), endo F (lanes C and F) or left untreated (lanes A and D). Cell lysates were separated on 8.5% polyacrylamide gels and analyzed by immunoblotting with a gl-specific monoclonal antibody cocktail. The positions of authentic gl (a-gl) and recombinant gl (b-gl) and the molecular weight shifts due to the various treatments are indicated with arrowheads.

stocks of BHV-1 strain Cooper were grown in MDBK cells as previously described (Babiuk et al., 1975). Spodoptera frugiperda (Sf9) cells were grown and maintained in TNM-FH medium (GIBCO) containing 10% FBS according to the procedures described by Summers and Smith (1987). Virus stocks of wild-type AcNPV and recombinant virus were prepared in Sf9 cells as described by Summers and Smith (1987). Monoclonal antibodies specific for gl were developed and characterized by van Drunen Littel-van den Hurk et al. (1984). The gl-specific monoclonal antibody mixture used for identification of recombinant gl consisted of equivalent amounts of 1B10 (epitope I), 3F3 (epitope II), 1E11 (epitope III), 1F8 (epitope IVa), 5G2 (epitope IVb), 3G11 (epitope IVb), 5G11 (epitope IVc), 6G11 (epitope IVc), 1F10 (epitope V), and 2C5 (epitope V).

Insertion of BHV-1 gl DNA into the transfer vector

A cassette of the gl glycoprotein gene has been prepared in plasmid pSV2Neo as previously described (Fitzpatrick et al., 1988). The plasmid was digested with restriction endonuclease BglII and the fragment representing the gl gene was purified by agarose gel electrophoresis and ligated into the BamHI site of the baculovirus transfer vector pVL941 to form the expression vector pVlglB. The pVlglB plasmid was purified for transfection by CsCl gradient centrifugation and two cycles of ethanol precipitation.

Transfection and selection of recombinant viruses

The purified plasmid was mixed with an equal amount of A. californica viral DNA and used to transfect subconfluent monolayers of Sf9 cells as outlined by Summers and Smith (1987). Recombinant baculoviruses were identified by plaque hybridization essentially as outlined by Summers and Smith (1987). The polyhedrin-negative recombinants were plaque purified three to four times on Sf9 cells to remove contaminating wild-type virus.

Preparation of cell lysates and immunoprecipitation

To analyze expression of recombinant gl, confluent monolayers of Sf9 cells on 35-mm petri dishes were infected with individual polyhedrin-negative recombinants at a m.o.i. of 5 and incubated for 48 hr at 28°C. The cells were scraped into PBS, pelleted at 150 g for 1 min, and resuspended in 50 µl of RIPA buffer (0.02 M Tris–hydrochloride [pH 8.0], 0.15 M NaCl, 1% sodium deoxycholate, 1% nonidet P-40, 10 mM ethylene diamine tetraacetic acid [EDTA], 10 mM phenylmethylsulfonylfluoride [PMSF]). The supernatant was collected and 5 µl was combined with reducing electro-
Fig. 4. Predicted amino acid sequence of BHV-1 gl and N-terminal amino acid sequence of the glb and glc subunits (bold). Cleavage sites are indicated by arrows and labeled.

### Analysis of carbohydrates

Proteins were digested with endoglycosidase H or glycopeptidase F as described by Ronin et al. (1987). Infected cells were collected by centrifugation and 2 x 10^6 cells were resuspended in 10 μl of appropriate enzyme incubation buffer and boiled for 2 min for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. For immunoprecipitation, BHV-1-infected MDBK cells were overlaid with methionine-free MEM containing 0% FBS, labeled at 6 hr postinfection with 50 μCi per ml of [L-35S]methionine (Amersham, Oakville, Ontario) and harvested at 24 hr post-infection. Bac-gl infected cells were overlaid at 16 hr postinfection with methionine-free TNM-FH medium containing 0% FBS, labeled with 100 μCi per ml of [L-35S]methionine 1 hr later, and harvested at 19 hr postinfection. Tunicamycin at 10 μg per ml was added with the methionine-free medium. Immunoprecipitation with a gl specific monoclonal antibody cocktail was performed as described previously (van Drunen Littel-van den Hurk et al., 1984).

**Fig. 4.** Predicted amino acid sequence of BHV-1 gl and N-terminal amino acid sequence of the glb and glc subunits (bold). Cleavage sites are indicated by arrows and labeled.
Protein sequencing

N-terminal sequencing of the glb and glc components of affinity-purified authentic and recombinant gl was performed by Edman degradation with an Applied Biosystems Model 470A gas phase sequenator equipped with an on-line model 120 PTH amino acid analyzer.

Immunofluorescence and flow cytometry

The expression of glycoprotein gl in recombinant-baculovirus-infected Sf9 cells was determined at 24, 48, and 72 hr postinfection. Briefly, cells were washed in PBS and cytosin smears were prepared and fixed in methanol. They were incubated for 30 min at 3˚C with a 1:100 dilution of a gl-specific monoclonal antibody mixture and washed in PBS and dH2O. They were stained with fluorescein-isothiocyanate-conjugated (FITC) rabbit anti-mouse IgG (Boehringer Mannheim) for 30 min at 37˚ and washed again before being mounted in PBS–glycerol for examination. For surface staining and flow-cytometric analysis, cells were suspended in PBSG containing 0.2% gelatin and 0.03% NaN3 (PBSG) at 4 × 10^6 cells/ml. They were then washed in PBSG and then incubated with FITC rabbit anti-mouse
Fig. 6. Immunofluorescence of Sf9 cells infected with Bac-gl. Cells were incubated with a gl-specific monoclonal antibody mixture, washed with cold PBS, and stained for fluorescence microscopy with FITC-labeled goat anti-mouse IgG. (a) Methanol-fixed Bac-gl-infected Sf9 cells. (b) Unfixed Bac-gl-infected Sf9 cells. Phase-contrast views of the corresponding cells are also shown (c and d). Magnification: ×1000.

IgG for 30 min at 4°C. After washing, the cells were fixed in 2% formaldehyde and analyzed with an EPICS CS (Coulter Electronics, Ltd.) flow cytometer as described elsewhere (Campos et al., 1989). The percentage of positive cells was calculated using the immuno-program (Coulter Electronics, Ltd., MDAPS system) for the analysis of immunofluorescence histograms.

Cell fusion assay

Monolayers of Sf9 cells in 24-well tissue culture plates were infected with recombinant virus at a m.o.i. of 5–10 PFU per cell. At 36 hr post-infection, the medium was replaced with TNM-FH medium, adjusted to a pH ranging from 5.0 to 6.5. Syncytia formation was
observed at pH 5.4 and monitored under a phase contrast microscope (Zeiss Model IM35; magnification 200×). Monospecific and monoclonal antibodies were added at a dilution of 1:100 at the time of pH shift.

**Immunization of cattle**

Glycoprotein gl was purified by immunoabsorbant chromatography from Bac-gl-infected Sf9 cells or BHV-1-infected MDBK cells as described in detail previously (van Drunen Littel-van den Hurk and Babiuk, 1985). Groups of eight animals each were immunized with 100 or 10 μg of affinity-purified recombinant or authentic gl in Emulsigen PLUS at a ratio of 7:3 (v/v) as outlined by the manufacturer (MVP Laboratories, Kaislton, NE). The animals were injected intramuscularly and they received a booster immunization 28 days...
EXPRESSION OF BHV-1 gl BY BACULOVIRUS

RESULTS

Production of recombinant gl glycoprotein in Sf9 cells

Recombinants containing the gl gene inserts were tested for their ability to produce BHV-1 glycoprotein I after infection of Sf9 cells. All of the gl recombinants directed the synthesis of a polypeptide with an apparent molecular weight of 116 kDa, which was visible on a Coomassie brilliant blue stained gel at 48 hr postinfection. This protein was missing in uninfected cells and cells infected with the parental baculovirus and probably represented recombinant glycoprotein gla. Three additional polypeptides appeared during infection with the gl recombinants. The 63- and 52-kDa polypeptides probably corresponded to the glb and glc components of the recombinant glycoprotein (Fig. 1). In order to confirm the identity of these glycoproteins, immunoblot analyses were performed on Bac-gl-infected Sf9 cells and BHV-1 infected MDBK cells (Fig. 2). A gl-specific monoclonal antibody mixture that recognized the 130k, 74k, and 55k components of authentic gl in BHV-1-infected MDBK cells, reacted with three polypeptides with apparent molecular weights of 116, 63, and 52 kDa in Bac-gl-infected Sf9 cells. The 38-kDa polypeptide, observed in Fig. 1, was either a gl fragment not detected by gl-specific monoclonal antibodies, or a baculovirus-specified polypeptide. These data suggest that terminal glycosylation of gl has not occurred in the recombinant virus-infected Sf9 cells. Although recombinant gl was cleaved in infected Sf9 cells, the efficiency of cleavage was lower than that of authentic gl.

Processing of gl in mammalian and insect cells

To further analyze the observed difference in molecular weight of the recombinant and authentic gl, Bac-gl-infected Sf9 cells, and BHV-1-infected MDBK cells were treated with tunicamycin, an inhibitor of N-linked glycosylation. In these cells only one polypeptide with an apparent molecular weight of 105k was observed (Fig. 3A), which corresponds to the previously identi-
fied polypeptide backbone of authentic gl (van Drunen Littel-van den Hurk and Babiuk, 1986b). This experiment proved that the decreased molecular weight of gl produced in insect cells was due to altered glycosylation. Due to the short labeling period, the recombinant glb and glc were not observed on this gel. To compare the type of carbohydrate attached to recombinant and authentic gl, both glycoproteins were subjected to digestion with endo H or endo F. Digestion with endo H resulted in a slight decrease in apparent molecular weight of authentic gla and glc, but had no effect on glb, which confirms previous studies (van Drunen Littel-van den Hurk et al., 1986b). The greater portion of recombinant gla and glc was sensitive to endo H, indicating the presence of high-mannose type oligosaccharides. In contrast, the recombinant glb was not
EXPRESSION OF BHV-1 gl BY BACULOVIRUS

TABLE 1
INHIBITION OF FUSION ACTIVITY MEDIATED BY gl EXPRESSED IN BACULOVIRUS

| Treatment | Fusion activity (%) |
|-----------|---------------------|
| TNM-FH, pH 5.4 | 80 |
| Trypsin | 80 |
| Normal Rabs | 80 |
| gl-specific Rabs | 0 |
| Control Mab | 80 |
| gl-specific Mabs mixture | 0 |
| 1Bl0 Mab (I) | 5 |
| 3F3 Mab (II) | 80 |
| 1E11 Mab (III) | 80 |
| 1F8 Mab (IVA) | 80 |
| 5G2 Mab (IVB) | 10 |
| 5G11 Mab (IVC) | 60 |
| 1F10 Mab (V) | 80 |

* Cell fusion was induced at 36 hr postinfection by replacing the cell culture medium with TNM-FH, pH 5.4. At the time of pH shift a final dilution of 1:100 of Rabs (rabbit serum) or Mabs (monoclonal antibodies) was added to the medium. The control Mab was specific for an unrelated, bovine rotavirus antigen. Treatment with 20 µg trypsin was carried out for 10 min, just before pH shift at 36 hr.

The cells were counted 8 hr after the pH shift. The percentage of fused cells was calculated on a total of 400 cells and rounded to the nearest decimal.

sensitive to endo H, suggesting that these oligosaccharides were trimmed. All of the recombinant and authentic forms of gl were sensitive to endo F, showing precursor molecules with similar apparent molecular weights in BHV-1 and Bac-gl-infected cells (Fig. 3B).

Authentic and recombinant gl are both cleaved during processing to the mature polypeptide. However, the cleavage process is incomplete in mammalian cells and even less efficient in insect cells. It has been proposed that the Arg–Arg–Ala–Arg–Arg sequence (501–505), which occurs in the region of nonsimilarity with HSV-1, may be the processing site for BHV-1 gl (Whitbeck et al., 1988). To confirm the position of the cleavage site of authentic as well as recombinant gl, we sequenced the N-terminus of the glc glycoprotein from infected MDBK and Sf9 cells. This analysis confirmed that the first 12 N-terminal amino acids of authentic and recombinant gl correspond to positions 506–517 (Fig. 4). Since recombinant gl was cleaved at the same site as authentic gl, the reduced cleavage efficiency is probably due to the presence of relatively low amounts of enzyme in baculovirus-infected cells, as compared to the large amounts of gl produced in these cells. N-terminal sequencing of the glb glycoprotein demonstrated that the same signal is cleaved in MDBK and Sf9 cells and that the amino terminal residue of authentic as well as recombinant gl is Arg-68.

Kinetics and level of expression of the recombinant gl glycoprotein

The amount of gl synthesized in recombinant baculovirus-infected Sf9 cells was quantitated by ELISA standardized with affinity-purified recombinant gl. Sf9 cells grown as monolayers in 35-mm petri dishes were infected with Bac-gl at a m.o.i. of 5, and aliquots of 1 X 10⁶ cells were harvested at various times postinfection. Immunoreactive gl could be detected as early as 24 hr after infection and maximal expression was observed between 36 and 48 hr, thereafter, a slight decrease in measurable glycoprotein occurred. This decline presumably reflects cell lysis and subsequent degradation of the glycoprotein. This analysis showed that, at maximal levels of expression, 28 µg of gl were produced per 10⁶ cells (Fig. 5A). In order to analyze the possibility of producing recombinant gl on a larger scale, Sf9 cells were grown in suspension cultures and infected with the recombinant baculovirus at a m.o.i. of 1. In addition to yield by ELISA, the viability of the cells and percentage of infected cells were determined. Figure 5B shows that the percentage of infected cells increased gradually, reaching peak levels of 85% at 72 hr after infection, when the viability of the cells was down to 25%. The viability of the cells was too low for flow

TABLE 2
REACTIVITY OF MONOCLONAL ANTIBODIES WITH AUTHENTIC AND RECOMBINANT gl

| Monoclonal Designation | Epitope Specificity | Neutralizing Activity | ELISA titer |
|------------------------|---------------------|-----------------------|-------------|
| 1Bl0 | I | ~ | 100 | 6400 |
| 3F3 | II | ± | 6400 | 25600 |
| 1E11 | III | ++ | 1600 | 6400 |
| 1F8 | IVa | + | 25600 | 6400 |
| 5G2 | IVb | + | 6400 | 6400 |
| 3G11 | IVb | + | 1600 | 1600 |
| 5G11 | IVc | + | 1600 | 100 |
| 6G11 | IVc | ++ | 400 | 100 |
| 1F10 | V | ± | 1600 | 1600 |
| 2C5 | V | ± | 6400 | 6400 |

a Monoclonal antibodies developed by van Drunen Littel et al. (1984).
b gl epitopes assigned by competitive binding assays (van Drunen Littel-van den Hurk et al., 1985).
c Neutralizing titers were determined for ascites fluids in the presence of guinea pig serum as a source of complement. ~, titer < 4; ±, titer < 100; +, titer > 100; ++, titer > 10,000 (van Drunen Littel-van den Hurk et al., 1985).
d Antigen titer was expressed as the reciprocal of the highest dilution of infected cells giving a reading of at least 0.05 OD (492 nm). A 1:100 dilution corresponds to 2 X 10⁴ cells.
cytometric analysis beyond this time point. Analysis by ELISA demonstrated that up to 36 μg of gl were produced per 10⁶ cells. This demonstrates the feasibility of growing the recombinant baculovirus on a larger scale and obtaining high yields of the glycoprotein.

Intracellular localization of recombinant gl in Sf9 cells

The intracellular distribution of the recombinant gl glycoprotein was examined by an indirect immunofluorescence assay. At 48 hr postinfection, recombinant gl was primarily localized in the perinuclear membranes of the infected Sf9 cells (Fig. 6a). To determine whether the recombinant gl was present on the surface of infected cells, immunofluorescence analysis was carried out on unfixed cells. Localization of gl on the cell surface was demonstrated by bright surface fluorescence (Fig. 6b). Wild-type AcNPV-infected control cells did not show any fluorescence with the gl-specific monoclonal antibody panel (not shown).

Fusogenic properties of recombinant gl in insect cells

It has been shown previously that one of the functional characteristics of gl is its ability to induce cell fusion in absence of other viral proteins (Fitzpatrick et al., 1988, 1990b). To determine whether the recombinant protein retained this functional property, Sf9 cells were infected with Bac-gl. Fusion of the insect cells was not evident under standard culture conditions (Fig. 7a), but after a shift to pH 5.4 fusion was apparent in Bac-gl-infected Sf9 cells within 2 hr (Fig. 7b). The syncytia formation observed in these cells continued to increase over 8 hr of observation (Fig. 7c). Fusion was not detected in cultures infected with wild-type AcNPV over the pH range examined (not shown). Inclusion of gl-specific rabbit serum or a mixture of gl-specific monoclonal antibodies completely inhibited fusion by gl (Table 1). When individual monoclonal antibodies were included in the media, fusion was almost completely inhibited by the monoclonal antibodies 1B10 (epitope I) and 5G2 (epitope IVb) and partially inhibited by 5G11 (epitope IVc). Inclusion of trypsin at the time of pH shift did not affect the fusion activity.

Antigenic and immunogenic properties of gl expressed in Sf9 cells

The antigenic properties of recombinant gl were evaluated using a panel of gl-specific monoclonal antibodies. The epitopes recognized by these monoclonal antibodies have been identified and characterized previously (van Drunen Littel-van den Hurk et al., 1985; Fitzpatrick et al., 1990a). The reactivity of these monoclonal antibodies in an ELISA (Table 2) indicated that all of the epitopes identified on the authentic glycoprotein were also present on the recombinant gl glycoprotein. The reaction between the monoclonal antibodies and two carbohydrate-dependent epitopes (IVa and IVc; van Drunen Littel-van den Hurk et al., 1990b) was weaker on recombinant gl than on authentic gl, which is in agreement with lack of terminal glycosylation of gl in Sf9 cells. Epitopes I, II, and III, however, appeared to

| Immunogen* | Dose (μg) | Antibody titer |
|------------|-----------|----------------|
| Authentic gl | 100 | 8,125 |
| Authentic gl | 10 | 2,560 |
| Recombinant gl | 100 | 10,240 |
| Recombinant gl | 10 | 1,280 |
| Placebo | N.A. | 10 |

* Animals received two intramuscular immunizations of authentic gl, recombinant gl, or PBS (placebo) in Emulsigen PLUS.

* ELISA titers were determined against affinity-purified gl and expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control value.
be more reactive on recombinant gl than on its authentic counterpart.

To compare the immunogenicity of recombinant and authentic gl, calves were immunized with affinity-purified glycoprotein from recombinant Bac-gl-infected Sf9 cells or BHV-1-infected MDBC cells. Two immunizations of recombinant or authentic gl in Emulsigen PLUS elicited antibodies that were reactive with gl from BHV-1 in an immunoblot assay (Fig. 8). The antibody titers induced by recombinant and authentic gl were very similar (Table 3).

**DISCUSSION**

The goal of this study was to determine whether the baculovirus system would produce gl in an authentic form and in sufficient quantities for further structural, functional, and immunological analyses. The level of gl synthesis attained by using the baculovirus expression vector was extremely high, about 36 µg per 10⁶ cells by 48-72 hr postinfection. This amount is at least 10-fold greater than the quantity of gl glycoprotein found in BHV-1-infected cells (van Drunen Littel-van den Hurk and Babiuk, 1985), which makes this system the most efficient means for producing gl.

BHV-1 gl has six potential receptor sites for N-linked glycosylation of which one is probably not used (Whitbeck et al., 1988). The apparent molecular weight of the gl complex was 116/63/52 kDa in insect cells, which corresponds in size to the cotranslationally glycosylated gl produced in BHV-1-infected cells (van Drunen Littel-van den Hurk and Babiuk, 1985), which makes this system the most efficient means for producing gl.

The recombinant gl glycoprotein was transported to and expressed on the surface of infected cells, and as such, was capable of inducing cell-to-cell fusion. This indicates that complete glycosylation is not needed for fusion activity. Although it is not known whether the gl molecules expressed on the surface of the infected cells are cleaved or not, addition of trypsin did not enhance syncytia formation, indicating that the fusion activity is not totally dependent upon cleavage of gl. Optimal conditions for fusion appeared to be more acidic in insect cells than in mammalian cells (Fitzpatrick et al., 1990b). This phenomenon has been observed for different fusion proteins expressed in insect cells, like the F protein of measles virus and the S glycoprotein of bovine coronavirus (Vialard et al., 1990; Yoo et al., 1990). The glycosylation differences observed in all cases may be responsible for conformational changes resulting in the altered optimum pH for fusogenicity. In addition, the membranes of insect and mammalian...
cells may differ as targets for fusion. That fusion was mediated by gl was confirmed by total inhibition in the presence of gl-specific polyclonal or a cocktail of monoclonal antibodies. Monoclonal antibody 1R10, specific for epitope I, inhibited fusion almost completely. Epitope I maps to a segment between residues 744-763, a highly conserved portion of the extracellular region of the carboxy terminal fragment (glc) of gl (Fitzpatrick et al., 1990a). This implies that one domain responsible for cell fusion is located on the glc subunit, between or in the vicinity of residues 744-163. Monoclonal antibody 5G2, specific for epitope IVb, and monoclonal antibody 5G11, specific for epitope IVc, partially inhibited fusion activity. These epitopes map to the amino terminus of gl between residues 60 and 110 (Fitzpatrick et al., 1990a). This suggests that domain IV is also involved in the fusion process. In analogy to the predicted secondary structure determined for gB (HSV) by Pellett et al. (1985), gl might also fold back, thereby positioning the amino terminus in close proximity to the membrane. Alternatively, fusion may be a complex process which requires the sequential or coordinated activity of at least two separate domains of gl. To exclude the possibility that the monoclonal antibodies specific for epitopes I and IV inhibit fusion indirectly, both fusogenic regions need to be confirmed by mutagenic analysis. This is the first report implying domains I and IV of BHV-1 gl, respectively. To exclude the possibility that the monoclonal antibodies specific for epitopes I and IV inhibit fusion indirectly, both fusogenic regions need to be confirmed by mutagenic analysis. This is the first report implying domains I and IV of BHV-1 gl, respectively. To exclude the possibility that the monoclonal antibodies specific for epitopes I and IV inhibit fusion indirectly, both fusogenic regions need to be confirmed by mutagenic analysis. This is the first report implying domains I and IV of BHV-1 gl, respectively. To exclude the possibility that the monoclonal antibodies specific for epitopes I and IV inhibit fusion indirectly, both fusogenic regions need to be confirmed by mutagenic analysis. This is the first report implying domains I and IV of BHV-1 gl, respectively.

In addition to the structural and functional analyses of recombinant gl, we studied the antigenicity by ELISA. BHV-1 gl has at least four neutralizing and three weakly or nonneutralizing epitopes, some of which are conformational in nature (van Drunen Littel-van den Hurk et al., 1990; Fitzpatrick et al., 1990a). All of these epitopes were recognized on recombinant gl in the fusion process. Recently, three domains involved in fusion activity of HSV-1 gl were identified (Navarro et al., 1992). Two of these, D1 and D5a, correspond to the amino terminal domain IV and the carboxy terminal domain I of BHV-1 gl, respectively.

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ACKNOWLEDGMENTS

The authors are grateful to Dr. D. Yoo for helpful discussions, Tammy Wolfe and Terry Beskorwayne for technical assistance, the animal support staff at VIDO for care and handling of the animals, and Marilee Hagen for preparing the manuscript. Financial support was provided by the Medical Research Council and the Natural Science and Engineering Council of Canada. D.R.F. was a recipient of a Commonwealth fellowship.

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