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Characterization of Envelope Proteins of Infectious Bovine Rhinotracheitis Virus (Bovine Herpesvirus 1) by Biochemical and Immunological Methods

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Ten glycoproteins of molecular weights of 180,000, 150,000, 130,000, 115,000, 97,000, 77,000, 74,000, 64,000, 55,000, and 45,000 (designated as 180K, 150K, etc.) and a single nonglycosylated 107,000-molecular-weight (107K) protein were quantitatively removed from purified bovine herpesvirus 1 (BHV-1) virions by detergent treatment. Immunoprecipitations with monospecific and monoclonal antibodies showed that three sets of coprecipitating glycoproteins, 180K/97K, 150K/77K, and 130K/74K/55K, were the major components of the BHV-1 envelope. These glycoproteins were present in the envelope of the virion and on the surface of BHV-1-infected cells and reacted with neutralizing monoclonal and monospecific antibodies. Antibodies to 150K/77K protein had the largest proportion of virus-neutralizing antibodies, followed by antibodies to 180K/97K protein. Monoclonal antibodies to 130K/74K/55K protein were neutralizing but only in the presence of complement; however, monospecific antisera produced with 55K protein did not have neutralizing activity. Analysis under nonreducing conditions showed that the 74K and 55K proteins interact through disulfide bonds to form the 130K molecule. Partial proteolysis studies showed that the 180K protein was a dimeric form of the 97K protein and that the 150K protein was a dimer of the 77K protein, but these dimers were not linked by disulfide bonds. The 107K protein was not glycosylated and induced antibodies that did not neutralize BHV-1. The 64K protein was not precipitated by anti-BHV-1 convalescent antisera, and monospecific antisera to this protein precipitated several polypeptides from uninfected cell lysates, suggesting that 64K is a protein of cellular origin associated with the BHV-1 virion envelope.

Bovine herpesvirus 1 (BHV-1), the causal agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis, is a member of the *alphaherpesvirinae* subfamily. It is an important agent of bovine respiratory disease, either directly (9) or as a predisposing factor for bacterial disease (26).

Up to 33 structural polypeptides, 11 of which are glycosylated, have been described for BHV-1 (22). The glycoproteins in the viral envelope are pivotal in the host-virus relationship since they are involved in the recognition, attachment, and penetration of herpesviruses into susceptible cells (18), in viral neutralization (10, 20), and in the immune destruction of infected cells (1, 3, 23).

BHV-1 glycoproteins have been reported to induce virus-neutralizing antibodies (6, 20, 24). The association of two glycoproteins with the BHV-1 envelope has been described previously (2). However, data from immunological and biophysical approaches to understanding the pathobiology of BHV-1 have not been correlated to date. The objective of this communication was to define the proteins associated with the BHV-1 envelope, describe their interactions, and correlate these data with immunological properties as determined by reaction with monospecific and monoclonal antibodies.

MATERIALS AND METHODS

Cells and viruses. Madin-Darby bovine kidney (MDBK) cells (ATCC CCL22) were grown in minimum essential medium (MEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% horse serum or fetal bovine serum (Hyclone Laboratories). The Cooper-1 (Colorado-1) strain of BHV-1 obtained from American Type Culture Collection (Rockville, Md.) at passage 10 in bovine kidney cells was plaqued purified once in MDBK cells, and a master stock was prepared. All the experiments were performed with virus at passages 14 to 16.

Virus purification. MDBK cells infected with 0.1 PFU of BHV-1 per cell were incubated at 37°C until 90 to 100% cytopathic effect was observed. The supernatant was cleared of cell debris at 10,000 × *g* for 10 min and frozen at −70°C until used. Virions were purified by ultracentrifugation as described by Misra et al. (22) with the exception that virus was pelleted through a 2- to 3-ml cushion of 40% sucrose in phosphate-buffered saline (PBS) (pH 7.2) before layering onto 20 to 50% potassium tartrate gradients.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the procedure of Laemmli (17). A 5% acrylamide stacking and a 7.5% acrylamide resolving slab gel were routinely used, unless otherwise indicated. All samples were prepared for electrophoresis by suspension in SDS-PAGE sample buffer containing 2% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.0001% bromophenol blue in 62.5 mM Tris hydrochloride. Samples were boiled for 3 to 5 min in this buffer before electrophoresis. When radiolabeled samples were used, the slab gels were dried and autoradiographed by standard methods (4).

Iodination of proteins. Iodination of cell surface proteins and virion proteins was done with 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, Ill.), using the method of Fraker and Speck (8) as described by Markwell and Fox (21). Briefly, purified virions suspended in PBS at 1 mg of protein per ml were incubated for 15 min at 25°C in Iodogen-coated glass vials with 200 μCi

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of $Na^{125}I$ (New England Nuclear Corp., Boston, Mass.). Labeled virus was pelleted through 40% sucrose, suspended in PBS, counted, and stored at −70°C until used. To label cell surface proteins, MDBK monolayers inoculated with 1 PFU of BHV-1 per cell or mock infected were incubated for 12 to 16 h at 37°C, washed once with MEM, and covered with 0.3 ml of MEM containing 200 μCi of $Na^{125}I$, and an Iodogen-coated cover slip was floated on top. After a 15-min incubation at 25°C, 1 ml of MEM containing 25 mM NaI was added to each well, and the cover slip was removed. Monolayers were washed with NET (0.15 M NaCl, 0.005 M EDTA, 0.05 M Tris [pH 7.5], 0.2 mM phenylmethylsulfonyl fluoride) and lysed with NET containing 0.5% Nonidet P-40 (NP-40) and 0.5% sodium deoxycholate (DOC) for 15 min on ice. Cell lysates were centrifuged at 10,000 × *g* for 10 min, followed by centrifugation at 100,000 × *g* for 60 min. Lysates were frozen at −70°C until used. Labeled proteins were analyzed by SDS-PAGE and autoradiography.

Intrinsic labeling. MDBK cells were infected with BHV-1 (10 PFU per cell) and incubated for 6 h at 37°C in MEM with 2% fetal bovine serum or horse serum. Infected- and control-cell monolayers to be labeled with [^{35}S]methionine were washed twice with methionine-free MEM, and methionine-free medium containing 25 μCi of [^{35}S]methionine (New England Nuclear Corp.) per ml was added. For [^{3}H]glucosamine labeling, cells were incubated in MEM containing 2% fetal bovine serum and 10 μCi of [^{3}H]glucosamine (New England Nuclear Corp.) per ml. After 24 h of incubation, virus was purified from supernatants. Cell monolayers were washed twice with PBS and disrupted with 0.5% NP-40–0.5% DOC in NET buffer. Cell lysates were cleared of cell debris at 10,000 × *g* for 20 min and cleared of subcellular and viral particles at 100,000 × *g* for 1 h (cleared lysates). Purified virions and cell lysates were stored at −70°C until used.

Preparation of viral envelopes. Viral envelopes were prepared basically as described by Vernon et al. (25). Potassium tartrate-purified virions were treated with 0.5% NP-40–0.5% DOC in NET buffer for 15 min at 4°C. Detergent-treated virions were layered onto a 40% sucrose cushion and centrifuged at 100,000 × *g* for 1 h. Solubilized envelope proteins were carefully removed from the top of the sucrose cushion and suspended in SDS-PAGE sample buffer. The pellet containing the nucleocapsids was similarly treated. Samples containing radioactivity were analyzed by SDS-PAGE and visualized by autoradiography.

Immunoprecipitation. The procedure used for immunoprecipitation was a modification of that described by Kessler (15). Formalin-fixed *Staphylococcus aureus* Cowan I (Staph-A) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was cleaned by boiling in PBS containing 10% 2-mercaptoethanol and 3% SDS for 5 min and washed once with washing buffer (NET, 0.5% NP-40) and once with suspension buffer (NET, 0.05% NP-40, 2% bovine serum albumin). Cleaned Staph-A was then incubated with rabbit antiserum for 30 min on ice and washed with suspension buffer. When rabbit anti-BHV-1 sera were used, the antibody-coated Staph-A was directly resuspended in either cleared lysates or in purified envelope proteins and incubated on ice for 1 h with occasional shaking. When monoclonal antibodies or bovine antisera were used, they were incubated on ice for 30 min with Staph-A coated with the respective rabbit antispecies antibody. Then the double-antibody-coated Staph-A was incubated with the lysates or envelopes. All the antigen preparations were preincubated with either Staph-A or rabbit antispecies-coated Staph-A, or with both, for 30 min to minimize nonspecific reactivity. After incubation with antigen, the Staph-A-antibody-antigen complexes were washed five times with buffer (NET, 0.05% NP-40), suspended in PAGE sample preparation buffer, and boiled for 3 to 5 min. When nonreducing conditions were desired, the 2-mercaptoethanol was omitted from the PAGE sample preparation buffer. Preparations were centrifuged, and the supernatants were analyzed by SDS-PAGE and autoradiography.

Preparation of monospecific antisera. Specific antisera to individual BHV-1 envelope proteins were produced by a procedure similar to that used by Eberle and Courtney (7). Viral envelope fractions prepared as described above, at a protein concentration of 1 mg/ml, were analyzed on SDS-PAGE gels and stained with Coomassie brilliant blue. One lane in the same gel was loaded with similarly prepared [^{35}S]methionine-labeled viral envelope proteins to serve as markers. Stained gels were soaked overnight in 10% methanol to remove the acetic acid and SDS. Protein bands corresponding to the [^{35}S]methionine labeled envelope proteins at 97,000, 77,000, 64,000, 55,000, and 45,000 molecular weight (MW) (97K protein, 77K protein, etc.) were cut out and injected subcutaneously in adult New Zealand White rabbits. Rabbits were given booster injections at least twice at weekly intervals. Serum samples were tested for virus neutralization and immunoprecipitation.

Production of hybridomas. Live BHV-1 (2×10^8 50% tissue culture infective doses [$TCID_{50}$] per ml) purified by sedimentation through a 40% sucrose cushion was mixed with an equal volume of complete Freund adjuvant and injected intraperitoneally (0.7 ml) into BALB/c mice. Neutralization-positive mice were boosted with an intravenous injection of 0.2 ml of the sucrose-purified virus 3 days before fusion of spleen cells with P3x63-Ag8.653 myeloma cells (ATCC CRL 1580) (13). Fusions were performed by a modification of the method of Kohler and Milstein (16) as described by Letchworth and Appleton (G. J. Letchworth and J. Appleton, U.S. Department of Agriculture Handbook no. 630, 1984). Hybridomas were screened for activity against whole virus in an enzyme-linked immunosorbent assay (ELISA), and positive hybridomas were expanded and frozen in liquid nitrogen. Hybridomas were cloned by limiting dilution.

ELISA. The labeled avidin-biotin ELISA technique was used. All ELISA reagents were purchased from Cappel Laboratories (Cochranville, Pa.) and used according to the instructions of the manufacturer, which are a modification of the method of Kendall et al. (14). In brief, polystyrene microELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated at 4°C overnight with sucrose-purified infectious bovine rhinotracheitis virus (500 ng of protein per well) in carbonate-bicarbonate buffer, pH 9.6. Negative control plates were coated with uninfected MDBK cell lysate. Remaining binding sites were blocked with 1% gelatin at 37°C, and wells were washed three times with PBS containing 0.5% Tween 20 and 0.5% gelatin. Hybridoma supernatants (100 μl) were added and incubated at 30°C for 1 h. The wells were again washed three times, and 100 μl of a 1:2,000 dilution of biotinylated sheep anti-mouse immunoglobulin G was added. After incubation for 2 h at 30°C, the wells were again washed three times with PBS-Tween. Avidin-peroxidase (100 μl) at a 1:20,000 dilution was then added at 30°C for another 2 h. Wells were washed five times with PBS-Tween, and 100 μl of citrate-phosphate buffer (pH 5.0) containing *o*-phenylenediamine (0.4 mg/ml) and 0.01% H_2O_2 was added. The reaction was terminated with 100 μl of

2.5 M H_2SO_4 per well when nonspecific reactions were first noted in negative control wells. The ELISAs were read visually or with a microplate reader at 490 nm.

Neutralization tests. Neutralization tests were carried out by standard methods with 100 and 1,000 $TCID_{50}$ of BHV-1. Monoclonal antibodies and monospecific antisera were tested for their ability to participate in complement-enhanced neutralization with rabbit complement at a dilution giving maximum enhancement of neutralization without cytotoxicity. In no case did complement or inactivated negative control serum neutralize BHV-1 in the absence of BHV-1-specific antibody. Virus and antibody were incubated at 37°C for 1 h before cells were added. Neutralization tests were read daily after the appearance of cytopathic effect.

Peptide mapping by limited proteolysis. ^{125}I-labeled BHV-1 envelope proteins were immunoprecipitated with monoclonal antibody 110604 (group 1), 180804 (group 2), or 510604 (group 3) (see Fig. 4 and 5) and separated by preparative 7.5% PAGE under nonreducing conditions. Individual protein bands were located in the gel by autoradiography and were cut out, placed into 15% polyacrylamide gels, digested with V-8 protease, and analyzed as described by Cleveland et al. (5).

RESULTS

Preparation of envelope proteins. We used NP-40, a nonionic detergent, and DOC, a slightly anionic detergent, to solubilize and remove the envelopes from potassium tartrate-gradient-purified BHV-1 virions. Viral nucleocapsids remained intact and most retained their tegument as demonstrated by electron microscopy and by the inability of this treatment to release radioactivity from nucleocapsids containing [^{3}H]thymidine-labeled DNA (data not shown).

Eleven proteins were removed by detergent treatment from [^{35}S]methionine-labeled virions. The MWs of these proteins estimated from multiple SDS-PAGE gels were 180,000, 150,000, 130,000, 115,000, 107,000, 97,000, 77,000, 74,000, 64,000, 55,000, and 45,000 (Fig. 1, lanes 1 to 3; Table 1). The 107K protein was present in large amounts in both envelope and nucleocapsid fractions. The 180K and 150K bands were faint and were difficult to visualize in some gels. When [^{3}H]glucosamine-labeled virions were treated in the same fashion, the removed proteins corresponded in MW with those removed from [^{35}S]methionine-labeled virions, except for the 107K protein which did not incorporate [^{3}H]glucosamine (Fig. 1, lanes 4 to 7). No detectable [^{3}H]glucosamine-labeled proteins remained associated with the nucleocapsid after detergent treatment (Fig. 1, lane 5). Conversely, no envelope proteins were detected in the envelope fractions in the absence of detergent treatment (Fig. 1, lane 6). This indicates that all the viral glycoproteins were in the envelope and were quantitatively removed from the virion by detergent treatment.

Surface labeling of virus-infected cells. Iodination with diphenylglycoluril was used to identify viral proteins exposed on the membranes of infected cells. Proteins of approximately 92,000, 77,000, 74,000, and 55,000 MW were heavily labeled (Fig. 2, lane 4; Table 1). Their viral origin is supported by their absence from membranes of uninfected cells (Fig. 2, lane 5), comigration with proteins iodinated on purified virions (Fig. 2, lanes 2 and 3), and immunoprecipitation from both virions and infected but not uninfected cells by antiviral monoclonal antibodies (see Fig. 5). Some proteins not demonstrated on infected-cell membranes were labeled on virions and either were not removed by detergent

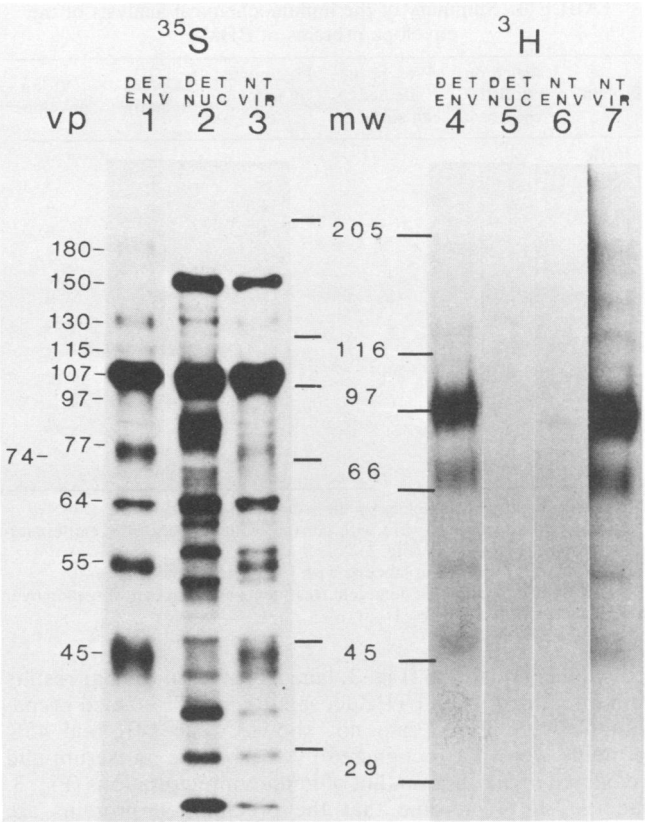

FIG. 1. NP-40/DOC detergent treatment (DET) was used to dissociate radiolabeled whole virions into envelope (ENV) and nucleocapsid (NUC) fractions. An autoradiograph is shown. Envelope proteins were found on top of the 40% sucrose cushion after detergent treatment and ultracentrifugation. Nucleocapsids and whole virions (VIR) pelleted through the cushion. Envelope proteins are shown in lanes 1 and 4. Nucleocapsid proteins are shown in lane 2. No [^{3}H]glucosamine-labeled proteins were found with the nucleocapsids (lane 5). Whole virions that were not detergent treated (NT) pelleted through 40% sucrose and are shown in lanes 3 and 7. No proteins were solubilized from whole virions and found in the envelope fraction in the absence of detergent treatment of [^{3}H]glucosamine-labeled or [^{35}S]methionine-labeled (data not shown) virus (lane 6). Numbers show viral proteins (vp) or marker protein MW ($\times 10^3$).

extraction (48K, 42K, and 33K proteins), suggesting that they were capsid proteins exposed by degradation of virion envelopes, or were removed by detergent treatment (107K, 64K, and 45K proteins), suggesting an undefined association with the virion membrane. Proteins of 180,000, 150,000, and 130,000 MW also were labeled on infected cells and virions and were removed from virions by detergent treatment, but were present in very small amounts difficult to photograph in autoradiograms. The 150K protein was glycosylated (data not shown) and thus apparently not identical to the 150K nonglycosylated protein described by Misra et al. (22).

Immune reactions of the envelope proteins with bovine convalescent antisera. To show that the proteins identified by the different isotopes were of viral origin and were recognized by the in vivo bovine immune response, purified viral envelope preparations radiolabeled with [^{35}S]methionine were immunoprecipitated with bovine BHV-1-specific antiserum. Eight proteins (150K, 130K, 115K, 107K, 97K, 77K, 74K, and 55K) were precipitated by the bovine convalescent

TABLE 1. Summary of the immunochemical analysis of the envelope proteins of BHV-1

Protein	Labeled on purified virions[a]	Present on infected-cell surface[a]	Precipitated by immune sera[b]	Glycosylation[c]	Virion envelope[d]
180K	+			+	+
150K	+		+	+	+
130K	+		+	+	+
115K			+	+	+
107K	+		+		±
97K	+	+	+	+	+
77K	+	+	+	+	+
74K	+	+	+	+	+
64K	+			+	±
55K	+	+	+	+	+
48K	+				
45K	±			+	±
42K	+				
33K	+				

[a] Determined by ^{125}I labeling by the iodogen method (Fig. 2).
[b] Immunoprecipitations were with convalescent antisera from cattle naturally infected with BHV-1 (Fig. 3).
[c] Determined by intrinsic labeling with [^{3}H]glucosamine (Fig. 4).
[d] Determined by nonionic detergent treatments and ultracentrifugation over a 40% sucrose cushion (Fig. 1).

polyclonal antiserum (Fig. 3, lane 2; Table 1). Similar results were obtained when [^{3}H]glucosamine- or ^{125}I-labeled preparations were used (data not shown). The 64K and 45K proteins were not recognized by the bovine antiserum and remained in the supernatant of immunoprecipitations (Fig. 3, lane 3). It is possible that the precipitated proteins are coprecipitating rather than precipitating individually. However, when envelope proteins were precipitated with monoclonal antibodies as follows, no coprecipitation was seen.

Immunoreactivity of envelope proteins with monospecific and monoclonal antibodies. Rabbit monospecific antisera against the 97K, 77K, 64K, and 55K polypeptides and 41 ELISA-positive monoclonal antibodies were chosen for this study. Their antigenic specificity was determined by immunoprecipitation and SDS-PAGE analysis of [^{35}S]methionine-, [^{3}H]glucosamine-, and ^{125}I-labeled antigens.

The monoclonal and monospecific antibodies precipitated antigens in four distinct patterns which were similar when [^{35}S]methionine-, [^{3}H]glucosamine-, or ^{125}I-labeled antigens were used (Fig. 4 and 5). All antibodies tested were placed onto one of these four groups based solely on antigenic specificity. The first group (group 1) of 1 monospecific and 14 monoclonal antibodies precipitated the 77K glycoprotein (Fig. 4, lanes 4 and 9). A faint 150K glycoprotein band could occasionally be seen in immunoprecipitations with ^{3}H- or ^{35}S-labeled envelopes, but not reproducibly. This 150K glycoprotein was apparent in immunoprecipitations with reduced amounts of NP-40 and ^{125}I-labeled proteins (Fig. 6, lane 3). Two additional nonspecific bands were visible in Fig. 6, lane 3, probably owing to the lower amount of detergents used in the immunoprecipitation procedure. Nine monoclonal antibodies and the monospecific antiserum from group 1 totally neutralized 1,000 $TCID_{50}$ of BHV-1 in the absence of complement. Two monoclonal antibodies delayed the appearance and spread of cytopathic effect and were considered "partially neutralizing" in the absence of complement. In the presence of complement, two monoclonal antibodies gained the ability to partially neutralize BHV-1 (Table 2).

The second group (group 2) of 11 monoclonal and 2 monospecific antibodies precipitated a glycoprotein of 97,000 MW and seemed to slightly increase the precipitation of a 107K nonglycosylated protein (Fig. 4, lanes 3 and 8; Fig. 5, lanes 5 and 6). However, this same 107K protein was precipitated nonspecifically by every antibody tested. Antibodies from this group had a range of neutralization capacities. Both monospecific antisera and a single monoclonal antibody were totally neutralizing with and without complement. Five hybridoma antibodies were partially neutralizing in the absence of complement. In the presence of complement, three additional monoclonal antibodies became totally neutralizing and one additional monoclonal antibody became partially neutralizing (Table 2). A faint 180K glycoprotein band could occasionally be seen in immunoprecipitations with ^{3}H- and ^{35}S-labeled envelopes, but not reproducibly. This 180K glycoprotein was apparent in immunoprecipitations with reduced amounts of NP-40 and ^{125}I-labeled proteins (Fig. 6, lane 2).

The third group (group 3) of 14 monoclonal antibodies precipitated a triplet of glycoproteins of 130,000, 74,000, and 55,000 MW (Fig. 4, lanes 2 and 7; Fig. 5, lanes 7 and 8). In addition to the monoclonal antibodies, two monospecific antiserums were prepared against only the 55K glycoprotein.

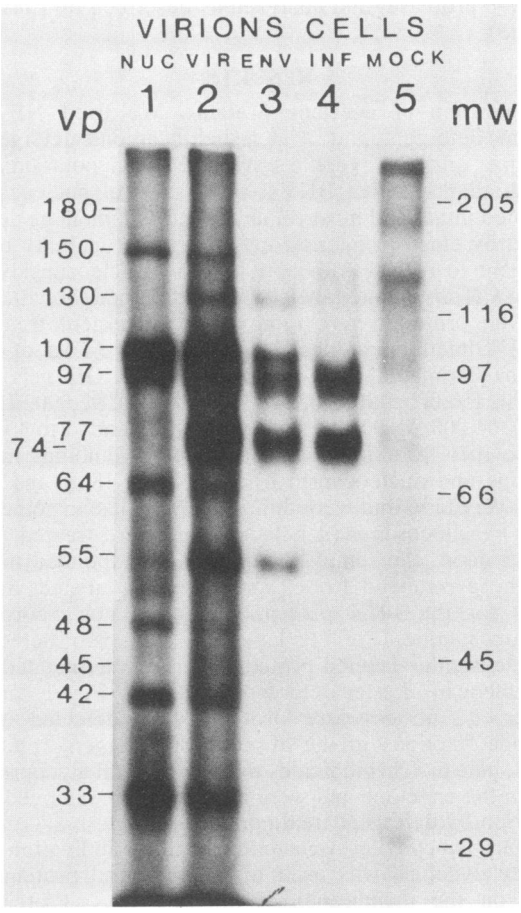

FIG. 2. Autoradiograph of the proteins on purified BHV-1 virions and BHV-1-infected or mock-infected cells ^{125}I-labeled by the Iodogen method. Labeled virions were treated with 0.5% NP-40–0.5% DOC, and the nucleocapsid (NUC) (lane 1) and envelope (ENV) (lane 3) fractions were obtained as described in the legend to Fig. 1. Labeled untreated virions (VIR) are shown in lane 2. Surface-labeled proteins from infected (INF) and mock-infected (MOCK) cells are shown in lanes 4 and 5, respectively. Numbers on left show viral proteins (vp); numbers on right show marker protein MW ($\times 10^{3}$).

These monospecific antisera also precipitated the 130K and 74K glycoproteins as well as the 55K protein. Only one monoclonal antibody from this group had partial neutralizing activity in the absence of complement. However, in the presence of complement six monoclonal antibodies were completely neutralizing, and three were partially neutralizing. The two monospecific and five remaining monoclonal antibodies were not neutralizing in the presence or absence of complement (Table 2).

The last group (group 4) of two monoclonal antibodies reproducibly increased the precipitation of the 107K nonglycosylated polypeptide (Fig. 4, lane 5). Monoclonal antibodies from this group were not neutralizing.

Monospecific antiserum to the 64K polypeptide precipitated only this polypeptide from purified envelope preparations and did not neutralize BHV-1 in the presence or absence of complement. Unlike the other monospecific antisera, it also precipitated several proteins from uninfected-cell lysates with the strongest bands at 235,000 and 59,000 MW (data not shown).

Analysis under reducing and nonreducing conditions. Several monoclonal and monospecific antibodies precipitated more than one polypeptide. Group 3 antibodies precipitated three polypeptides of 130,000, 74,000, and 55,000 MW when analyzed by SDS-PAGE under reducing conditions (Fig. 7, lane 3). This could be attributed to disulfide-bond linkages, shared antigenic determinants between the glycoproteins, or noncovalent protein-protein interactions. To study these possibilities, we analyzed purified BHV-1 envelopes and immunoprecipitations with monoclonal antibodies by SDS-PAGE in the absence of 2-mercaptoethanol. The monoclonal antibodies precipitated diminished amounts of the 130K, 74K, and 55K proteins under these conditions. In addition, a new band appeared at 145,000 MW (Fig. 7, lane 4). The purified envelopes analyzed by SDS-PAGE under reducing conditions showed strong bands at 74,000 and 55,000 MW and a weak band at 130,000 MW (Fig. 7, lane 1). The purified envelopes analyzed under nonreducing conditions showed diminished bands at 74,000 and 55,000 MW, but strong bands were observed at 130,000 and 145,000 MW (Fig. 7, lane 2). Since disulfide-bond linkages are retained under these nonreducing conditions, this indicates that the 74K and 55K glycoproteins are linked by disulfide bonds to form the 130K/145K glycoprotein(s).

The 77K/150K and the 97K/180K proteins, precipitated with group 1 and group 2 antibodies, respectively, were also analyzed under nonreducing conditions. However, no increase in the intensity of the high-MW bands was seen (data not shown).

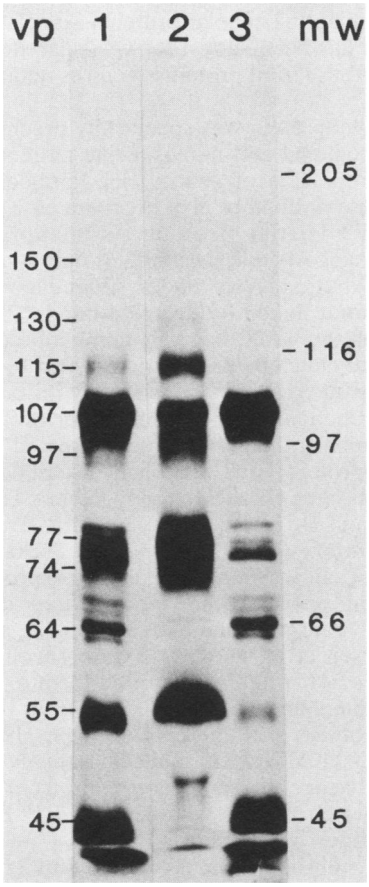

FIG. 3. Autoradiograph of [^{35}S]methionine-labeled envelope proteins precipitated by antibodies found in bovine convalescent sera. Purified envelope proteins are shown in lane 1. Proteins precipitated by bovine anti-BHV-1 sera are shown in lane 2. Proteins remaining in the supernatant after immunoprecipitation are shown in lane 3. The proteins in lane 3 that are not recognized by the bovine humoral immune response are thought to be nonessential in recovery from infection. Numbers are as defined in the legend to Fig. 2.

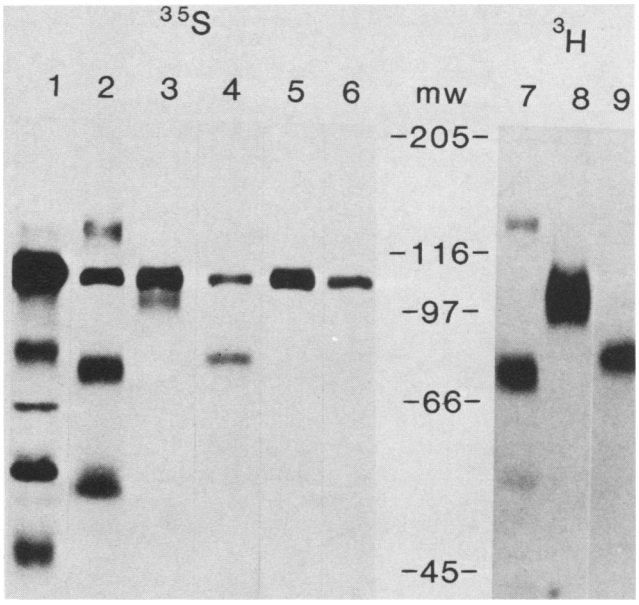

FIG. 4. Autoradiograph of immunoprecipitation of radiolabeled viral proteins with monoclonal antibodies. Lanes 1 through 6 show [^{35}S]methionine-labeled envelope proteins. Lanes 7 through 9 show [^{3}H]glucosamine-labeled envelope glycoproteins. Lane 1 shows BHV-1 envelope proteins. Lanes 2 and 7 show precipitation of 130K, 74K, and 55K envelope proteins with 510604, a group 3 monoclonal antibody. Lanes 3 and 8 show precipitation of 97K envelope protein with 180804, a group 2 monoclonal antibody. Lanes 4 and 9 show precipitations of 77K envelope protein with 110604, a group 1 monoclonal antibody. Lane 5 shows the reproducibly increased precipitation of nonglycosylated 107K protein with 102102, a group 4 monoclonal antibody. Lane 6 shows the nonspecific precipitation of the 107K protein with rabbit anti-mouse antibody-coated Staph A cells. Note that this nonglycosylated protein is nonspecifically precipitated in every lane of the ^{35}S autoradiograph. MW, $\times 10^3$.

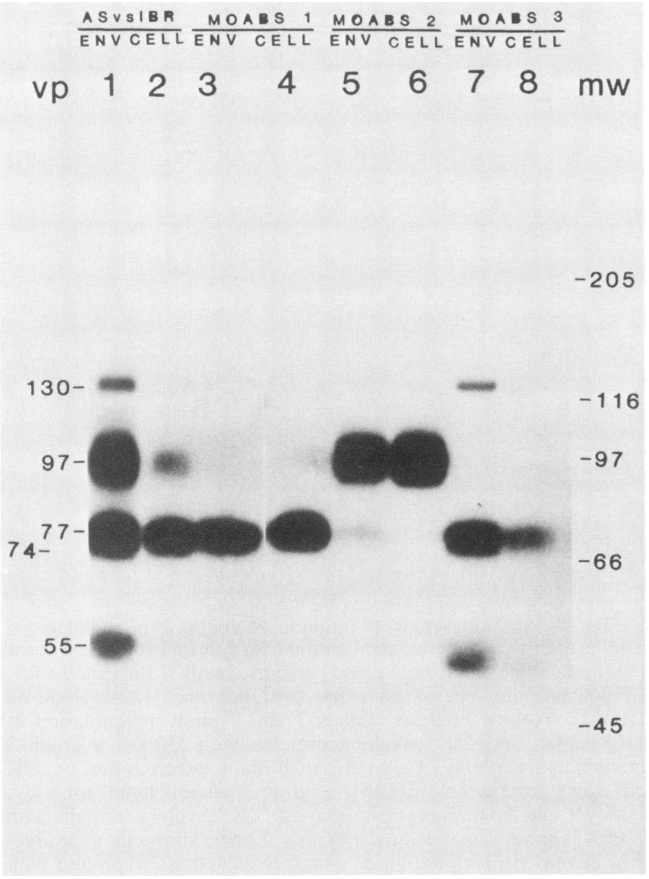

FIG. 5. Autoradiograph of the immunoprecipitation patterns of ^{125}I-surface-labeled proteins from purified virions and infected cells. Envelope proteins (ENV) from surface-labeled virions (lanes 1, 3, 5, 7) or cell lysates (CELL) of surface-labeled infected cells (lanes 2, 4, 6, and 8) were used as antigens in immunoprecipitations. Antibodies tested were anti-BHV-1 rabbit antisera (AS vs IBR; lanes 1 and 2), monoclonal antibodies 110604, a group 1 monoclonal antibody (MOABS 1; lanes 3 and 4), 180804, a group 2 monoclonal antibody (MOABS 2; lanes 5 and 6), and 510604, a group 3 monoclonal antibody (MOABS 3; lanes 7 and 8). MW, $\times 10^3$. vp, Viral proteins.

Peptide mapping by limited proteolysis. The MW of the proteins coprecipitating with monoclonal antibodies from groups 1 and 2 suggested that the high-MW proteins could be dimers of the low-MW peptides. Since no increase in the intensity of the 180K and 150K bands was observed under nonreducing conditions, we hypothesized that these dimers were held together by noncovalent interactions. Indirect support for this idea came from the fact that the 180K and 150K proteins were most evident in ^{125}I-surface-labeled virions when immunoprecipitation procedures were done in the presence of 0.05% NP-40 rather than 0.5% NP-40 (Fig. 6a, lanes 1 to 3). To test this possibility, we performed peptide mapping by limited proteolysis with V-8 protease. The 150K and 77K proteins had similar patterns (Fig. 6b, lanes 150 and 77), although the small amounts of 150K protein available precluded absolute conclusions. The 180K and 97K proteins had identical peptide maps (Fig. 6, lanes 180 and 97). The disulfide-bond-linked polypeptide was also analyzed by peptide mapping. The 130K protein was a broad band running at 124,000 to 145,000 MW in the preparative gel; therefore, the 130K and 145K proteins were not distinguishable. The pattern of this broad band was a combination of the patterns of the 74K and 55K proteins (Fig. 6, lanes 130, 74, and 55).

DISCUSSION

Our results show that 180,000-, 150,000-, 130,000-, 115,000-, 107,000-, 97,000-, 77,000-, 74,000-, and 55,000-MW viral proteins are associated with the envelope of BHV-1 as determined by their removal from virions with nonionic detergents (Table 1). Predominant molecules on the virion surface appear to be the 97K protein and its dimer, 180K, the 77K protein and its dimer, 150K, and a 130,000-MW disulfide-linked heterodimer of 74K and 55K. The 107K protein was a major component of the virion but was found with both the envelope and nucleocapsid fractions after detergent treatment. With the exception of the 107K protein, all are glycosylated, and with the exception of the 107K and 115K proteins, all appeared to be exposed on both the envelope of the virion and the surface of virus-infected cells, as demonstrated by their susceptibility to iodination. All these proteins precipitated specifically with bovine convalescent antisera and both monoclonal and monospecific antibodies. Additional glycoproteins of 64,000 and 45,000 MW were removed from the virions by detergent treatment; however, they did not precipitate with convalescent antisera, suggesting that they might be cellular proteins associated with viral envelopes, as has been shown for vesicular stomatitis virus (27). Each of these viral proteins requires additional discussion.

The 77K glycoprotein was specifically precipitated by the group of monoclonal and monospecific antibodies that had the largest proportion of neutralizing antibodies (Table 2, group 1). These antibodies also precipitated a faint band at 150,000 MW. Partial proteolysis mapping of these polypeptides suggests that the 150K protein is a dimer of the 77K protein, but analysis under nonreducing conditions indicates it is not linked by disulfide bonds. The increased appearance of the 150K band in immunoprecipitations of surface-labeled envelope preparations when the NP-40 concentration was decreased is consistent with our hypothesis that 150K is a noncovalently linked dimer of 77K.

Although monoclonal antibodies directed against this BHV-1 glycoprotein have not been described previously, this protein has been characterized by others. Lum and Reed (M. A. Lum and D. E. Reed, p. 510. *In* R. W. Loan (ed.), *Bovine Respiratory Disease Symposium*, Texas A&M University Press, College Station, Texas, 1984) produced monospecific rabbit antisera that precipitated a 77,000- to 81,000-MW glycoprotein and had neutralizing activity. Similarly, Gregersen et al. (11) made monospecific antibodies reactive with a 74K protein and found them to be neutralizing without complement. It is apparent from our work and the work of others that there is a single glycoprotein of 77,000 to 82,000 MW that induces strongly neutralizing antibodies. Absence of this glycoprotein from the list published by Misra et al. (22), who described 25 BHV-1 structural polypeptides, is puzzling.

The 97K glycoprotein was precipitated by group 2 (Table 2) antibodies. Most of the monoclonal antibodies required the presence of complement for neutralization. A 180,000- to 190,000-MW glycoprotein appeared above our 97K band in many immunoprecipitations, especially when ^{125}I-labeled antigens were used. Partial proteolysis patterns of the 97K and 180K polypeptides were similar, suggesting that 180K is a dimeric form of 97K. Although unaffected by reducing conditions, the 180K band increased in immunoprecipitations of envelope preparations from virions iodinated with

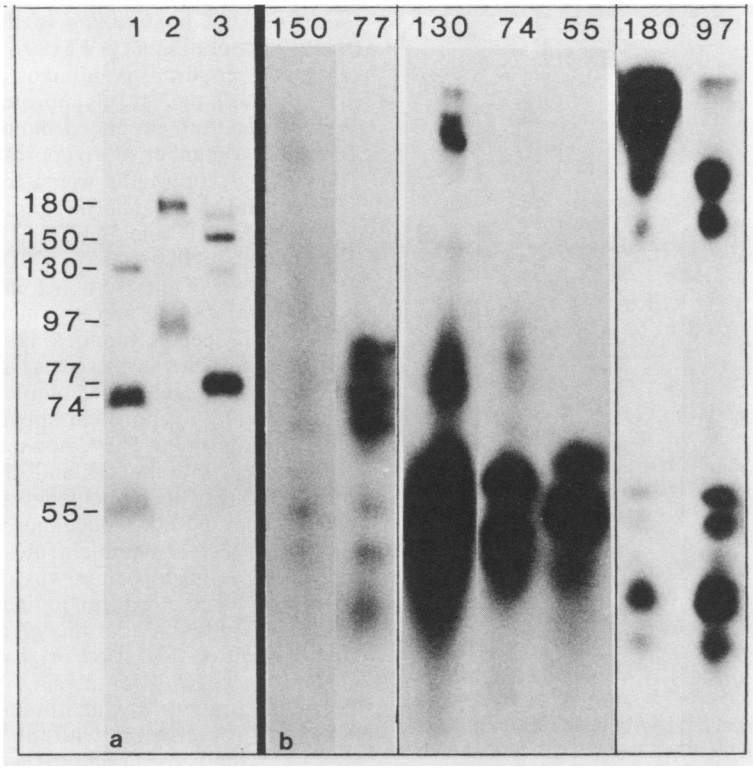

FIG. 6. Peptide mapping of ^{125}I-labeled envelope proteins by partial V-8 proteolysis in SDS-PAGE as described by Cleveland et al. (5). Envelope proteins were immunoprecipitated with monoclonal antibodies from group 1 (a, lane 3), group 2 (a, lane 2), and group 3 (a, lane 1). Individual bands of the 150K, 77K, 130K, 145K, 74K, 55K, 180K, and 97K glycoproteins were digested with V-8 protease and are shown in their respective lanes in panel b.

decreased NP-40 concentrations, suggesting that the 180K protein is a noncovalently linked dimer of the 97K protein.

It could be argued that formation of the 150K and 180K dimers was an artifact of the surface-labeling procedure. However, we could not reproduce this effect by subjecting ^{35}S-labeled virions to the same iodination procedure with nonradioactive NaI (data not shown).

The 97K glycoprotein has been described previously. Van Drunen Littel-Van Den Hurk et al. (24) described gp3/gp9 (180K/91K) that coprecipitated with monoclonal antibodies. Our data correlate well with that study, despite small disagreements in MW (gp91 versus gp97) that could be attributed to using different MWs for the proteins used as standards. However, our analysis under nonreducing conditions did not increase the intensity of the high-MW band, and we could not agree that the 180K glycoprotein was a disulfide-linked dimeric form of the 97K glycoprotein. Gregersen et al. (11) found a BHV-1 glycoprotein with an MW of 93,000 that induced neutralizing monospecific antibodies. Collins et al. (6) have also produced neutralizing monoclonal antibodies that are specific for an 82K glycoprotein, but they could not see a 180K protein by autoradiography. Two of their monoclonal antibodies have been tested in our laboratory and are identical to our 97K/180K-specific monoclonal antibodies.

A triplet of glycoproteins with MWs of 130,000, 74,000, and 55,000 was precipitated by group 3 antibodies (Table 2). Monoclonal antibodies of this group required complement for complete neutralization. Two monospecific antisera produced against the 55K protein were not neutralizing in the presence or absence of complement. Data from im-

TABLE 2. Neutralization activities of monoclonal and monospecific antibodies to BHV-1 envelope proteins[a]

Group	Protein specificity[b]	No. of monoclonal antibodies						No. of monospecific antibodies (neutralization[c])	
		Neutralization with complement			Neutralization without complement				
		Total	Partial	None	Total	Partial	None	Total	None
1	150K/77K	9	4	1	9	2	3	1	0
2	180K/97K	4	6	1	1	5	5	2	0
3	130K/74K/55K	6	3	5	0	1	13	0	2
4	107K	0	0	2	0	0	2	NA[d]	NA

[a] Tested against 100 $TCID_{50}$ of BHV-1.
[b] As determined by immunoprecipitation assay.
[c] Neutralization ability did not change in the presence or absence of complement.
[d] NA, Not available.

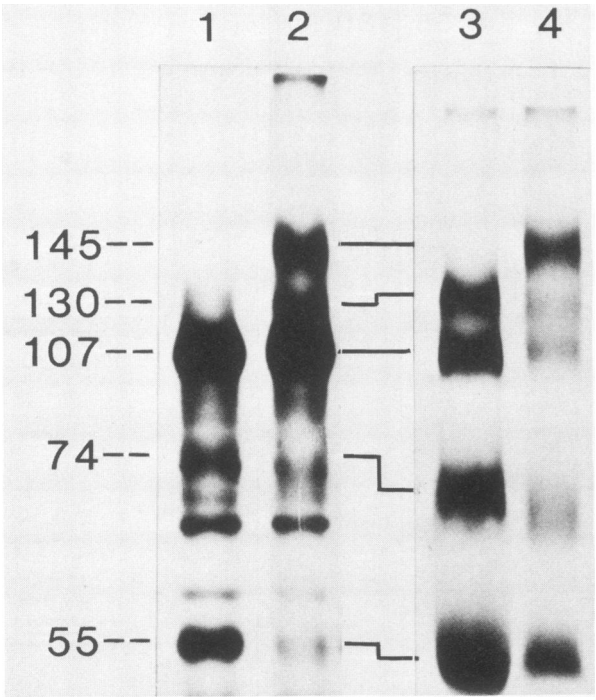

FIG. 7. SDS-PAGE analysis of [^{35}S]methionine-labeled envelope glycoproteins under reducing and nonreducing conditions. Lanes 1 and 2 are purified envelopes analyzed under reducing and nonreducing conditions, respectively. Lanes 3 and 4 are immunoprecipitations of envelope proteins with 510604, a group 3 monoclonal antibody. The immunoprecipitations were analyzed under reducing and nonreducing conditions, respectively. Numbers on left show viral proteins.

munoprecipitations analyzed under nonreducing conditions were consistent with the proposal of Van Drunen Littel-Van Den Hurk et al. that the 74K and 55K proteins are disulfide-linked subunits of the 130K protein (24). However, under nonreducing conditions, we detected an additional protein (145,000 MW). The 145K protein is most likely identical to the 130K protein but with an intact intrachain disulfide bond since the partial proteolysis pattern of 130K and 145K proteins cut from preparative gels as a single band was a combination of those from the 74K and 55K polypeptides. This suggests that 130K/145K protein is a disulfide-linked complex of 74K and 55K proteins and would explain why monospecific antiserum prepared against the 55K protein also precipitated the 74K and 130K proteins. Interestingly, recent reports describing the envelope proteins of pseudorabies virus (herpesvirus suis I), identified a complex of envelope proteins (125K, 74K, 58K) linked covalently by disulfide bridges (12, 19).

The 130K, 74K, and 55K glycoproteins also have been reported previously. Van Drunen Littel-Van Den Hurk et al. (24) produced similar monoclonal antibodies. These had neutralizing activity and an immunoprecipitation pattern identical to those of our antibodies. However, their neutralizing monoclonal antibodies were all reactive with the 74K glycoprotein in Western blots, suggesting that only this protein contains important neutralizing epitopes. Our monoclonal antibody work does not confirm their supposition that this group of glycoproteins is the most significant in neutralization or that this group of glycoproteins is most likely to contain the viral attachment site, since 14 of our 16 antibodies were not neutralizing in the absence of complement. However, preliminary Western blot analysis in our laboratory does confirm that all neutralizing monoclonals are also reactive with the 74K glycoprotein. Collins et al. (6; personal communication) produced monoclonal antibodies that precipitated a number of glycoproteins; the most predominant of these glycoproteins was a triplet with MWs of 120,000, 69.000, and 55,000. (These MWs were previously reported as 102,000, 69,000, and 55,000 [6] but have since been revised to 120,000, 69,000, and 55,000; Collins, personal communication. Two of their monoclonal antibodies were tested in our laboratory and had specificities identical to our 130K-, 74K-, 55K-specific monoclonal antibodies). Several monoclonal antibodies in this group could neutralize BHV-1 but only in the presence of complement. Gregersen et al. (11) found a 69K glycoprotein apparently corresponding to our 74K glycoprotein. They, however, could not correlate this 69K protein with the 55K and 130K proteins since they were not working with monoclonal antibodies. They found that this protein did not induce antibodies that neutralized virus in the absence of complement.

The 115K glycoprotein was removed by detergent treatment; however, it was not iodinated on the virion or infected cells. It is possible that this glycoprotein is not exposed on the surface of the virion or that it does not have tyrosine residues accessible for labeling. The fact that it was the only envelope protein to which monoclonal antibodies were not identified is consistent with the former possibility.

The nonglycosylated 107K protein appears in large amounts with both the envelope and the nucleocapsid fractions after detergent treatment. It is not labeled on the surface of infected cells, suggesting that it is not an exposed component of the viral envelope. The distribution of this protein in both the envelope and nucleocapsid fractions and its apparent absence on the surface of infected cells supports the hypothesis that it is a component of the viral tegument. Immunoprecipitation with polyclonal antiserum specific to BHV-1 did not remove all the 107K protein from the envelope preparations. This could be explained by the large amount of this protein present in both envelope and nucleocapsid, low amounts of anti-107K specific antibodies in the serum, or the presence of more than one protein at 107,000 MW. Two monoclonal antibodies reproducibly increased the precipitation of this protein but were not neutralizing. This 107K protein appeared nonspecifically in all immunoprecipitations. Collins et al. (6; personal communication) reported the isolation of a monoclonal antibody that precipitated a nonglycosylated 115K protein and also precipitated nonspecifically in every lane of their immunoprecipitations. Their monoclonal antibodies were neutralizing, whereas ours were not. One of their monoclonal antibodies was tested in our laboratory and found to be identical in specificity to our 107K-specific antibodies.

In summary, our results suggest that the major BHV-1 glycoproteins involved in viral neutralization in vitro are the 150K/77K and 180K/97K proteins and to a lesser extent the 130K/74K/55K protein. This correlates well with those viral proteins surface labeled on infected cells and associated with the virion envelope. The role of the individual envelope glycoproteins in viral pathogenesis is currently under investigation. Panels of antibodies such as produced here and the identification of individual epitopes on the envelope proteins will enable expanded studies of the immunological and functional importance of each viral envelope protein. Such data will be useful for both the design and evaluation of future subunit vaccines.

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