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Synthesis, Cellular Location, and Immunogenicity of Bovine Herpesvirus 1 Glycoproteins gI and gIII Expressed by Recombinant Vaccinia Virus†

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Two of the major glycoproteins of bovine herpesvirus 1 (BHV-1) are gI, a polypeptide complex with apparent molecular weights of 130,000, 74,000, and 55,000, and gIII (a 91,000-molecular-weight [91K] glycoprotein), which also exists as a 180K dimer. Vaccinia virus (VAC) recombinants were constructed which carry full-length gI (VAC-I) or gIII (VAC-III) genes. The genes for gI and gIII were each placed under the control of the early VAC 7.5K gene promoter and inserted within the VAC gene for thymidine kinase. The recombinant viruses VAC-I and VAC-III retained infectivity and expressed both precursor and mature forms of glycoproteins gI and gIII. The polypeptide backbones, partially glycosylated precursors, and mature gI and gIII glycoproteins were indistinguishable from those produced in BHV-1-infected cells. Consequently, they were apparently cleaved, glycosylated, and transported in a manner similar to that seen during authentic BHV-1 infection, although the processing efficiencies of both gI and gIII were generally higher in recombinant-infected cells than in BHV-1-infected cells. Immunofluorescence studies further demonstrated that the mature gI and gIII glycoproteins were transported to and expressed on the surface of cells infected with the respective recombinants. Immunization of cattle with recombinant viruses VAC-I and VAC-III resulted in the induction of neutralizing antibodies to BHV-1, which were reactive with authentic gI and gIII. These data demonstrate the immunogenicity of VAC-expressed gI and gIII and indicate the potential of these recombinant glycoproteins as a vaccine against BHV-1.

Bovine herpesvirus 1 (BHV-1) is responsible for a variety of disease conditions in cattle, including respiratory infection, conjunctivitis, vulvovaginitis, abortion, and, less commonly, encephalitis and generalized systemic infection (15). It is one of the most important agents of bovine respiratory disease, either directly or as a predisposing factor for bacterial infection, specifically with *Pasteurella haemolytica* (58).

Viral glycoproteins are the major structural components present in the envelope of herpesviruses. They play an important role in the virus-host relationship, since they are involved in the recognition, attachment, and penetration of the virus into susceptible cells (13, 21, 24, 41), in virus neutralization (16, 53), and in immune destruction of virus-infected cells (4, 7, 32, 40).

The immune responses mediating recovery from a BHV-1 infection have been well documented (39). However, only limited studies have been conducted with respect to the specific glycoproteins involved in the infection and recovery process from BHV-1. BHV-1 specifies more than 25 structural polypeptides, of which 11 are glycosylated (6, 27). By using monoclonal antibodies, four unique glycoproteins have been identified and further characterized: gI, a complex of three glycoproteins with apparent molecular weights of 130,000, 74,000, and 55,000; gII (a 108,000-molecular-weight glycoprotein [108K glycoprotein]); gIII (91,000), which also occurs as a dimer with a molecular weight of 180,000; and gIV (71,000), with a dimer with a molecular weight of 140,000 (10, 25, 50, 52). The precursors to each of these four glycoprotein families have been identified in pulse-chase and inhibitor studies (47, 50). In addition, gene mapping and DNA sequence data have indicated that gI, gII, gIII, and gIV are, respectively, homologous to gB, gE, gC, and gD of herpes simplex virus type 1 or type 2 (HSV-1 and HSV-2) (20, 29, 56; T. Zamb, manuscript in preparation).

The use of monoclonal antibodies enabled us to better understand the roles of three individual BHV-1 glycoproteins in the humoral immune response to a BHV-1 infection. Glycoproteins gI, gIII, and gIV all stimulate the production of neutralizing antibodies in mice, rabbits, and cattle (9, 48, 49, 52). The neutralizing activity of gI was localized in at least six different epitopes on the gIb subunit (51). Monoclonal and monospecific antibodies to gI, gIII, and gIV were also able to mediate antibody-dependent, complement-mediated cytolysis of virus-infected cells. Glycoprotein gIII was particularly important in this function, with at least six different epitopes involved (51). Most important, glycoproteins gI, gIII, and gIV were the main proteins recognized by cattle infected with BHV-1 (49), and they all were able to protect cattle from lethal BHV-1-*P. haemolytica* challenge (1).

The objective of the present study was to obtain a tool to investigate the involvement of individual BHV-1 glycoproteins in the cell-mediated immune response and to study the practical application of these glycoproteins as a vaccine. The use of vaccinia virus (VAC) as an expression vector has a number of advantages for these purposes. It is a double-stranded DNA virus that is able to stably maintain foreign

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DNA inserts; its promoter permits the expression of foreign genes in cell culture; and it has a broad host range. A number of VAC recombinants have been constructed by the insertion of foreign genes for the expression of proteins, such as influenza A virus haemagglutinin (34, 44), hepatitis B surface antigen (35, 43), HSV-1 glycoprotein D (11, 35), rabies virus glycoprotein G (18), vesicular stomatitis virus glycoproteins G and N (22), *Plasmodium knowlesi* sporozoite antigen (42), Sindbis virus structural proteins (36), respiratory syncytial virus glycoprotein G (3, 12), feline leukemia virus envelope protein (33), and human T cell lymphotropic virus type III envelope protein (8, 17). Immunization of (laboratory) animals with these recombinants induced high titers of neutralizing antibodies against each agent and protected the animals from challenge with corresponding wild-type pathogen (5, 12, 22, 31, 55, 57).

In the present paper we describe the construction and characterization of recombinant VAC vectors containing the genes for BHV-1 glycoproteins gI and gIII. Initial studies on the recombinants showed that the synthesis, maturation, and cell surface expression of glycoproteins gI and gIII were similar in cells infected with recombinants and in those infected with BHV-1 itself, although the processing efficiency was generally higher in recombinant-infected cells than in BHV-1-infected cells. Most important, VAC recombinants expressing gI or gIII induced a neutralizing antibody response to BHV-1 in cattle.

MATERIALS AND METHODS

Cells and viruses. Strain P8-2 of BHV-1 was propagated in Georgia bovine kidney (GBK) cells and quantitated by plaquing in microdilution plates with an antibody overlay as described previously (38). VAC (WR strain) and recombinant VAC were propagated in BSC-1 or BSC-40 cells. Madin-Darby bovine kidney (MDBK) cells, BSC-1 and BSC-40 cells, bovine fibroblasts (BFB), bovine tracheal cells (BTB), and human thymidine kinase-negative (TK^-) 143 cells were grown as monolayers in Eagle minimal essential medium (MEM) (GIBCO, Grand Island, N.Y.), supplemented with 10% fetal bovine serum (GIBCO).

Preparation of DNA. All DNA used for ligations and transfections was CsCl gradient purified (23) and set to concentrations of 1 μg/ml in 10 mM Tris (pH 7.5)–1 mM EDTA.

Transfection and isolation of recombinant viruses. Recombinant VAC were selected by marker rescue as previously described (54). Approximately 3×10^6 BSC-40 cells (thymine kinase positive [TK^+]) were infected with wild-type VAC (WR strain) at a multiplicity of infection of 0.03 PFU/cell. At 4 h postinfection, approximately 15 μg of $CaCl_2$-precipitated (125 mM) linearized plasmid DNA, i.e., pgIvax (the gI gene cloned downstream from the 7,000-molecular-weight VAC gene promoter) or pgIIIvax (the gIII gene cloned downstream from the 7.5K gene promoter), was added to the infected BSC-40 cells. After 4 days of incubation at 37°C, viruses were harvested from cell supernatants following two cycles of freezing and thawing. Several dilutions of sonicated virus supernatants were plated on TK^- 143 cells and then overlaid with 1% agarose in growth medium containing 5-bromo-2′-deoxyuridine (25 μg/ml) to select for TK^- virus. After 3 days, individual TK^- plaques were removed, and virus from these plaques was plated on BSC-40 cells. Putative recombinant viruses were repurified by plaquing on BSC-40 cells. Individual plaques were amplified by growth on BSC-40 cells and virus supernatants were tested for the presence of gI and gIII proteins by enzyme-linked immunosorbent assay (ELISA) using polyclonal rabbit antiserum specific for either gI or gIII.

Preparation of radiolabeled cell lysates. BSC-1, MDBK, BFB, or BTB cells were infected with BHV-1, VAC, VAC-I, or VAC-III at a multiplicity of infection of 10. After adsorption of the virus for 1 h, the monolayers were overlaid with methionine-free MEM (GIBCO) containing 2% fetal bovine serum and further incubated at 37°C. At 6 h after infection, 50 μCi of L-[^{35}S]methionine (Amersham Corp., Oakville, Ontario, Canada) per ml was added to the cultures. At 24 h postinfection, the cells were harvested and washed with phosphate-buffered saline (0.01 M NaH_2PO_4/Na_2HPO_4, 0.15 M NaCl, pH 7.4). In time course experiments, BHV-1-, VAC-, VAC-I-, or VAC-III-infected BSC-1 cells were labeled with L-[^{35}S]methionine immediately after virus adsorption and harvested at various times after infection. To increase the incorporation of isotopically labeled methionine, the cells were grown in methionine-free MEM for 6 h before infection. In pulse-chase experiments, the cells were overlaid with methionine-free MEM after virus adsorption. At 6 or 12 h postinfection, the cells were pulse-labeled for 15 min with 200 μCi of L-[^{35}S]methionine in Hanks balanced salt solution (GIBCO). Either the cells were harvested immediately or the label was first chased for 2 h by washing and incubating the cells in MEM containing 100 μg of cycloheximide per ml. To prepare lysates, the cells were suspended in modified RIPA buffer (0.02 M Tris hydrochloride [pH 8.0], 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40), left on ice for 15 min, and sonicated for 15 s at a setting of 100 on a Sonifier cell disrupter (model 1510 Braunsonic; Braun Melsungen AG, Federal Republic of Germany). The suspensions were clarified by centrifugation at 20,000 rpm for 15 min at 30°C in an A100 rotor at room temperature (Airfuge; Beckman Instruments, Inc., Fullerton, Calif.). The supernatants were used immediately for immunoprecipitation.

Immunoprecipitation. The procedure for immunoprecipitation has been described in detail previously (47, 52).

Enzyme treatments. Immunoprecipitated polypeptides were eluted from protein A-Sepharose (Pharmacia, Montreal, Quebec, Canada) by boiling for 5 min in 0.8% sodium dodecyl sulfate (SDS) for endoglycosidase H (endo H; Miles Laboratories, Inc., Elkhart, Ind.) treatment or in 0.5% SDS containing 0.1 M 2-mercaptoethanol for *N*-glycanase (*N*-Gly; Genzyme Corp., Boston, Mass.) treatment. Protein A-Sepharose beads were removed by centrifugation. To remove high-mannose-type oligosaccharides, the supernatants were adjusted to pH 5.5 with 0.125 M sodium citrate, containing 1 mM phenylmethylsulfonyl fluoride, and treated with 125 mU of endo H per ml. To digest all classes of N-linked carbohydrates, the supernatants were adjusted to 0.17% SDS, 0.2 M sodium phosphate (pH 8.6), 10 mM 1,10-phenanthroline hydrate, 1.25% Nonidet P-40 and treated with 14 U of *N*-Gly per ml. Control samples were left untreated. After incubation for 20 h at 37°C, the polypeptides were precipitated with ice-cold acetone. The precipitates were collected by centrifugation, and the pellets were suspended in electrophoresis sample buffer (0.0625 M Tris hydrochloride [pH 6.8], 1.25% SDS, 12.5% glycerol, 0.15 M 2-mercaptoethonol, 0.00125% bromophenol blue) and boiled for 5 min prior to electrophoresis.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 7.5% polyacrylamide discontinuous gels (19) as described previously (47, 50). Electrophoresis was carried out under reducing conditions. Samples

containing ^{35}S were analyzed by autoradiography of the gels on 3M X-ray film (Picker, Saskatoon, Saskatchewan, Canada). The molecular weights of the polypeptides were estimated from the molecular weight markers (Bio-Rad Laboratories, Mississauga, Ontario, Canada) that were electrophoresed in parallel with the samples.

ELISAs. To identify recombinant VAC expressing gI or gIII, an indirect ELISA was performed essentially as described previously (52). Microdilution plates were coated with cell extracts prepared from recombinant TK^- virus-infected BSC-40 cells and reacted with gI- or gIII-specific rabbit sera. Affinity-purified, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG; Boehringer Mannheim, Dorval, Quebec, Canada) was used at a dilution of 1:2,000 for detection.

A sandwich ELISA was used to compare the yields of glycoproteins gI and gIII from recombinant-infected cells to those from BHV-1-infected cells. Microdilution plates were coated with 200 μl per well of a mixture of monoclonal IgG as the captive antibody and then incubated with 200 μl per well of lysate from recombinant- or BHV-1-infected cells. Cell lysates were prepared by suspending 1.5×10^7 cells in 1.5 ml of modified RIPA buffer. The cells were left on ice for 15 min and sonicated for 15 s at a setting of 100 on a Sonifier cell disrupter before use in the ELISA. A mixture of HRPO-conjugated monoclonal antibodies with a different epitope specificity was used for detection at 200 μl per well.

To determine the antibody responses of cattle immunized with the recombinant VACs, the ELISA was performed essentially as described previously (52). However, affinity-purified, peroxidase-conjugated rabbit anti-bovine IgG (Zymed Laboratories Inc., South San Francisco, Calif.) at a dilution of 1:3,000 was used as the detecting antibody.

Cell surface immunofluorescence. BSC-1 cells were infected with BHV-1, VAC, VAC-I, or VAC-III at a multiplicity of infection of 10. After 20 h at 37°C, the cells were removed by mild trypsinization, and 10^6 cells were suspended in 250 μl of 1:20 diluted rabbit antiserum specific for gI or gIII. After reaction for 45 min on ice, the cells were washed three times in Hanks balanced salt solution and incubated with a 1:10 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antiserum (Organon Teknika, West Chester, Pa.). After further reaction for 45 min on ice, the cells were washed three times in Hanks balanced salt solution and finally suspended in 10% glycerol-phosphate-buffered saline, mounted on glass slides, and observed with the aid of a fluorescence miscroscope.

Immunization of cattle. Groups of three to five calves each were immunized by intradermal as well as intramuscular inoculation with VAC-I, VAC-III, or VAC. The intradermal inoculation consisted of 10^9 PFU of virus in 1 ml, whereas the intramuscular inoculation contained 10^7 PFU in a 10-ml volume. The animals were boosted twice at 21-day intervals with equal amounts of virus. Animals were bled weekly for assessment of antibody responses.

Neutralization test. The neutralization titers of the bovine sera were determined as described previously (2), and each was expressed as the reciprocal of the dilution causing a 50% reduction of plaques relative to the virus control.

RESULTS

Construction of VAC insertion plasmids. The gI gene maps between 0.422 and 0.443 genome equivalents (Zamb, in preparation), which is within the BHV-1 *Hin*dIII A fragment described by Mayfield et al. (26). Our map position and the DNA sequence for the gI gene are consistent with previously published data (29, 56). A *Kpn*I-*Acc*I partial digestion of the *Hin*dIII A fragment produced a 3,255-base-pair (bp) subfragment which contains the entire gI gene coding sequence. DNA sequence analyses placed an *Acc*I site 20 bp 5′ to the ATG start codon, while the *Kpn*I site is 420 bp 3′ to the TGA stop codon. This fragment was inserted into a synthetic DNA polylinker present between the *Eco*RI and *Sal*I sites of PBR328 (i.e., ppo126) to produce pgI complete (Fig. 1A). The *Acc*I asymmetric end of the 3,255-bp fragment was first blunted with Klenow enzyme, and the gI fragment was then ligated to the *Hpa*I and *Kpn*I sites of ppo126 to give pgI complete. *Hpa*I and *Kpn*I sites are within the polylinker of ppo126 and are flanked, respectively, by a *Bgl*II and a *Bam*HI site. The gI gene was then transferred from pgI complete as a 3,260-bp *Bgl*II-*Bam*HI fragment to the *Bam*HI site of the VAC insertion vector pGS20 (30; Fig. 1B) to generate pgIvax (plasmid pGS20 carrying the gI gene).

The BHV-1 gIII gene maps between 0.120 and 0.131 genome equivalents (Zamb, in preparation) which is within the BHV-1 *Hin*dIII I fragment (26). The entire gene is contained within a 3,090-bp *Bgl*II-*Eco*RI subfragment of *Hin*dIII I which was cloned into the *Eco*RI and *Bam*HI sites of ppo126 to yield p113 EcoRI-BglII 3.0 (Fig. 2). The gIII gene was transferred to the *Bam*HI and *Sma*I sites of pGS20 as a 2,550-bp *Eco*RI-*Bam*HI subfragment of p113 EcoRI-BglII 3.0 to generate pgIIIvax (plasmid pGS20 carrying the gIII gene). The *Bam*HI site of the gIII gene subfragment is 50 bp upstream from the ATG start codon, while the *Eco*RI site, which was blunted with Klenow enzyme prior to ligation, is 920 bp downstream from the TAG stop codon (Fitzpatrick et al., manuscript in preparation).

Construction of recombinant VAC-I and VAC-III. The two plasmids pgIvax and pgIIIvax were then used to transfect BSC-40 cells infected with wild-type VAC (WR strain). Homologous recombination between VAC TK sequences in the plasmid and virus genome resulted in the insertion of the gI or gIII gene into VAC. Recombinant VACs putatively expressing BHV-1 gI or gIII were selected as TK^- plaques produced on TK^- 143 cells in the presence of 5-bromodeoxyuridine, following recovery of recombinant virus from the initial BSC-40 cell infection. Recombinant VAC actually expressing BHV-1 gI (VAC-I) or gIII (VAC-III) was identified by screening TK^- virus in an ELISA. Infected-cell extracts from recombinant TK^- virus were immobilized on microdilution plates and reacted with serial dilutions of gI- or gIII-specific rabbit serum. ELISA-positive infected-cell extracts were used for further studies.

Analysis of recombinant virus DNA. To insure proper gene insertion, putative recombinant virus DNA was isolated, digested with restriction endonucleases known to cut within the BHV-1 gene inserts, run on agarose gels, and transferred to nitrocellulose by the methods of Southern (45). Southern transfers were then probed with ^{32}P-labeled nick-translated gI and gIII gene fragments. The order and size of the fragments generated from the recombinant viruses were consistent with those predicted by the DNA sequence analyses of the gI and gIII genes (29, 56; Zamb, in preparation) (data not shown).

Analysis of glycoproteins made in recombinant-infected cells. To examine the protein products translated in vitro from the BHV-1-specific transcripts, BSC-1 cells were infected with BHV-1, VAC WR, recombinant VAC-I, or recombinant VAC-III and labeled with L-[^{35}S]methionine. The radiolabeled proteins were immunoprecipitated with gI-specific monoclonal antibody 1E11 or gIII-specific mono-

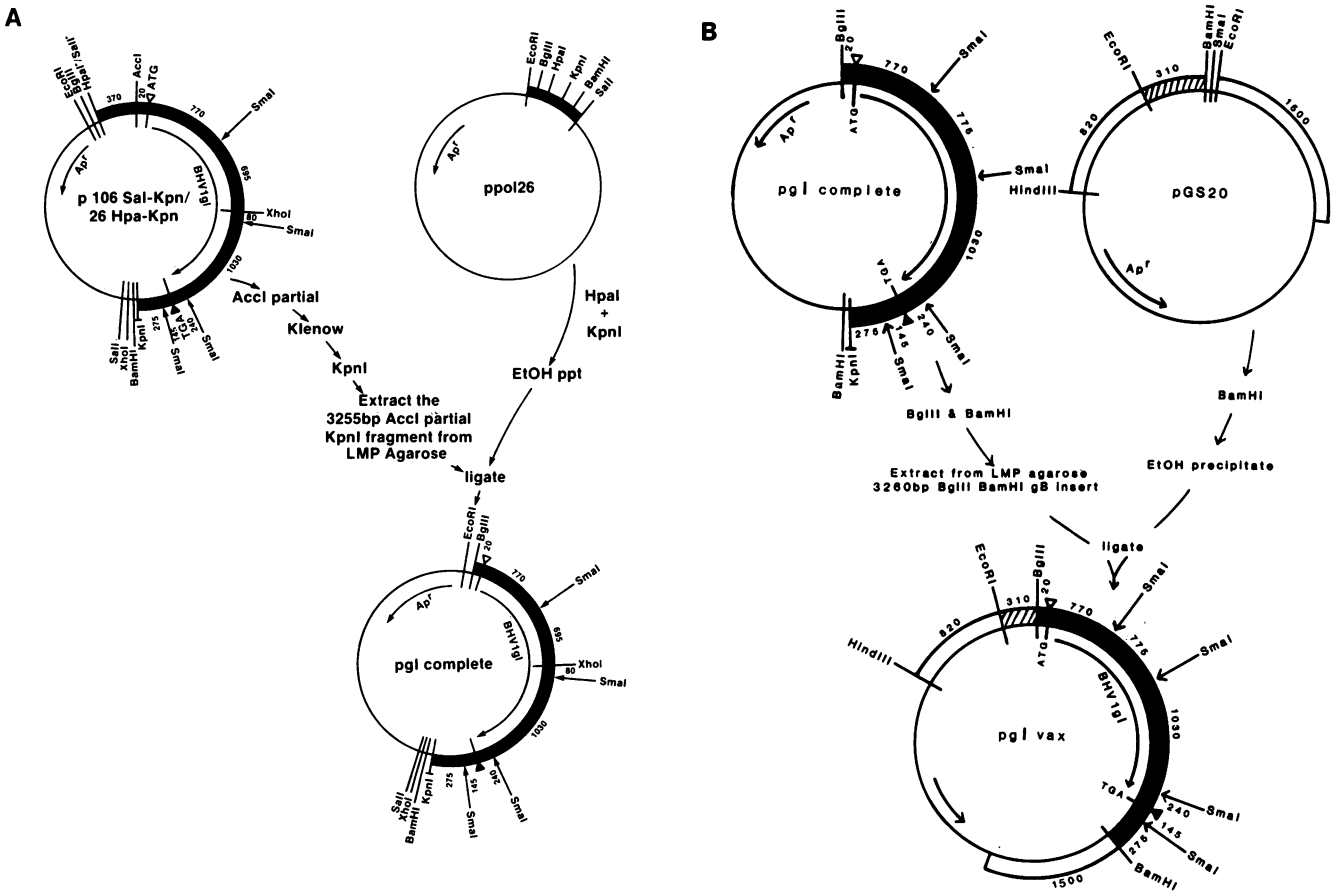

FIG. 1. Construction of recombinant plasmid pgIvax. ■, BHV-1 DNA (the arrow indicates the direction and extent of the gI gene coding sequences, which are partially homologous to the HSV gB genes); ▨, VAC transcriptional regulatory sequence, including RNA start site; □, flanking VAC DNA, including interrupted TK gene; ——, *Escherichia coli* plasmid DNA.

clonal antibody 1D6 (52) and analyzed by SDS-PAGE under reducing conditions.

Monoclonal antibody 1E11 precipitated three major glycoproteins from BSC-1 cells infected with recombinant VAC-I. These glycoprotein species comigrated exactly with authentic BHV-1 glycoproteins pgIa (apparent molecular weight, 117,000), gIb and gIc (55,000; Fig. 3A). BHV-1 glycoprotein gIa, the uncleaved counterpart of gIb and gIc (50), was not found in recombinant VAC-I-infected cells, indicating a difference in the efficiency of processing. Glycoproteins gIa and gIb, which have apparent molecular weights of 130,000 and 74,000, respectively, in MDBK or GBK cells (50, 52), appeared to have slightly lower molecular weights of 127,000 and 71,000, respectively, in BSC-1 cells. Similarly, monoclonal antibody 1D6 precipitated a unique glycoprotein from BSC-1 cells infected with recombinant VAC-III, which comigrated with authentic BHV-1 glycoprotein gIII. Although this glycoprotein has an apparent molecular weight of 91,000 in MDBK and GBK cells (50, 52), it appeared to have a molecular weight of 85,000 in BSC-1 cells. The observed shifts in apparent molecular weights were probably due to a difference in the extent of glycosylation. Monoclonal antibody 1E11 also precipitated a band with an apparent molecular weight of about 65,000 from VAC-infected BSC-1 cells, the nature of which is presently unknown.

Several other cell lines, both permissive and nonpermissive for VAC replication, were tested for the production of BHV-1 glycoproteins after infection with VAC-I or VAC-III. BFB and BTB cells, permissive for VAC replication, both produced the same species of BHV-1 glycoproteins as the BSC-1 cells did when infected with recombinant VAC-I or VAC-III (Fig. 3B and C). In addition to gIII, its precursor pgIII (molecular weight, 69,000) was detected in BHV-1-infected BFB and BTB cells but not in VAC-III-infected cells, indicating that in these cells recombinant-produced gIII is processed more efficiently than its authentic counterpart. However, in MDBK cells, which are nonpermissive for VAC growth, no expression of the glycoproteins was observed (Fig. 3D).

Comparison of glycoprotein production in recombinant- and BHV-1-infected cells. To compare the amounts of recombinant glycoprotein produced in different cell lines, a sandwich ELISA was performed. Cell lysates were prepared from cells infected with BHV-1, VAC-I, or VAC-III and assayed with respect to production of glycoproteins gI and gIII. Table 1 shows that MDBK is the cell line of choice for producing large quantities of BHV-1 glycoproteins, followed by BTB, BFB, and BSC-1, in that order. In contrast, BSC-1 is the better cell line for VAC-I and VAC-III, followed by BFB and BTB. MDBK cells infected with VAC-I or VAC-III did not produce any glycoproteins, which is in accordance with the nonpermissiveness of this cell line for VAC replication. A comparison of the best-producing cell lines for

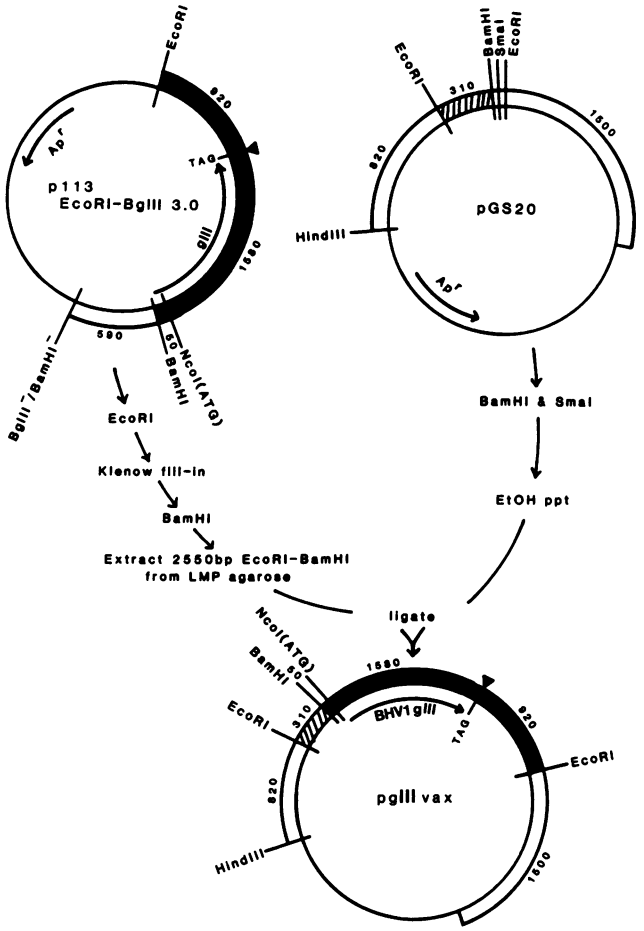

FIG. 2. Construction of recombinant plasmid pgIIIvax. ▬, BHV-1 DNA (the arrow indicates the direction and extent of the gIII gene coding sequences, which are partially homologous to the HSV gC genes; ▨, VAC transcriptional regulatory sequence, including RNA start site; ▭, flanking VAC DNA, including interrupted TK gene; ——, *E. coli* plasmid DNA.

each virus, i.e., MDBK for BHV-1 and BSC-1 for VAC-I and VAC-III, showed that authentic gI and gIII were produced in approximately a sixfold excess over recombinant gI and gIII.

Since the highest quantities of recombinant gI and gIII, as well as sufficient amounts of authentic gI and gIII, were produced in BSC-1 cells, BSC-1 cells were used for all subsequent experiments.

Posttranslational modifications of BHV-1 glycoproteins. BHV-1 gI and gIII proteins are glycosylated proteins. We previously showed that gI contains N-linked carbohydrates, whereas gIII has both N- and O-linked oligosaccharides (47). Since the authentic and recombinant glycoproteins gI and gIII comigrated in one-dimensional polyacrylamide gels (Fig. 3), they appear to be glycosylated to the same extent. To support this observation, two additional experiments were performed.

First, we investigated the order and time course of synthesis of gI and gIII. BSC-1 cells were infected with BHV-1, VAC-I, or VAC-III, labeled with L-[^{35}S]methionine immediately after virus adsorption, and harvested at 2-h intervals after infection. Cell lysates were prepared and precipitated with monoclonal antibody 1E11 or 1D6. This experiment demonstrated that both recombinant and authentic gI were synthesized as early as 2 h postinfection. Recombinant gIII was also detected at 2 h after infection, but authentic gIII was not present until 8 h postinfection (data not shown). This is in agreement with gI being a β protein, whereas gIII is a γ protein.

Subsequently, in an attempt to identify the polypeptide backbones of gI and gIII, the susceptibilities of these glycoproteins and their precursors to the action of the endoglycosydases endo H and N-Gly were examined. BHV-1-, VAC-I-, or VAC-III-infected BSC-1 cells were pulse-labeled for 15 min with L-[^{35}S]methionine and either harvested immediately or after the pulse was chased for 2 h. Based on the appearance of the glycoproteins in the infected cell, the pulse-chase experiments were performed at 6 h postinfection for analysis of gI and recombinant gIII and at 12 h postinfection for analysis of authentic gIII. Cell lysates were prepared and immunoprecipitated with the monoclonal antibodies used above. One-third of the immunoprecipitates was treated with endo H, one-third was incubated with N-Gly, and the remaining one-third served as a control.

As shown in the control samples (Fig. 4A, Con), monoclonal antibody 1E11 precipitated pgIa (molecular weight, 117,000), which was chased into gIb (71,000) and gIc (55,000), both in VAC-I- and in BHV-1-infected cells. In accordance with previous reports (50), a portion of gI remained uncleaved in BHV-1-infected cells and was visible as gIa (127,000). After incubation of the immunoprecipitates with endo H, instead of pgIa a new polypeptide with an apparent molecular weight of 105,000 (pI) was observed. Following endo H treatment, gIa, gIb, and gIc showed a slight shift in electrophoretic mobility, resulting in 120K, 68K, and 52K species. In the presence of N-Gly, pI(105) and two polypeptides with apparent molecular weights of 56,000 (nonglycosylated gIb) and 50,000 (nonglycosylated gIc) were detected. These results demonstrated the following. (i) The nonglycosylated precursor polypeptide pI(105), which has been described previously (50), is identical in VAC-I- and BHV-1-infected BSC-1 cells, and consequently, glycosylation and processing of recombinant and authentic gI as described below (ii and iii) are identical with respect to the mechanism, although different with respect to the efficiency. (ii) The cotranslational addition of sugars by N linkage converts pI(105) to the partially glycosylated intermediate pgIa (117,000), which has high-mannose-type sugars. (iii) The final product gI has both high-mannose- and complex-type oligosaccharides, the latter being in the majority and mostly localized in gIb.

In the control samples (Fig. 4B, Con) monoclonal antibody 1D6 precipitated pgIII (69,000), which was chased into gIII (85,000), both in VAC-III- and BHV-1-infected cells. gIII was not affected by the action of endo H, but instead of pgIII, a new polypeptide with a molecular weight of 61,000 (pIII) was identified. After treatment of the immunoprecipitates with N-Gly, pIII(61) was detected in the pulse sample, whereas a new polypeptide with an apparent molecular weight of 77,000 was found in the chase sample. These data showed the following. (i) The nonglycosylated polypeptide backbone pIII(61), which has been described previously (50), is identical in VAC-III- and BHV-1-infected BSC-1 cells. (ii) pIII(61) is converted to the partially glycosylated intermediate pgIII (69,000) by the addition of N-linked sugars of the high-mannose type. (iii) The final product gIII has both N-linked complex and O-linked oligosaccharides. The presence of O-linked carbohydrates was inferred from these data, but confirmed by incubation of gIII with neuraminidase and *O*-glycanase (unpublished results).

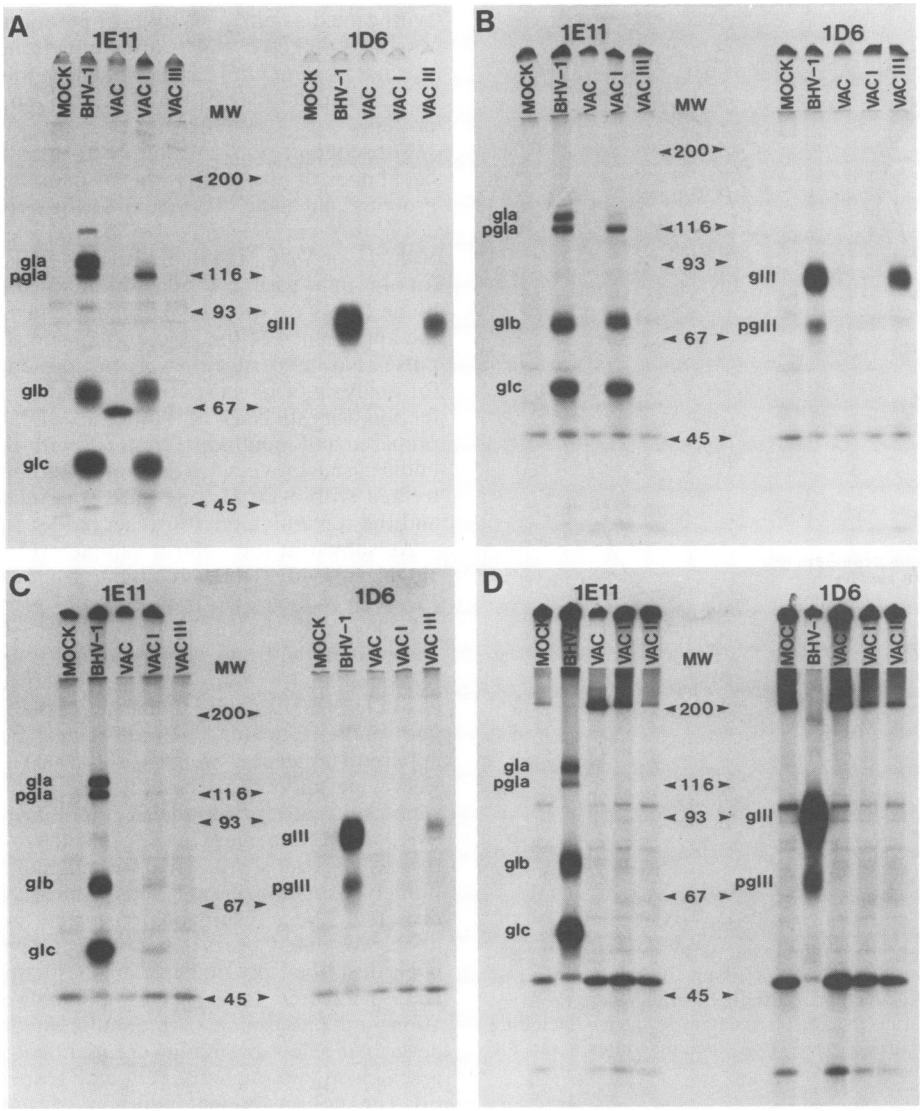

FIG. 3. Expression of glycoproteins gI and gIII. BSC-1 (A), BFB (B), BTB (C), or MDBK (D) cells were mock-infected or infected with BHV-1, VAC, VAC-I, or VAC-III recombinant virus. The cells were labeled with L-[^{35}S]methionine, and polypeptides were prepared and immunoprecipitated with gI-specific monoclonal antibody 1E11 or gIII-specific monoclonal antibody 1D6. The precipitated polypeptides were analyzed by SDS-PAGE (7.5%) and are identified in the left margins. Molecular weight (MW) markers (10^3) are shown in the middle.

Cell surface expression of BHV-1 glycoproteins. Expression of glycoproteins gI and gIII on the cell surface was examined by indirect immunofluorescence of recombinant- or BHV-1-infected live BSC-1 cells (Fig. 5). At 20 h postinfection, the cells were incubated with either gI- or gIII-specific rabbit serum. The recombinant-derived glycoproteins (Fig. 5A and B) had a patchy appearance over the entire cell surface, which was similar to the pattern observed for BHV-1-infected cells (Fig. 5C and D).

Immunogenicity of glycoproteins gI and gIII expressed by recombinant virus. Since we were interested in ascertaining whether vaccination with recombinant virus would result in a neutralizing antibody response to BHV-1, three groups of calves were inoculated with VAC-I, VAC-III, or VAC. Following two immunizations, animals immunized with VAC-I or VAC-III developed a high titer of serum neutralizing antibodies to BHV-1 (Fig. 6). Following a third immu-

TABLE 1. Comparison of the amounts of glycoproteins gI and gIII synthesized in recombinant- and BHV-1-infected cells

Virus	ELISA titer[a] in cell line:							
	BSC-1		MDBK		BFB		BTB	
	gI	gIII	gI	gIII	gI	gIII	gI	gIII
None	16	16	4	16	16	16	4	4
BHV-1	102	42	2,000	1,200	170	25	256	48
VAC	4	4	4	16	4	4	<4	4
VAC-I	320	4	16	16	200	4	85	4
VAC-III	<4	190	4	16	4	30	<4	25

[a] Recombinant- or BHV-1-infected cells were lysed in modified RIPA buffer, and microdilution plates, coated with 200 μl per well of monoclonal IgG, were incubated with 200 μl per well of cell lysate. The reaction was detected by incubation with 200 μl per well of horseradish peroxidase-conjugated monoclonal antibodies. The ELISA titer is expressed as the reciprocal of the highest dilution that still gave a reading of at least 0.1.

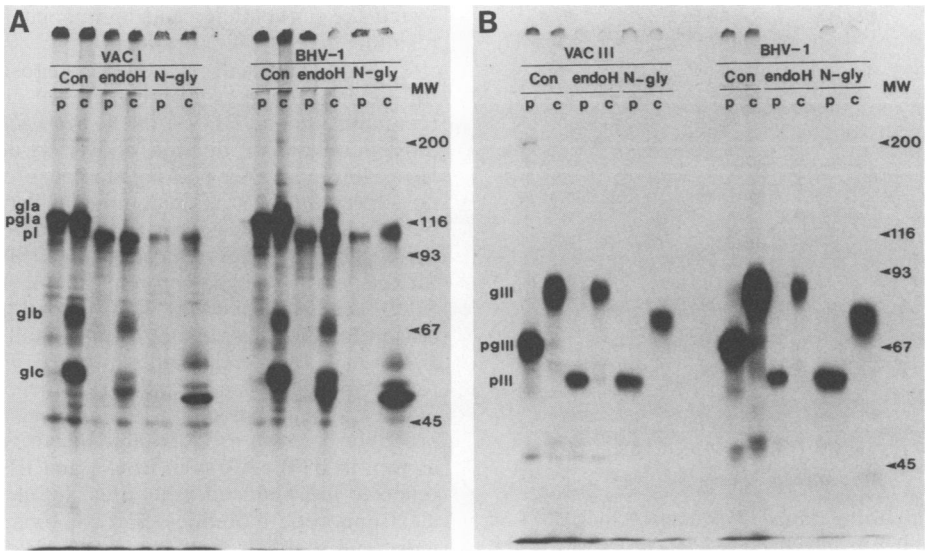

FIG. 4. Kinetics of gI and gIII expression. BSC-1 cells infected with BHV-1, VAC-I, or VAC-III were pulse-labeled with L-[^{35}S]methionine for 15 min and harvested immediately (p) or chased for 2 h (c). Cell lysates were prepared and immunoprecipitated with monoclonal antibody 1E11 (A) or 1D6 (B). One-third of the precipitates was treated with 125 mU of endo H per ml, one-third was treated with 14 U of *N*-Gly per ml, and the remaining one-third served as a control (Con). The precipitates were analyzed by SDS-PAGE (7.5%). The polypeptides are identified in the left margin. Molecular weight (MW) markers (10^3) are shown on the right.

nization, there was no further increase in the level of neutralizing antibodies to BHV-1 induced by the recombinant viruses. The sera of the animals that were inoculated with VAC did not show any neutralizing activity. To further measure the specificity of the immune response, the sera were tested in an ELISA, using either gI or gIII as the antigen. Animals immunized with VAC-I developed antibodies to gI, whereas animals inoculated with VAC-III recognized gIII (Fig. 7), indicating that the virus-neutralizing activity was specific for the respective glycoproteins. Animals that received VAC did not react with either gI or gIII.

DISCUSSION

Previously, we demonstrated the biological importance of three of the major BHV-1 glycoproteins, gI, gIII, and gIV,

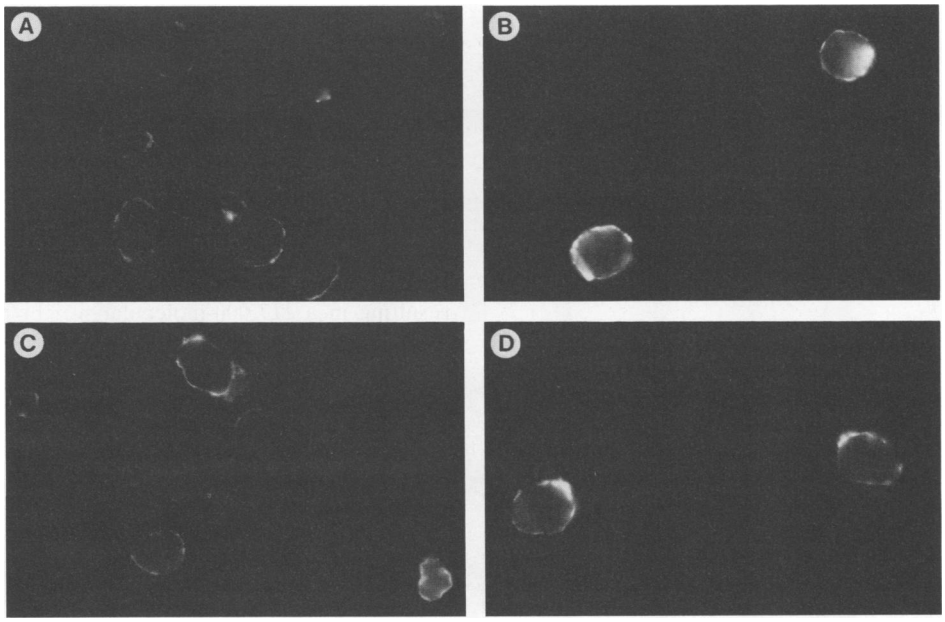

FIG. 5. Cell surface expression of glycoproteins gI and gIII. BSC-1 cells were infected with VAC-I (A), VAC-III (B), or BHV-1 (C and D). Cell surface immunofluorescence was demonstrated by using monospecific rabbit serum against gI (A and C) or gIII (B and D) in an indirect assay.

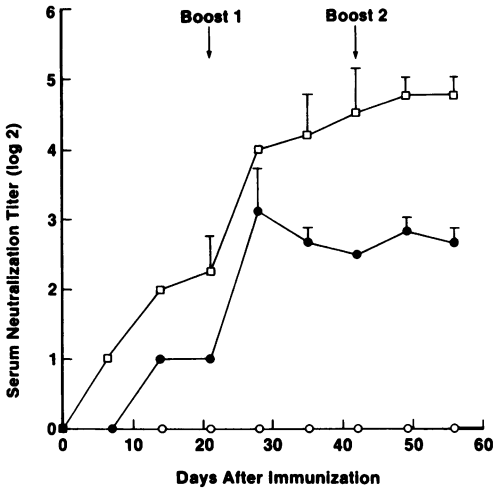

FIG. 6. Serum neutralizing antibody responses to BHV-1 of animals immunized with VAC recombinants expressing gI (VAC-I; ●) or gIII (VAC-III; □) or with VAC (○). The animals received booster immunizations 3 and 6 weeks later. Titers were determined by a 50% endpoint using 100 PFU of BHV-1.

specifically in the humoral immune response to a BHV-1 infection (48, 49). We also showed the potential of each of these glycoproteins as a subunit vaccine (1). Presently, we intend to study the specificity of the cell-mediated immune response to a BHV-1 infection and to investigate a practical form of a glycoprotein vaccine. We have chosen to express individual BHV-1 glycoproteins in recombinant VAC vectors. Since VAC recombinants are infectious and VAC has a wide host range, this strategy allows the expression of individual glycoproteins both in tissue culture and in animals. In this study, we constructed VAC recombinants and

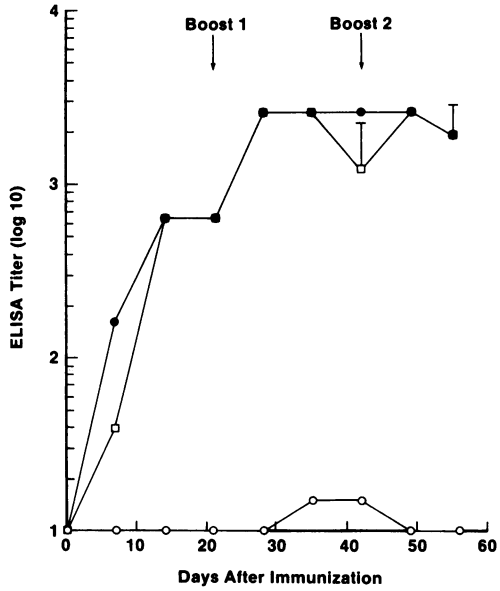

FIG. 7. ELISA titers of animals immunized with VAC-I (●), VAC-III (□), or VAC (○). The animals received booster immunizations 3 and 6 weeks later. The microdilution plates were coated with gI (0.25 μg/ml; ●), gIII (0.25 μg/ml; □), or gI plus gIII (0.25 μg/ml each; ○). The ELISA titer is expressed as the reciprocal of the dilution that still resulted in a reading of 0.1.

tested the authenticity and immunogenicity of each of the resulting glycoprotein products.

To find the optimal cell system, we tested several cell lines for the expression of glycoproteins gI and gIII by the VAC recombinants and BHV-1 itself. MDBK cells supported the most abundant production of BHV-1-derived gI and gIII. However, even after prolonged exposure of the fluorograph, no expression of VAC recombinant-derived gI or gIII was detected. This is in agreement with the nonpermissiveness of these cells for VAC replication, even though in one report Sindbis virus-specific proteins have been described in MDBK cells infected with VAC recombinant virus (36). The reason for this discrepancy is not readily apparent. BSC-1, BFB, and BTB cells all expressed BHV-1- as well as recombinant-derived gI and gIII. However, the production of recombinant gI and gIII was very low in BTB cells, probably because the recombinant viruses do not replicate as well in BTB cells as in BSC-1 and BFB cells, which was reflected in a reduced virus titer. In all three cell lines, the electrophoretic mobilities of recombinant gI and gIII were indistinguishable from those of authentic gI and gIII. BSC-1 cells were used in all following experiments on the basis of two observations. First, processing of gIII, produced by recombinant VAC virus or BHV-1, appeared to be identical in BSC-1 cells but not in BFB and BTB cells, where the precursor pgIII was detectable at 24 h after BHV-1 infection but not after VAC-III infection. Secondly, BSC-1 cells produced the greatest amounts of recombinant gI and gIII as well as sufficient quantities of authentic glycoproteins. The quantitative comparison also showed that in the most productive cell line for each system, the authentic glycoproteins were produced in a sixfold excess over the recombinant-derived glycoproteins. This is within the range reported by other investigators. In recombinant-infected cells, Sindbis glycoprotein G was expressed at 10% of the level observed in Sindbis-infected cells (36). In contrast, under most favorable conditions, the level of expression of respiratory syncytial virus (RSV) G protein in recombinant-infected cells was approximately equivalent to that in RSV-infected cells (3, 46).

The results discussed above show that recombinant- and BHV-1-derived glycoproteins gI and gIII have identical apparent molecular weights, suggesting that the polypeptide backbones and glycosylation patterns are identical. To confirm this hypothesis, these glycoproteins were subjected to the action of endo H or N-Gly, which showed an identical 105K nonglycosylated precursor polypeptide pI(105) in VAC-I- and BHV-1-infected cells. Furthermore, the cotranslational addition of N-linked sugars appeared to occur in a similar fashion in recombinant- and BHV-1-infected cells, resulting in a 117,000-molecular-weight polypeptide which was further processed into the mature glycoprotein gI. Similarly, the 61,000-molecular-weight polypeptide backbones of recombinant and authentic gIII appeared to be identical as shown by endo H and N-Gly digestion. This polypeptide was further processed by cotranslational addition of N-linked carbohydrates into a 69,000-molecular-weight partially glycosylated intermediate. Final maturation to gIII occurred by modification of N-linked sugars and addition of *O*-linked oligosaccharides. Incubation of gIII with N-Gly, which removes all N-linked carbohydrates, resulted in a 77,000-molecular weight partially glycosylated polypeptide. Consequently, O-linked sugars account for 16,000 of the molecular weight of gIII, whereas complex N-linked carbohydrates make up the remaining difference between pIII(61) and gIII(85K), i.e., 8,000.

The polypeptide backbones and mature products of gI and gIII were shown to be identical in recombinant- and BHV-1-infected cells. However, differences in the processing efficiencies of both gI and gIII were observed. First, the majority of gI is processed by proteolytic cleavage in BHV-1-infected cells, whereas in VAC-I-infected cells, all of gI was found as the cleaved forms gIb and gIc. The processing is apparently more efficient in recombinant-infected cells, perhaps because more cellular protease remains available during VAC-I infection than during BHV-1 infection, which completely shuts down host protein synthesis. The results presented here also show that cleavage of gI can occur in the absence of BHV-1 RNA replication or expression of its nonstructural proteins. Secondly, the efficiency of processing of pgIII to gIII by the addition of N-linked and O-linked sugars was higher in VAC-III-infected BFB and BTB cells than in BHV-1-infected cells. Apparently, more cellular glycosyltransferases remain available during the course of a VAC-III infection than during a BHV-1 infection, which results in complete shutdown of host protein synthesis.

Interestingly, the apparent molecular weights of gI and gIII in BSC-1 cells were different from those in MDBK or GBK cells (50), indicating a difference in the extent of glycosylation. Since the polypeptide backbones and high-mannose intermediates of these glycoproteins were identical in the three cell lines, this difference appears to be generated during posttranslational processing. This observation supports the hypothesis that BHV-1, like all other enveloped viruses (14), uses cellular enzymes for glycosylation (47).

Although the in vitro levels of expression of recombinant gI and gIII appeared to be lower than those of their authentic counterparts, the levels were sufficient for the induction of serum neutralizing antibodies in cattle. These antibodies recognized authentic gI and gIII, confirming the specificity of the neutralizing antibody response. It is important to note that the recombinant glycoproteins gI and gIII, even though in competition with numerous VAC antigens, induced neutralizing antibody titers comparable to those reached after two immunizations of affinity-purified authentic gI and gIII (1). Previously, the levels of these titers were found to be high enough for the induction of protection against challenge with BHV-1 and *P. haemolitica* in cattle (1), indicating the potential usefulness of VAC for the development of a vaccine against BHV-1.

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