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Synthesis and Processing of Bovine Herpesvirus 1 Glycoproteins

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Four unique glycoproteins or glycoprotein complexes were recognized by a panel of monoclonal antibodies to bovine herpesvirus 1 (BHV-1), i.e., GVP 6/11a/16 (130,000-molecular-weight glycoprotein [130K glycoprotein]/74K/55K), GVP 7 (108K), GVP 3/9 (180K/91K), and GVP 11b (71K). The absence of any antigenic or structural relationship between GVP 11a and GVP 11b, which were previously identified as one glycoprotein, GVP 11, demonstrated that these two GVP 11 species are unique glycoproteins. GVP 3 and GVP 9 showed complete sequence homology, as shown by the identity of their antigenic determinants and by partial peptide mapping. This observation, as well as the ratio of their apparent molecular weights, indicated that GVP 3 (180K) is a dimeric form of GVP 9 (91K). GVP 6 and GVP 11a, as well as GVP 6 and GVP 16, showed at least partial sequence homology, since they shared several antigenic determinants and peptides. In addition, GVP 6, GVP 11a, and GVP 16 were derived from one primary precursor. These results, as well as the ratio of their apparent molecular weights, indicated that the GVP 6/11a/16 complex consists of two forms: one in which GVP 6 (130K) is uncleaved and the other one in which GVP 6 is cleaved and composed of GVP 11a (74K) and GVP 16 (55K), linked by disulfide bridges. An antigenically distinct precursor to each of the four BHV-1 glycoproteins or glycoprotein complexes was identified by monoclonal antibodies. These precursors, pGVP 6 (117K), pGVP 11a (62K), pGVP 7 (100K), pGVP 9 (69K), and pGVP 11b (63K) were sensitive to endo-β-*N*-acetylglucosaminidase H treatment, indicating that they represent the partially glycosylated high-mannose-type intermediate forms generated by cotranslational glycosylation of the primary, unglycosylated precursors to GVP 6/11a/16, GVP 7, GVP 3/9, and GVP 11b, which were identified as having apparent molecular weights of 105,000, 90,000, 61,000, and 58,000, respectively. A new nomenclature for the BHV-1 glycoproteins, based on roman numerals, is proposed.

Like most herpesviruses studied to date (7, 8, 42, 43), bovine herpesvirus 1 (BHV-1) specifies more than 25 structural polypeptides (4, 25). Among these, 11 glycosylated species were identified, more than have been reported for other herpesviruses (14, 40, 41, 44, 45, 47, 53). Previous studies, mainly on the prototype herpesvirus, herpes simplex virus, have shown that these glycoproteins become incorporated into the plasma membrane of the host cell (10, 35) and ultimately constitute part of the virion envelope (30, 37). In that capacity, they have important biological and immunological functions, e.g., they effect attachment and penetration of the virus into the cell (24, 37) and mediate the immune responses of the host to the infection (28, 29, 50). The different responses of the bovine immune system to a BHV-1 infection, which may result in virus neutralization or cell lysis, have been studied in great detail (1, 12, 13, 36). Consequently, we wished to determine the involvement of each of the BHV-1 glycoproteins in these interactions between the virus and the immune system, but the large number of BHV-1 glycoproteins greatly hampered these studies. However, the availability of monoclonal antibodies, which recognize single antigenic determinants, would have allowed us to (i) analyze the biological and immunological functions of the BHV-1 glycoproteins, (ii) demonstrate unique identity and interrelationships among these glycoproteins, and (iii) determine precursor-product relationships between different proteins. To this end, we recently produced a series of monoclonal antibodies to BHV-1. These monoclonal antibodies had four different specificities, i.e., they recognized GVP 6 (130,000-molecular-weight glycoprotein [130K glycoprotein]), GVP 11a (74K), and GVP 16 (55K); GVP 7 (108K); GVP 3 (180K) and GVP 9 (91K); or GVP 11b (71K) (48, 49). These glycoproteins were all present in virions and were expressed on the plasma membranes of the virus-infected cells. The monoclonal antibodies also reacted with some additional polypeptides in the infected cells, which were not present in the virion or on the plasma membrane and, thus, may be the precursors to the glycoproteins. Preliminary data on the relationships among the glycoproteins in the GVP 6/11a/16 and GVP 3/9 complexes indicated that GVP 6, GVP 11a, and GVP 16 are linked by disulfide bonds and that GVP 3 is a dimer of GVP 9 (49). Initial studies on the biological functions of these glycoproteins demonstrated that GVP 6/11a/16, GVP 3/9 (49), and GVP 11b (26) participated to different extents in virus neutralization and antibody-complement-mediated lysis of virus-infected cells.

In the present study we used the monoclonal antibodies and peptide mapping to demonstrate the uniqueness of the four BHV-1 glycoproteins or glycoprotein complexes, as well as the interrelationships among the glycoproteins within the GVP 6/11a/16 and GVP 3/9 complexes. The monoclonal antibodies were further used to identify the precursors to these four BHV-1 glycoproteins or glycoprotein complexes. Finally, the oligosaccharide structures of the glycoproteins and their precursors were characterized by using endo-β-*N*-acetylglucosaminidase H (endo H). The presence of high-mannose-type oligosaccharides can be determined by the susceptibility of a glycoprotein to the action of endo H because this enzyme cleaves between the two proximal *N*-acetylglucosamine residues of the high-mannose-type oligosaccharides, but not between those of the complex type (52). A primary precursor and a high-mannose intermediate

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to each of the four major BHV-1 glycoproteins were identified. GVP 3/9 and GVP 11b were shown to have only complex-type oligosaccharides, whereas 6/11a/16 and GVP 7 contained high-mannose- and complex-type carbohydrate side chains.

MATERIALS AND METHODS

Virus propagation and purification. The P8-2 strain of BHV-1 was grown in Georgia bovine kidney (GBK) cells, concentrated by ultracentrifugation, and purified on potassium tartrate gradients, as described previously (1, 25).

Preparation of radiolabeled cell and virus lysates. To prepare cell lysates, GBK cells were infected with BHV-1 at a multiplicity of infection of 10. After adsorption of the virus for 1 h, the monolayer was overlaid with either methionine-free or glucose-free Eagle minimal essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) containing 2% fetal bovine serum (GIBCO) and further incubated at 37°C. Six hours after infection, 25 μCi of L-[^{35}S]methionine, 50 μCi of [^{3}H]mannose, or 50 μCi of [^{3}H]glucosamine (Amersham, Oakville, Ontario, Canada) per ml was added to the cultures. At 24 h postinfection the cells were harvested, washed with phosphate-buffered saline (0.01 M Na_2HPO_4-NaH_2PO_4, 0.15 M NaCl [pH 7.4]), and suspended in modified RIPA buffer (0.02 M Tris hydrochloride [pH 8.0], 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P-40). The cell lysates were left on ice for 15 min, sonicated for 15 s at a setting of 4 on a Sonifier cell disrupter (model W14OD; Ultrasonics, Plainsview, N.Y.), and clarified by centrifugation at 80,000 rpm for 15 min in a 30° A100 rotor at room temperature (Airfuge; Beckman Instruments, Inc., Fullerton, Calif.) or at 25,000 rpm for 1 h in an SB405 rotor at 4°C (IEC model B-60 ultracentrifuge). The supernatants were used immediately for immunoprecipitation. To prepare virus lysates, BHV-1 was labeled with L-[^{35}S]methionine and purified as described previously (25). The purified virus was suspended in TNE (0.01 M Tris hydrochloride [pH 7.5], 0.15 M NaCl, 0.001 M EDTA) containing 1% Nonidet P-40 and left on ice for 15 min. After sonication for 15 s at a setting of 4 on a Sonifier cell disrupter (model W14OD; Ultrasonics), extracted proteins were separated from insoluble material by ultracentrifugation at 25,000 rpm for 2 h at 4°C in an SB405 rotor (IEC model B-60) and used immediately for immunoprecipitation.

Time course and pulse-chase experiments. In time course experiments BHV-1-infected 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 BHV-1-infected 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). The label was chased for 2 h by washing and incubating the cells in MEM containing 100 μg of cycloheximide per ml. Cell lysates for immunoprecipitation were prepared as usual.

Immunoprecipitation. The procedure for immunoprecipitation was described in detail previously (48, 49).

Endo H treatment. Immunoprecipitated polypeptides were eluted from protein A-Sepharose (Pharmacia, Montreal, Quebec, Canada) by boiling for 5 min in 0.8% sodium dodecyl sulfate (SDS). The protein A-Sepharose was removed by centrifugation, and the supernatants were adjusted to pH 5.5 with 0.125 M sodium citrate containing 1 mM phenylmethylsulfonyl fluoride. One half of the samples was treated with 60 mU of endo H (Miles Laboratories, Inc., Elkhart, Ind.) per ml, and the other half served as controls. After incubation at 37°C for 20 h, 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-mercaptoethanol, 0.00125% bromophenol blue) and boiled for 1 min for analysis by polyacrylamide gel electrophoresis (PAGE).

PAGE. SDS-PAGE was performed on 7.5% polyacrylamide slab gels with a discontinuous buffer system (21). Unless otherwise noted, electrophoresis was performed under reducing conditions. To visualize molecular weight markers, the gels were stained with Coomassie blue after electrophoresis. For autoradiography, the gels were dried and placed in contact with 3M X-ray film (Picker, Saskatoon, Saskatchewan, Canada). For fluorography, the gels were impregnated with En^{3}Hance (New England Nuclear Corp., Lachine, Quebec, Canada) or Amplify (Amersham), dried, and placed in contact with film at −70°C. The molecular weights of the polypeptides were estimated from the molecular weight markers (Bio-Rad, Mississauga, Ontario, Canada) that were electrophoresed in parallel with the samples.

Peptide mapping. For partial peptide mapping, GVP 6/11a/16 and GVP 11b were labeled with L-[^{35}S]methionine as usual, whereas GVP 3/9 was labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine (Amersham). The radiolabeled glycoproteins were precipitated with the appropriate monoclonal antibodies and subjected to electrophoresis on 7.5% polyacrylamide gels as described above. Rabbit anti-mouse immunoglobulin G (IgG) Immunobeads (Bio-Rad) were used to precipitate immune complexes. Peptide maps were obtained by cleavage with *N*-chlorosuccinimide (NCS; Sigma Chemical Co., St. Louis, Mo.), as described by Lischwe and Ochs (23). After electrophoresis, the gel was thoroughly rinsed with distilled water and autoradiographed while wet. The glycoprotein bands were localized by alignment of the autoradiogram with the wet gel, excised, and stored at −20°C. Digestion of the glycoprotein-containing gel slices was performed in large vials on a shaker at room temperature. The gel slices were first washed with 20 ml of distilled water for 20 min with one change and then with 10 ml of urea-distilled H_2O-CH_3COOH (1 g/1 ml/1 ml) for 20 min with one change. The glycoprotein-containing slices were digested for 30 min in 5 ml of 0.015 M NCS in urea-distilled H_2O-CH_3COOH. Subsequently, the slices were washed with distilled water, as described above, and equilibrated in 10 ml of 0.0625 M Tris hydrochloride (pH 6.8)–3% SDS–10% glycerol–15% 2-mercaptoethanol for 1.5 h with three changes. Finally, the gel slices were loaded onto a 4.5% stacking–15% resolving gel to analyze the digests.

Western blot analysis. The Western blotting technique of Burnette (5) was used as described previously (49). Briefly, Nonidet P-40-extracted viral glycoproteins were separated by electrophoresis in denaturing, reducing gels and transferred to nitrocellulose paper. The nitrocellulose was incubated with a 1:1,000 dilution of ascites fluid and then with a 1:1,000 dilution of rabbit anti-mouse IgG (Cooper Biomedical, Inc., West Chester, Pa.) and 5×10^5 to 1×10^6 cpm of ^{125}I-labeled protein A (Pharmacia). Finally, the nitrocellulose strips were dried and analyzed by autoradiography on 3M X-ray film.

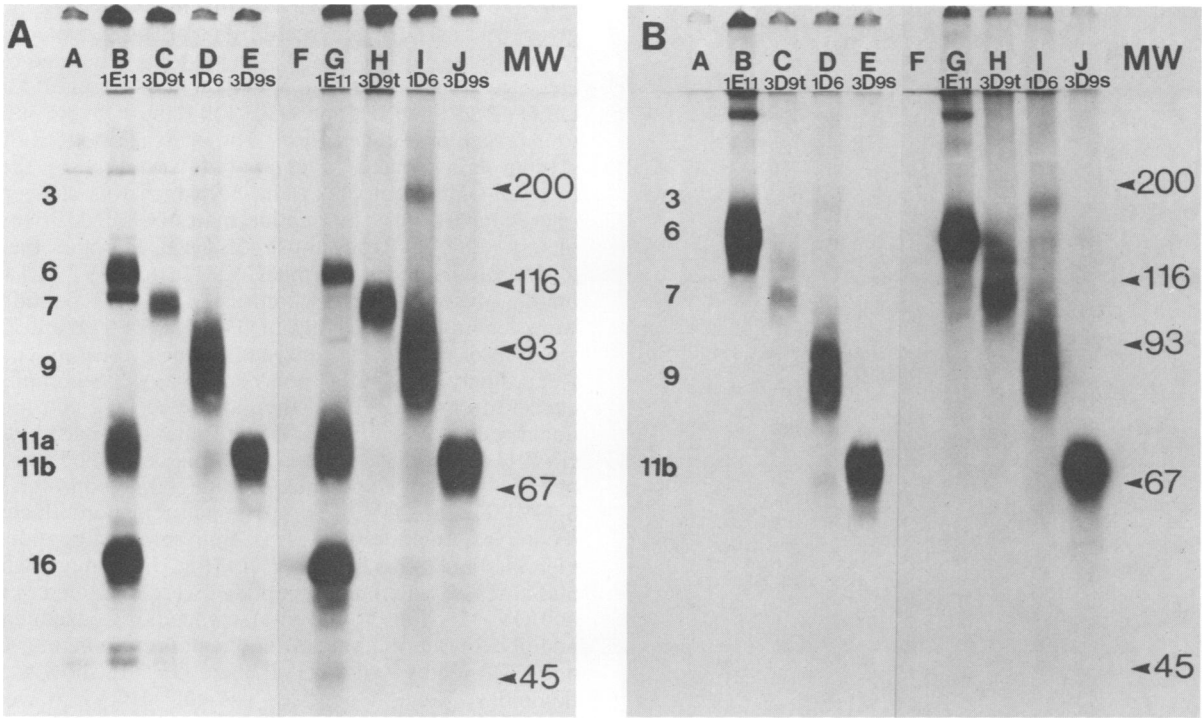

FIG. 1. L-[^{35}S]methionine-labeled polypeptides immunoprecipitated from virus-infected cell (lanes A to E) or virus (lanes F to J) lysates and analyzed by SDS-PAGE (7.5%) under reducing (A) or nonreducing (B) conditions. The lysates were prepared from mock-infected (lanes A and F) or BHV-1-infected (lanes B to E and G to J) cells and precipitated by monoclonal antibody 1E11 (lanes A, B, F, and G), 3D9T (lanes A, C, F, and H), 1D6 (lanes A, D, F, and I), and 3D9S (lanes A, E, F, and J). The precipitated glycoproteins are identified in the left margin. Molecular weight markers (10^3) are shown in the right margin.

RESULTS

BHV-1 glycoproteins identified by monoclonal antibodies. We recently produced a series of monoclonal antibodies to BHV-1 (49). The glycoproteins, precipitated by these monoclonal antibodies from L-[^{35}S]methionine-labeled infected-cell and virus lysates, were analyzed by SDS-PAGE under reducing conditions.

The results of this analysis are illustrated for selected antibodies in Fig. 1A. One group of monoclonal antibodies reacted with GVP 6 (130K), GVP 11a (74K), and GVP 16 (55K) (lanes B and G), one monoclonal antibody was specific for GVP 7 (108K) (lanes C and H), another group of monoclonal antibodies precipitated GVP 3 (180K) and GVP 9 (91K) (lanes D and I), and one monoclonal antibody recognized GVP 11b (71K) (lanes E and J). In addition to GVP 6, GVP 11a, and GVP 16, a 117K polypeptide was precipitated from the infected-cell lysate (lane B), but not from the virus lysate (lane G). This polypeptide may be the precursor to these glycoproteins. Similarly, a potential precursor form of GVP 9, with an apparent molecular weight of 69,000, was identified (lane D). The 63K polypeptide (lane E) may be the precursor to GVP 11b.

These potential precursor forms were not only labeled with L-[^{35}S]methionine (Fig. 1) but also, like the glycoproteins, with [^{3}H]mannose (Fig. 2) and [^{3}H]glucosamine (data not shown).

The immunoprecipitates were also analyzed by electrophoresis under nonreducing conditions (Fig. 1B). Whereas identical patterns were observed for GVP 7, GVP 3/9, and GVP 11b, monoclonal antibodies that precipitated GVP 6, GVP 11a, and GVP 16 under reducing conditions now only precipitated a nonreduced GVP 6 complex (148K). These results suggest that GVP 6, GVP 11a, and GVP 16 are linked by disulfide bridges.

When the BHV-1 glycoproteins were studied without the aid of monoclonal antibodies, one glycoprotein with an apparent molecular weight of 71,000 to 72,000 was identified and named GVP 11 (25). However, the absence of any antigenic relatedness between GVP 6/11a/16 and GVP 11b, as shown by the reaction of the monoclonal antibodies, as well as the slight difference in apparent molecular weight between complexed GVP 11 and individual GVP 11, suggested that there may actually be two unique GVP 11 species, one of which is part of the GVP 6/11a/16 complex. To differentiate these two glycoproteins, we introduced the names GVP 11a and GVP 11b.

Antigenic relationships among the glycoproteins in the GVP 6/11a/16 and GVP 3/9 complexes. To determine whether the glycoproteins in the GVP 6/11a/16 and GVP 3/9 complexes were antigenically related, they were solubilized, electrophoretically separated under denaturing and reducing conditions, and transferred to nitrocellulose. Subsequently, the reactivity of the individual glycoproteins with the various monoclonal antibodies was determined in an immunoblot assay. As illustrated for selected antibodies (Fig. 3), GVP 6/11a/16-specific monoclonal antibodies reacted with GVP 6 and GVP 11a (lane A) or GVP 6 and GVP 16 (lane B). Similarly, GVP 3/9-specific monoclonal antibodies recognized GVP 3 (lane E) and GVP 9 (lanes D and E). In contrast, the GVP 11b-specific monoclonal antibody reacted only with GVP 11b (lane C). These results show that GVP 3 and GVP 9, GVP 6 and GVP 11a, and GVP 6 and GVP 16 share several antigenic determinants.

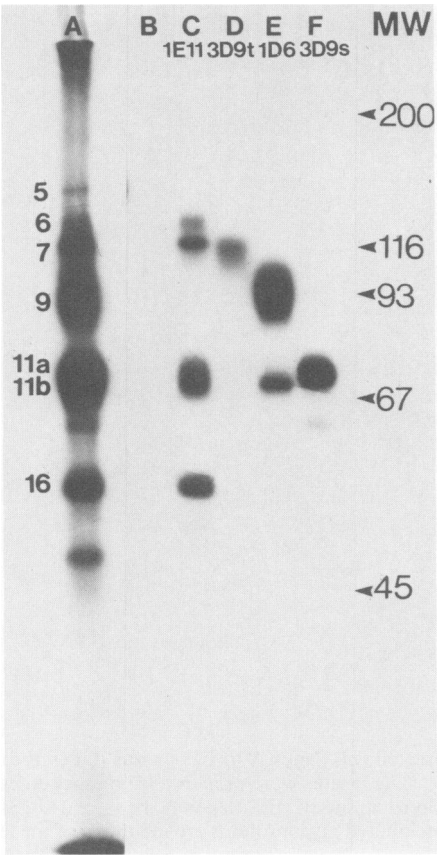

FIG. 2. [^{3}H]mannose-labeled polypeptides immunoprecipitated from mock-infected (lane B) or BHV-1-infected (lanes C to F) cells by monoclonal antibodies 1E11 (lanes B and C), 3D9T (lanes B and D), 1D6 (lanes B and E), and 3D9S (lanes B and F). The total BHV-1-infected cell lysate is shown in lane A. The immunoprecipitates were analyzed by SDS-PAGE (7.5%). The viral glycoproteins are identified in the left margin. Molecular weight markers (10^3) are shown in the right margin.

Absence of antigenic relatedness between GVP 11a and GVP 11b. It was shown that monoclonal antibody 1E11 precipitated GVP 6/11a/16, whereas monoclonal antibody 3D9S precipitated GVP 11b (Fig. 1A and 2), indicating that GVP 11a and GVP 11b are two different glycoproteins. The difference in their behavior under nonreducing conditions (Fig. 1B) supports this concept. To further prove the uniqueness of GVP 11a and GVP 11b, cross-immunoprecipitation experiments were performed (Fig. 4). First, L-[^{35}S]methionine-labeled cell lysates were incubated with monoclonal antibody 1E11, which precipitated GVP 6/11a/16. Similarly, GVP 11b was precipitated from cell lysates with monoclonal antibody 3D9S. Subsequently, the supernatants of the precipitates were divided into two aliquots and incubated with homologous or heterologous antibody. 1E11 precipitated GVP 6/11a/16 from the supernatant of the 3D9S precipitate but not from the 1E11-treated supernatant, whereas 3D9S precipitated GVP 11b from the supernatant of the 1E11 precipitate but not from the 3D9S-treated supernatant (Fig. 4).

Structural comparison of GVP 6, GVP 11a, GVP 16, and GVP 11b. To obtain more evidence for the previously suggested interrelationship among GVP 6, GVP 11a, and GVP 16, as well as for the supposed difference between GVP 11a and GVP 11b, partial peptide maps of each of these glycoproteins were generated by using NCS, which cleaves tryptanophyl peptide bonds. The peptides generated by NCS cleavage are shown in Fig. 5. GVP 6 (lane B), GVP 11a (lane C), GVP 16 (lane D), and GVP 11b (lane E) were subjected to cleavage in reduced form. The cleavage pattern of these glycoproteins was also compared with that of the nonreduced GVP 6 complex (lane A), which was cleaved in its nonreduced state. The peptide map of GVP 11b was completely different from those of GVP 11a and the other glycoproteins, indicating that GVP 11a and GVP 11b are two unique glycoproteins. In contrast, all of the peptides that were generated by cleavage of GVP 6, GVP 11a, and GVP 16 were also present in the nonreduced GVP 6 complex. Accordingly, when the nonreduced GVP 6 complex was excised from the gel and then analyzed by electrophoresis under reducing conditions, it was found to consist of GVP 6, GVP 11a, and GVP 16 (Fig. 6). This confirmed our earlier observation that the combined reduced glycoproteins GVP 6, GVP 11a, and GVP 16 correspond to the nonreduced GVP 6 complex. In addition, at least four common peptides were identified in GVP 6 and GVP 11a (Fig. 5, open arrowheads), and at least two common peptides were identified in GVP 6 and GVP 16 (Fig. 5, closed arrowheads). Consequently, in addition to sharing several antigenic determinants, GVP 6 and GVP 11a as well as GVP 6 and GVP 16 show sequence homology. Several peptides present in GVP 6 were not identified in GVP 11a or GVP 16, and vice versa. They probably are the peptides generated around the cleavage site, which differ in length between GVP 6 and GVP 11a or GVP 16.

Structural relationship between GVP 3 and GVP 9. GVP 3, which often coprecipitated with GVP 9 (Fig. 1), also shares a number of antigenic determinants with GVP 9 (Fig. 3). The

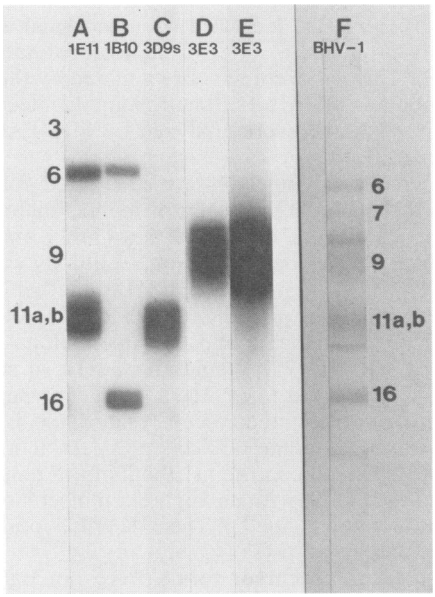

FIG. 3. Reactivity of monoclonal antibodies 1E11 (lane A), 1B10 (lane B), 3D9S (lane C), and 3E3 (lanes D and E) with BHV-1 glycoproteins that were solubilized with Nonidet P-40, electrophoretically separated under denaturing and reducing conditions, and transferred to nitrocellulose. GVP 3 was only visible after prolonged exposure of the nitrocellulose strip (lane E). The BHV-1 glycoproteins were visualized by staining one nitrocellulose strip with amido black (lane F). The viral glycoproteins are identified in the margins.

ratio of the respective molecular weights of GVP 3 (180,000) and GVP 9 (91,000) suggests that these two glycoproteins may have a dimer-monomer relationship. To obtain further evidence for this hypothesis, partial peptide maps of these glycoproteins were generated by cleavage with NCS. The peptide maps of GVP 3 and GVP 9 were identical (Fig. 7), strongly supporting the concept that GVP 3 is a dimer of GVP 9.

Synthesis and processing of BHV-1 glycoproteins. To study the order and time course of synthesis of GVP 6/11a/16, GVP 7, GVP 3/9, and GVP 11b, BHV-1-infected cells were labeled with L-[^{35}S]methionine immediately after virus adsorption and harvested at 2-h intervals after infection. Cell lysates were prepared and were precipitated with the four monoclonal antibodies used above so that each individual glycoprotein could be clearly identified. These experiments demonstrated that GVP 6/11a/16, GVP 7, and GVP 11b were synthesized as early as 2 h postinfection, whereas GVP 3/9 was not detected until 8 h postinfection (data not shown).

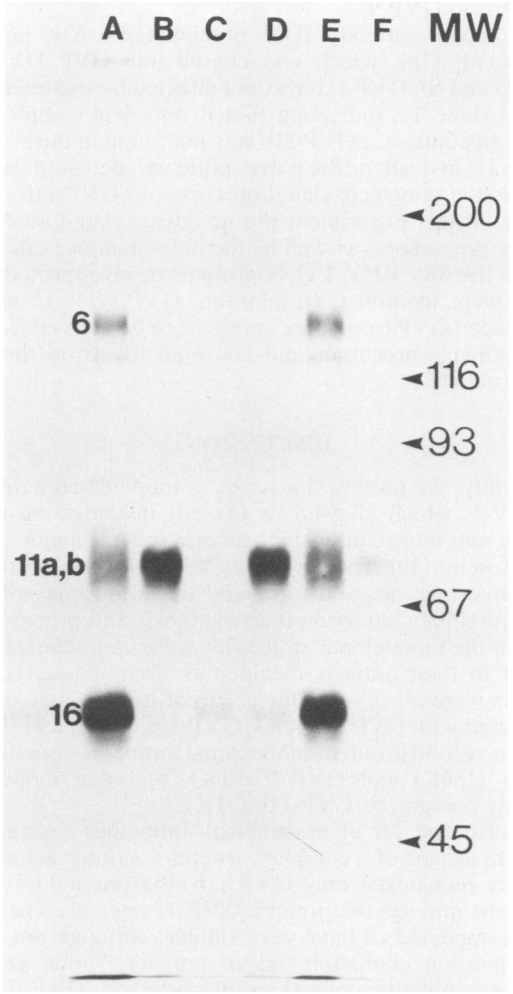

FIG. 4. L-[^{35}S]methionine-labeled polypeptides immunoprecipitated from BHV-1-infected cells by monoclonal antibody 1E11 (lane A), 3D9S (lane B), 1E11 followed by 1E11 (lane C), 1E11 followed by 3D9S (lane D), 3D9S followed by 1E11 (lane E), and 3D9S followed by 3D9S (lane F). The immunoprecipitates were analyzed by SDS-PAGE (7.5%). The precipitated glycoproteins are identified in the left margin. Molecular weight markers (10^3) are shown in the right margin.

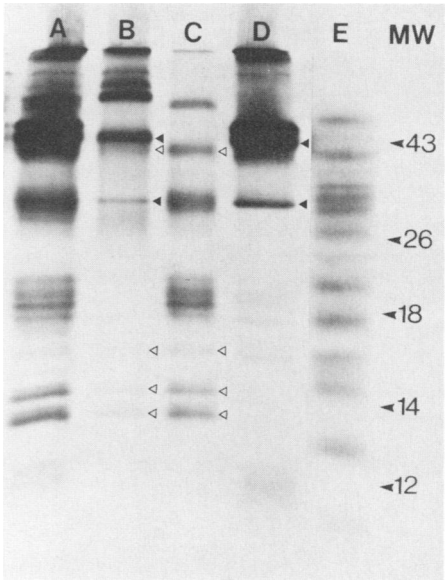

FIG. 5. NCS (0.015 M) cleavage patterns of L-[^{35}S]methionine-labeled nonreduced GVP 6 complex (lane A), GVP 6 (lane B), GVP 11a (lane C), GVP 16 (lane D), and GVP 11b (lane E), analyzed by SDS-PAGE (15%). All glycoproteins were precipitated with the appropriate monoclonal antibodies and separated in 7.5% polyacrylamide gels under nonreducing (nonreduced GVP 6 complex) or reducing (GVP 6, GVP 11a, GVP 16, and GVP 11b) conditions before cleavage. The positions of the molecular weight markers (10^3) are shown in the right margin. Open arrowheads indicate common peptides in GVP 6 and GVP 11a; closed arrowheads indicate common peptides in GVP 6 and GVP 16.

Because no monospecific antisera against BHV-1 glycoproteins were available, it was not possible to identify the precursors to these glycoproteins. The availability of monoclonal antibodies against BHV-1 glycoproteins allowed us to define precursor forms of these glycoproteins and to study the conversion of these procursors into the products. To characterize the oligosaccharide side chains of the six BHV-1 glycoproteins and their precursors in particular, we investigated their susceptibility to the action of endo H. BHV-1-infected cells were pulse-labeled for 15 min with L-[^{35}S]methionine and harvested immediately or after the label was chased for 2 h. Cell lysates were prepared and precipitated with the monoclonal antibodies used above. One half of the immunoprecipitates was treated with endo H,

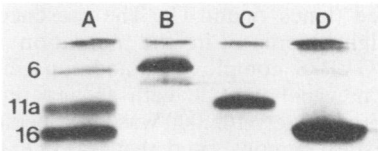

FIG. 6. PAGE (15%) of L-[^{35}S]methionine-labeled nonreduced GVP 6 complex (lane A), GVP 6 (lane B), GVP 11a (lane C), and GVP 16 (lane D). The glycoproteins were precipitated with monoclonal antibody 1E11 and separated in 7.5% polyacrylamide gels under nonreducing (nonreduced GVP 6 complex) or reducing (GVP 6, GVP 11a, and GVP 16) conditions. After electrophoresis, the gels were autoradiographed while wet. The glycoprotein bands were then localized by alignment of the autoradiograms with the wet gels, excised, and analyzed by electrophoresis in a 15% reducing gel. The positions of the glycoproteins on this gel are shown in the left margin. Only the upper portion of the gel is presented.

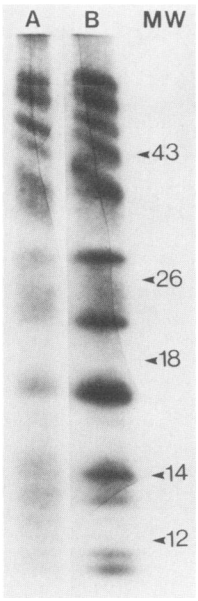

FIG. 7. NCS (0.015 M) cleavage patterns of L-[^{35}S]methionine- and L-]^{35}S]cysteine-labeled GVP 3 (lane A) and GVP 9 (lane B), analyzed by SDS-PAGE (15%). The glycoproteins were precipitated with monoclonal antibody 1D6 and separated in a 7.5% polyacrylamide gel under reducing conditions before cleavage. The positions of the molecular weight markers (10^3) are shown in the right margin.

and the other half served as controls. Based on the time of appearance of the four glycoproteins or glycoprotein complexes in the infected cell, the pulse-chase experiments were performed at 6 h postinfection for analysis of GVP 6/11a/16, GVP 7, and GVP 11b and at 12 h postinfection for analysis of GVP 3/9 (Fig. 8).

Monoclonal antibody 1E11 precipitated GVP 6 (130K), GVP 11a (74K), and GVP 16 (55K), as well as a 117K polypeptide (pGVP 6), which was chased into GVP 6, GVP 11a, and GVP 16, and a 62K polypeptide (pGVP 11a), which was chased into GVP 11a (Fig. 8, lanes E and G). In the presence of endo H (lanes F and H), pGVP 6, pGVP 11a, and GVP 16 were not detectable; instead, three new polypeptides with apparent molecular weights of 105,000, 58,000, and 52,000 were observed. In contrast, GVP 6 and GVP 11a were not affected. When the 1E11 immunoprecipitate was analyzed by electrophoresis under nonreducing conditions, the nonreduced GVP 6 complex, as well as a 125K polypeptide, which was chased into the nonreduced GVP 6 complex, were identified (lanes A and C). The presence of endo H caused a slight increase in the migration rate of the nonreduced GVP 6 complex. In addition, instead of its precursor, a new polypeptide with an apparent molecular weight of approximately 105,000 was identified (lanes B and D). These results demonstrated that (i) GVP 16, pGVP 6, pGVP 11a, and nonreduced pGVP 6 have N-linked oligosaccharides of the high-mannose type; (ii) GVP 6 and GVP 11a lack the high-mannose-type sugars and probably have oligosaccharides of the complex type; (iii) the nonreduced GVP 6 complex probably has oligosaccharides of the complex and high-mannose types; (iv) the 105K polypeptide is probably the nonglycosylated precursor to GVP 6/11a/16, as well as to the nonreduced GVP 6 complex; and (v) the cotranslational addition of sugars (20, 51) by N linkage converts the 105K polypeptide to a partially glycosylated intermediate with an apparent molecular weight of 117,000 and 125,000 under reducing and nonreducing conditions, respectively.

Monoclonal antibody 3D9T precipitated a 100K polypeptide (pGVP 7), which was chased into GVP 7 (108K) (lanes I and K). When endo H was present, GVP 7 showed a slight increase in electrophoretic mobility (lane L), indicating that it has oligosaccharides of the complex and high-mannose types. In addition, instead of pGVP 7, a new polypeptide with an apparent molecular weight of 90,000 was detected (lane J), which is probably the nonglycosylated precursor to GVP 7.

Monoclonal antibody 1D6 precipitated GVP 9 (91K), as well as a 69K polypeptide (pGVP 9), which was chased into GVP 9 (lanes M and O). GVP 9 was not affected by the action of endo H (lane P), suggesting that it lacks high-mannose-type sugars and probably has oligosaccharides of the complex type. However, pGVP 9 was not detectable in the presence of endo H, although a new polypeptide with an apparent molecular weight of 61,000 was identified (lane N). This 61K polypeptide is presumably the nonglycosylated precursor to GVP 9.

Monoclonal antibody 3D9S precipitated a 63K polypeptide (pGVP 11b), which was chased into GVP 11b (71K) (lanes Q and S). GVP 11b was not affected by treatment with endo H (lane T), indicating that it only has complex-type sugars. In contrast, pGVP 11b was not found in the presence of endo H. Instead, a 58K polypeptide was detected (lane R), which is the nonglycosylated precursor to GVP 11b.

Thus, in this experiment the nonglycosylated analogs or primary precursors, as well as the high-mannose intermediates, to the four BHV-1 glycoproteins or glycoprotein complexes were identified. In addition, GVP 6/11a/16 and the nonreduced GVP 6 complex appeared to be derived from the same primary precursor and consequently from the same gene product.

DISCUSSION

Recently, we produced a series of monoclonal antibodies to BHV-1, which allowed us to start investigating unique identity and interrelationships among several major BHV-1 glycoproteins (49). The purpose of this study was to continue these investigations and to extend our knowledge of these glycoproteins by studying their synthesis and processing.

When the monoclonal antibodies were characterized with respect to their antigen specificities, four unique reaction patterns were observed. One group of monoclonal antibodies reacted with GVP 6 (130K), GVP 11a (74K), and GVP 16 (55K), a second group of monoclonal antibodies precipitated GVP 3 (180K) and GVP 9 (91K), and one monoclonal antibody recognized GVP 11b (71K).

Because one set of monoclonal antibodies reacted with GVP 11a as part of a complex, whereas another monoclonal antibody recognized only GVP 11b, the possibility existed that there may be two unique GVP 11 species. These two species appeared to have very similar, although not identical, migration characteristics in polyacrylamide gels and thus were initially defined as one species, GVP 11 (25). Based on the apparent absence of antigenic relationship between these two glycoproteins, we introduced the names GVP 11a and GVP 11b for the 74K and 71K species, respectively. Further evidence for the absence of any antigenic relationship between the two GVP 11 species was obtained by cross-immunoprecipitation studies. Finally, there was no structural relationship between these glycopro-

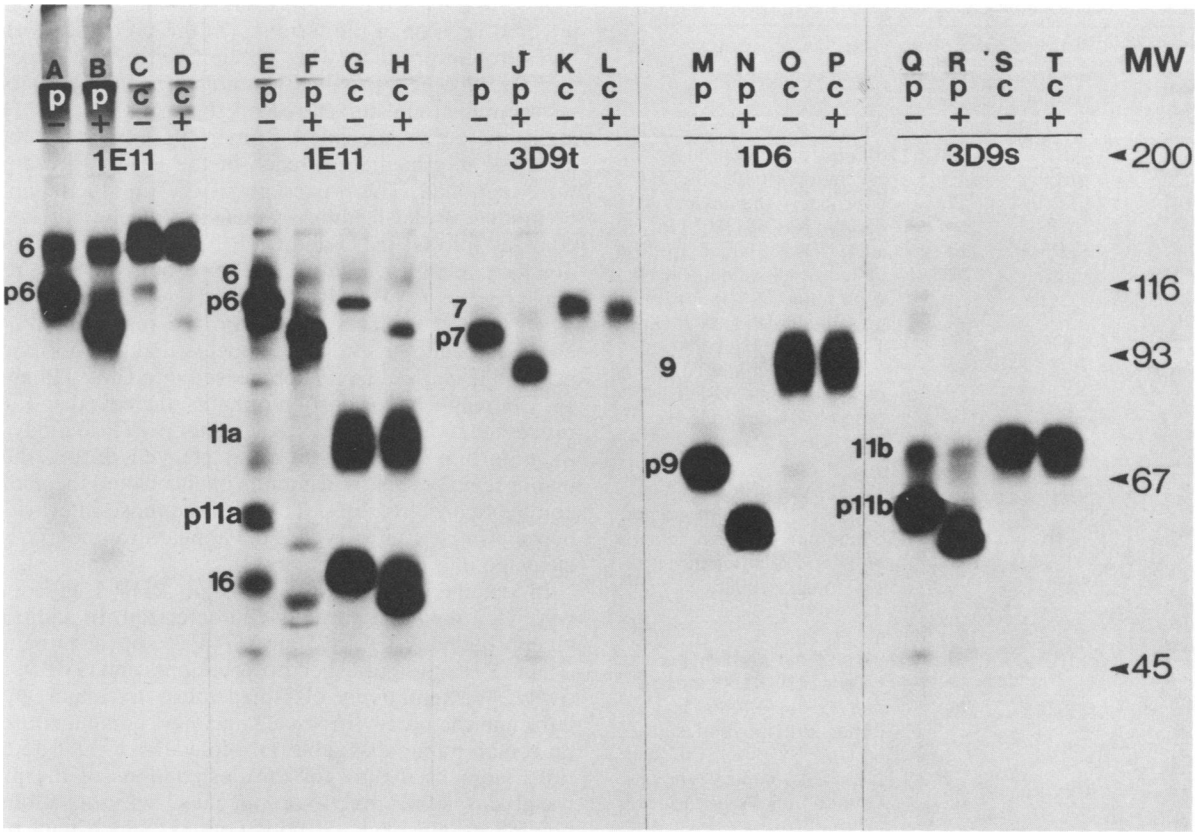

FIG. 8. Endo H treatment of BHV-1 glycoproteins. BHV-1-infected cells were pulse-labeled with L-[^{35}S]methionine for 15 min and harvested immediately (p) (lanes A, B, E, F, I, J, M, N, Q, and R) or chased for 2 h (c) (lanes C, D, G, H, K, L, O, P, S, and T). Cell lysates were prepared and precipitated with monoclonal antibodies 1E11 (lanes A to H), 3D9T (lanes I to L), 1D6 (lanes M to P), and 3D9S (lanes Q to T). One half of the samples was treated with 60 mU of endo H per ml (+) (lanes B, D, F, H, J, L, N, P, R, and T) and the other half served as controls (−) (lanes A, C, E, G, I, K, M, O, Q, and S). The precipitates were analyzed by electrophoresis in 7.5% polyacrylamide gels under reducing (lanes E to T) or nonreducing (lanes A to D) conditions. The precipitated polypeptides are identified in the margins. Molecular weight markers (10^3) are shown in the right margin.

teins, since the peptide map of GVP 11b did not show any similarity to those of GVP 11a, GVP 6, or GVP 16, proving that GVP 11a and GVP 11b arise from two separate gene products and, thus, are two unique glycoproteins.

Previous studies demonstrated that monoclonal antibodies directed against herpes simplex virus types 1 and 2 (2, 3, 32, 34, 54), human cytomegalovirus (33), Epstein-Barr virus (46), varicella-zoster virus (15, 27), and pseudorabiesvirus (18) can precipitate several polypeptides with different electrophoretic mobilities. Because a similar phenomenon occurred with BHV-1, several approaches were used to analyze the relationships among those BHV-1 glycoproteins that appeared to be complexed.

The ratio of the respective molecular weights of GVP 3 (180,000) and GVP 9 (91,000) indicated a dimer-monomer relationship between these two glycoproteins. This hypothesis was supported by the observation that even after treatment with 2-mercaptoethanol and SDS, GVP 3 and GVP 9 were both recognized by a series of different monoclonal antibodies, showing that GVP 3 and GVP 9 share several antigenic determinants. More importantly, the peptide maps of GVP 3 and GVP 9 were identical, which is consistent with the concept that GVP 3 is a dimeric form of GVP 9. Similar observations were reported for the herpes simplex virus glycoproteins gB, gC, and gD, which were shown to exist in disulfide-linked multimeric forms (6, 9, 38).

The relationships among GVP 6, GVP 11a, and GVP 16 appeared to be more complex. Analysis of the immunoprecipitates by electrophoresis under nonreducing conditions showed one 148K nonreduced GVP 6 complex instead of the individual glycoproteins GVP 6, GVP 11a, and GVP 16, indicating that these glycoproteins are linked by disulfide bridges. GVP 6 and GVP 11a as well as GVP 6 and GVP 16 showed extensive homologies. Even after the GVP 6/11a/16 complex was disrupted by treatment with 2-mercaptoethanol and SDS, a number of different monoclonal antibodies reacted with GVP 6 and GVP 11a or GVP 6 and GVP 16, indicating that GVP 6 and GVP 11a as well as GVP 6 and GVP 16 are antigenically related. GVP 6 and GVP 11a as well as GVP 6 and GVP 16 also appeared to be structurally related, as shown by partial peptide mapping. In addition, the combined peptides that were generated by cleavage of GVP 6, GVP 11a, and GVP 16 corresponded to those present in the nonreduced GVP 6 complex. The ratio of the respective molecular weights of GVP 6, GVP 11a, and GVP 16 suggested that GVP 11a and GVP 16 may represent a cleaved form of GVP 6, linked by disulfide bonds. This implies that the nonreduced GVP 6 complex may consist of two forms, one in which GVP 6 is uncleaved and the other one in which GVP 6 is cleaved into GVP 11a and GVP 16. This hypothesis is supported by the identification of a common 105K primary precursor to the nonreduced GVP 6 complex and GVP

TABLE 1. Tentative classification of BHV-1 glycoproteins

Glycoprotein and precursor		Apparent mol wt (10^3)[a]	Properties[b]
Previous nomenclature[c]	Proposed nomenclature[d]		
GVP 6	gIa	130	Derived from one primary precursor, pI(105). GVP 6 is probably the uncleaved counterpart of GVP 11a and GVP 16, which are linked by disulfide bridges. Also found as a multimer, previously designated GVP 1 (mol wt, >330,000) (25). Contains N-linked complex- and high-mannose-type oligosaccharides.
GVP 11a	gIb	74	
GVP 16	gIc	55	
pGVP 6	pgIa	117	
pGVP 11a	pgIb	62	
GVP 7	gII	108	Derived from primary precursor pII(90). A monomer containing N-linked complex- and high-mannose-type oligosaccharides.
pGVP 7	pgII	100	
GVP 9	gIII	91	Derived from primary precursor pIII(61). A monomer, but also found as a dimer, previously designated GVP 3 (180K). Contains N-linked complex-type and O-linked oligosaccharides.
pGVP 9	pgIII	69	
GVP 11b	gIV	71	Derived from primary precursor pIV(58). A monomer containing N-linked complex-type and O-linked oligosaccharides.
pGVP 11b	pgIV	63	

[a] The molecular weight estimations for the fully glycosylated products and the partially glycosylated intermediates are approximate because of anomalous SDS binding by glycosylated polypeptides (11).

[b] The presence of N- and O-linked oligosaccharide side chains on the BHV-1 glycoproteins was determined in previous studies by using the inhibitor tunicamycin (48).

[c] The previous nomenclature was introduced by Misra et al. (25) and modified by van Drunen Littel-van den Hurk et al. (49) and van Drunen Littel-van den Hurk and Babiuk (48).

[d] Those glycoproteins of BHV-1 that were identified by monoclonal antibodies were classified into four unrelated groups.

6/11a/16. The reason for the difference in the apparent molecular weights of the partially glycosylated intermediate and the fully glycosylated product under reducing and nonreducing conditions is presently unclear. It may be due to anomalous SDS binding by the glycosylated polypeptides (11), which may influence their electrophoretic mobility more strongly under nonreducing than under reducing conditions. This is currently being investigated. Although disulfide-linked glycoproteins have been recognized as biologically important constituents of enveloped RNA viruses (19, 22, 31, 39), among the herpesviruses, only pseudorabiesvirus (17, 18) and varicella-zoster virus (16) appear to have interchain disulfide linkages between major viral glycoproteins.

Several polypeptides with lower apparent molecular weights coprecipitated with the major BHV-1 glycoproteins from virus-infected cell lysates. The identification of these polypeptides as precursors was based on several observations: (i) they were not present on the surface of virus-infected cells or in the virions; (ii) the precursor forms and glycoprotein products were precipitated by the same monoclonal antibodies, proving the antigenic relatedness of these polypeptides; and (iii) based on the effect of endo H treatment, the nonglycosylated, primary precursor and the high-mannose intermediate to each of the BHV-1 glycoproteins were identified. The processing of GVP 6/11a/16 appears to be unique in that it involves cleavage of one portion of the complex into GVP 11a and GVP 16, which remain linked by disulfide bridges, whereas the other portion remains uncleaved as GVP 6. It is presently not known where and when cleavage occurs. Based on the results of the pulse-chase and endo H studies, it appears that a major portion of pGVP 6 is cleaved as it is processed into GVP 11a and GVP 16, whereas a minor portion remains uncleaved and, thus, is processed to GVP 6. The presence of pGVP 11a may be due to a portion of pGVP 6 being cleaved during the high-mannose stage, before trimming of sugars and the addition of complex carbohydrates. The high-mannose intermediate to herpes simplex virus type 2 gG appears to undergo peptide cleavage in a similar fashion (3).

In conclusion, six unique major BHV-1 glycoproteins were identified and partially characterized. In addition, two glycoproteins, GVP 1 (25) and GVP 3, appear to be multimers of GVP 6 (unpublished observations) and GVP 9, respectively. We tentatively classified these six major glycoproteins and the precursors by using a new nomenclature based on roman numerals (Table 1). Molecular weight determinations were used only for the designations of the primary, nonglycosylated precursors because of anomalous SDS binding by glycosylated polypeptides, which may result in inaccurate molecular weight values. Although a similar nomenclature was proposed recently for the glycoproteins of pseudorabiesvirus (18), any functional or serological analogies between the glycoproteins of pseudorabiesvirus and BHV-1 remain to be identified.

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