Characterization of Envelope Proteins of Alcelaphine Herpesvirus 1

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Alcelaphine herpesvirus 1 is a gammaherpesvirus which causes malignant catarrhal fever, an acute lymphoproliferative disorder of cattle and other susceptible Bovidae, which is almost invariably fatal. A preliminary analysis of proteins induced by the virus indicated that as many as six glycoproteins and one nonglycosylated molecule might be present in the virus envelope. Monoclonal antibodies selected for recognition of virion envelope proteins included two that recognized a complex of infected cell proteins, designated the gp115 complex, and neutralized virus infectivity in the absence of complement. The gp115 complex consisted of five glycoproteins of 115, 110, 105, 78, and 48 kilodaltons (kDa), and all except the 48-kDa species reacted with antibody in Western blots (immunoblots). Pulse-chase experiments analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions suggested that the 110-kDa protein was the precursor molecule which was processed by addition of sugars to 115 kDa. The 115-kDa protein was cleaved to form a disulfide-linked heterodimer of 78 and 48 kDa, which was the mature form of the molecule incorporated into the virion envelope. The glycoprotein contained N-linked sugars, but little or no O-linked sugar was present. The relative abundance of the mature protein and its ability to induce neutralizing antibodies suggest that it will prove useful to studies aimed at elucidating the biology and pathogenesis of alcelaphine herpesvirus 1.

Alcelaphine herpesvirus 1 (AHV-1) is a gammaherpesvirus carried by African antelope of the subfamily Alcelaphinae; similar or related viruses are carried worldwide by sheep and goats (15). Carrier animals are rarely affected clinically, but when the virus is passed to cattle or other susceptible Bovidae, it causes malignant catarrhal fever, an acute generalized disease which is almost invariably fatal (16). Economic losses in Africa due to malignant catarrhal fever are considerable (14), and the virus has decimated valuable collections of exotic Bovidae in zoological parks (2, 20-22, 25).

Although replication of AHV-1 in tissue culture has been studied only in cells of endothelial origin, the virus has been classified as a gammaherpesvirus because of its association with lymphoid tissue *in vivo*. Malignant catarrhal fever is characterized by widespread lymphoproliferation and an angitis which affects many arteries and veins. The virus has been isolated from buffy coat cells of diseased cattle, and lymphoblastoid cell lines with the characteristics of large granular lymphocytes have been cultured from experimentally infected rabbits and rats (17). Rats inoculated with sublethal doses of virus develop tumorlike nodules with cell morphology described as resembling that of naturally occurring leukemia (9, 10). The appropriateness of the classification of AHV-1 as a gammaherpesvirus has recently been confirmed by analyses of the physical properties of the viral genome which indicate similarities with members of the gamma-2-herpesviruses such as herpesvirus saimiri, herpesvirus atles, and herpesvirus sylvilagus (4). It consists of one unique region of DNA of approximately 130 kilobases and approximately 30 kilobases of additional tandem direct repeat sequences with a high G+C content. AHV-1 is thus not only of considerable interest to zoo and wildlife veterinarians and a potential target for livestock vaccination in parts of Africa, but it is also of potential significance as an animal

model of gammaherpesvirus-induced lymphoproliferative disease. It is the only known herpesvirus that induces lymphoproliferation in rodents, animals which are uniquely amenable to genetic and immunologic manipulation and which may provide an economic model to evaluate strategies for vaccination against human disease.

Relatively little has been reported concerning proteins of AHV-1 beyond the fact that they share antigenic epitopes with other bovine herpesviruses which confound serologic diagnosis of carrier animals (19). As part of a study to identify unique AHV-1 proteins which might be of importance to diagnosis, prophylaxis, and an understanding of the pathobiology of the virus, we sought to identify those molecules that are associated with the envelope of the virion. We report here on a preliminary characterization of AHV-1 envelope glycoproteins and a detailed analysis of the processing of a glycoprotein complex which carries an epitope capable of inducing antibodies that neutralize virus infectivity.

MATERIALS AND METHODS

Cells and virus. Early passage (passages 3 to 7) bovine embryonic lung cells were grown to confluency in Dulbecco minimal essential medium supplemented with 5% heat-inactivated fetal calf serum, 100 IU of penicillin, and 300 µg of glutamine per ml. Stocks of AHV-1 (strain 1982; Zoological Society of San Diego, San Diego, Calif.) were prepared by infecting cells at a multiplicity of infection of 5 to 10 PFU per cell. Virus was adsorbed for 2 h and then overlaid with Dulbecco minimal essential medium supplemented with 2% fetal calf serum. Infection was allowed to proceed for 72 h at 34°C, at which time cytopathic effect was extensive. Cells and medium were harvested, frozen and thawed twice, and stored at -70°C. Neutralization assays and initial studies with polyclonal sera used the prototype strain WC11. This strain was grown in Vero cells and harvested from infected cells at 10 to 12 days postinfection.

Radiolabeling. Bovine embryonic lung cells were infected

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with strain 1982 of AHV-1 at a multiplicity of infection of 5 to 10 PFU per cell, and Vero cells were infected with a similar multiplicity of WC11. For long-term labeling (24 to 48 h for strain 1982 and 240 to 264 h for WC11), cells were incubated in Hanks balanced salt solution containing 40 μ Ci of [35 S]methionine (Amersham Corp., Arlington Heights, Ill.) per ml, 2% dialyzed fetal bovine serum, and 1/10th the normal concentration of methionine or containing 100 μ Ci of [3 H]glucosamine per ml, 2% dialyzed fetal bovine serum, and 1/10th the normal concentration of glucose. Pulse-chase experiments were done only with strain 1982 since cytopathic effect did not become extensive in cells infected with WC11 until 10 days postinfection. Preliminary experiments with the 1982 strain indicated that a pulse-label at 24 h failed to achieve good discrimination of virus-induced proteins against a background of host-cell protein synthesis. Viral cytopathic effect was extensive by 48 h postinfection, and thus pulse-chase experiments were initiated at 36 h postinfection. Cells were labeled for 15 min at 36 h postinfection with [35 S]methionine at a concentration of 100 μ Ci/ml and chased in medium containing 100 times the normal concentration of unlabeled methionine and cycloheximide (50 μ g/ml) for 15, 30, 45, 60, or 120 min or 24 h. At the end of each chase period, cells were scraped from the flask with a rubber policeman into ice-cold phosphate-buffered saline, pelleted by centrifugation, washed two times in phosphate-buffered saline, and stored at -20°C .

Virus that was to be extrinsically labeled with ^{125}I was concentrated from 50 ml of spent culture medium by centrifugation at $100,000 \times g$, suspended in 0.5 ml of phosphate-buffered saline, and labeled with 0.5 to 1.0 mCi of ^{125}I by the use of Iodogen (tetrachlorodiphenylglycouril; Pierce Chemical Co., Rockford, Ill.) (6). Free iodine was removed by sieving on a desalting column (P6-DG; Bio-Rad Laboratories, Richmond, Calif.), and labeled virus was repelleted before solubilization.

Immunoprecipitation and electrophoresis. Immunoprecipitation was done as previously described (1). Briefly, cells or virus were solubilized with radioimmunoprecipitation buffer (RIPA) containing 0.05 M Tris hydrochloride (pH 7.2), 0.15 M NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.1 mM phenylmethylsulfonyl fluoride, and 100 U of aprotinin per ml, sonicated, and centrifuged at $100,000 \times g$ for 1 h. Supernatants were mixed with antibody and protein A-agarose beads (Genzyme Corp., Boston, Mass.). The precipitates were washed, dissociated by boiling in sample buffer with or without 2-mercaptoethanol, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (11) in 9% acrylamide cross-linked with 0.24% *N,N'*-diallyltartardiamide. Molecular weight markers (Sigma Chemical Co., St. Louis, Mo.) were electrophoresed in parallel channels. Gels containing proteins labeled with ^{125}I were stained, destained, dried on filter paper, and placed in contact with XAR film (Eastman Kodak Co., Rochester, N.Y.) at -70°C for fluorography (3). Gels containing [35 S]methionine or [3 H]glucosamine were infused with 2,5-diphenyloxazole before drying.

Western immunoblotting. Cell lysates were electrophoresed in 9% acrylamide and then electrotransferred onto nitrocellulose membranes (0.45- μm pore size; Schleicher & Schuell, Inc., Keene, N.H.). The transferred sheets were treated for 3 h with blocking buffer (10 mM Tris hydrochloride [pH 7.2], 0.15 M NaCl, 5% skim milk, 0.05% sodium azide) and reacted for 3 h with blocking buffer containing monoclonal antibody at 40 $\mu\text{g}/\text{ml}$. They were then washed five or six times with wash buffer (10 mM Tris hydrochloride

[pH 7.2], 0.15 M NaCl, 0.3% Tween 20) for 10 min each followed by an overnight wash. The washed sheets were reacted with alkaline phosphatase-conjugated goat antimouse antibodies (Hyclone, Logan, Utah) for 3 h, and the bound anti-mouse antibodies were detected by reacting with substrate (5-bromo-4-chloro-3-indophosphate and Nitro Blue Tetrazolium; Sigma).

Monoclonal antibodies. BALB/c mice (4 to 6 weeks old) received three weekly subcutaneous injections of virus pelleted from culture medium and emulsified with an equal volume of Freund adjuvant. Three days before fusion, the mice were boosted intraperitoneally with pelleted virus. Spleen cells were harvested, fused with SP2/0 Ag.14 myeloma cells, and plated in hypoxanthine-aminopterin-thymidine medium as previously described (1). Culture supernatants were initially screened in an enzyme-linked immunosorbent assay (ELISA) for the ability to react with AHV-1-infected cells. Supernatant fluids that reacted with virus-infected but not with uninfected cell lysates in the ELISA were further screened for reactivity with the membranes of infected and uninfected cells that had been briefly fixed with 0.1% paraformaldehyde (23). Supernatants that reacted only with virus-infected cell membranes were further evaluated for the ability to immunoprecipitate radiolabeled virus proteins. All hybridomas were repeatedly monocloned by limiting dilution before being grown as ascites in BALB/c mice. Antibody was purified from ascites fluid by chromatography on protein A-agarose before use. Antibody E8D2, which reacts with an Epstein-Barr virus-induced membrane protein (1), was used as a negative control antibody in some experiments.

ELISA. Lysates of infected and uninfected cells were made by scraping cells from flasks into a carbonate-bicarbonate buffer (pH 9.6) and freeze-thawing them three times. Equal amounts of lysate protein (3 μg) were added to wells of 96-well flat-bottomed plates and incubated overnight at 37°C . Plates were then incubated sequentially with 5% skim milk in phosphate-buffered saline-Tween 20, hybridoma supernatants, goat anti-mouse antibody conjugated to horseradish peroxidase (Organon Teknika, Malvern, Pa.), and the substrate *ortho*-phenylenediamine (Sigma). Plates were washed between incubations with 0.05% Tween 20 in 0.85% NaCl. The final enzyme reaction was stopped with 2 N H_2SO_4 , and the color change was measured at 492 nm.

Polyclonal antibodies. Adult New Zealand White rabbits were initially immunized with 2 ml of infected cell lysate emulsified in Freund complete adjuvant. Three subsequent inoculations were given at weekly intervals with 2 ml of cell lysate emulsified with incomplete Freund adjuvant, and blood was collected 2 weeks later. Immune sera were extensively absorbed with uninfected cells, and antibody was purified by chromatography on protein A-agarose.

N-Glycanase digestion. Immunoprecipitated proteins bound to protein A-agarose beads were eluted by boiling the pellets for 3 min in 0.5% SDS-0.2 M sodium phosphate (pH 8.6)-0.7% 2-mercaptoethanol and then incubated overnight at 37°C in 0.55 M sodium phosphate (pH 8.6) containing 7.5% Nonidet P-40, 250 U of *N*-glycanase (Genzyme) per ml, and 100 mM 1,10-phenanthroline hydrate in a final volume of 100 μl . Control samples without *N*-glycanase were incubated in parallel. After incubation, 100 μl of sample buffer (2 $\times$) was added to each and the samples were boiled for 3 min before analysis by SDS-PAGE.

Reduction and alkylation. Proteins separated by electrophoresis under nonreducing conditions were electroeluted from gels and reduced and alkylated as previously described

FIG. 1. SDS-PAGE in 9% polyacrylamide of proteins immunoprecipitated by rabbit anti-AHV-1 antibody from uninfected Vero (lane 1) and bovine embryonic lung (lane 4) cells, from bovine embryonic lung cells infected with AHV-1 strain 1982 (lanes 2 and 5), from Vero cells infected with WC11 (lane 3), and from AHV-1 (lane 6). Cells were metabolically labeled with [35 S]methionine (lanes 1, 2, and 3) or [3 H]glucosamine (lanes 4 and 5), and virus was labeled extrinsically with 125 I. Arrows on the right indicate virus-induced proteins and their apparent molecular weights ($\times 1,000$ [K]).

(26) with slight modifications. In brief, proteins were denatured by the addition of 5 mM guanidine hydrochloride–0.1% EDTA (pH 8.5)–20 mM dithiothreitol and incubated in a nitrogen atmosphere for 4 h at room temperature. Iodoacetamide was added to 50 mM, and incubation continued in a nitrogen atmosphere for another 30 min. Samples were dialyzed against 10 mM Tris hydrochloride–150 mM NaCl (pH 7.3) and analyzed by SDS-PAGE under reducing conditions.

Virus neutralization. The ability of antibody to neutralize virus infectivity was assayed in a standard plaque reduction assay. A known amount of virus was incubated for 1 h at 37°C with monoclonal antibody diluted in a fixed amount of normal serum proteins and then allowed to absorb onto monolayers of susceptible cells. One hour later, monolayers were overlaid with Dulbecco minimal essential medium containing carboxymethyl cellulose and supplemented with 2% fetal calf serum, glutamine, and antibiotics. At 10 days postinfection, the number of plaques was counted by visual inspection of cells.

RESULTS

Proteins immunoprecipitated by polyclonal antibodies. A preliminary characterization of virus-induced proteins was done by comparing molecules immunoprecipitated by polyclonal rabbit antibody from infected and uninfected cells which had been labeled with [35 S]methionine or with [3 H]glucosamine and from virus that had been labeled with 125 I. Our goal was to identify those proteins that are found in the membranes of infected cells and in the envelope of the virion, and it was assumed that the majority of such molecules would be both glycoproteins and accessible to extrinsic

labeling. A total of eight virus-induced proteins ranging in molecular weight from 48,000 to 145,000 were identifiable in cells infected with either strain 1982 or WC11 and labeled with [35 S]methionine and were not seen in uninfected cell lysates even after long exposure of gels. Only those molecules consistently seen in multiple analyses were included in these eight and are identified by arrows in Fig. 1, lanes 2 and 3. The prominent bands in lane 2 at approximately 200 and 45 kilodaltons (kDa) are cell proteins that label with various intensities and bind to protein A (1). Ten virus-induced proteins ranging in molecular weight from 46,000 to 145,000 were immunoprecipitated from [3 H]glucosamine-labeled cells (Fig. 1, lanes 4 and 5). In addition to molecules with masses similar to those of glycoproteins labeled with [35 S]methionine, virus-induced glycoproteins were seen at 62 and 46 kDa. Nothing corresponding to the 62-kDa glycoprotein could be identified with the [35 S]methionine label, but a protein that might correspond to the 46-kDa glycoprotein is tentatively identified in lanes 2 and 3 of Fig. 1. Eight proteins ranging in molecular weight from 46,000 to 130,000 were immunoprecipitated from iodinated virion lysates (Fig. 1, lane 6). A summary of AHV-1-induced proteins identified by polyclonal antibodies is given in Table 1. Since the patterns of proteins induced by strain 1982 and WC11 were similar and replication of strain 1982 was much faster than that of WC11 (3 days as opposed to 10 days), a majority of subsequent experiments were done with this strain.

Monoclonal antibodies. Identification of virus-induced proteins with polyclonal sera can only be tentative. More precise definition of the biochemical characteristics of AHV-1-induced membrane proteins and determination of identity and possible precursor-product relationships between mole-

FIG. 2. SDS-PAGE in 9% polyacrylamide of [³⁵S]methionine-labeled proteins immunoprecipitated from AHV-1-infected cells by rabbit anti-AHV-1 antibody (lane 1) or monoclonal antibodies 14B6 (lane 2), 12B5 (lane 3), and 11C3 (lane 4). Arrows on the right indicate virus-induced proteins specifically immunoprecipitated by monoclonal antibodies and their molecular weights (×1,000 [k]).

FIG. 3. SDS-PAGE in 9% polyacrylamide of [³H]glucosamine-labeled proteins immunoprecipitated from AHV-1-infected cells by rabbit anti-AHV-1 antibody (lane 1) or monoclonal antibodies 14B6 (lane 2), 12B5 (lane 3), and 11C3 (lane 4). Arrows on the right indicate virus-induced proteins specifically immunoprecipitated by monoclonal antibodies and their molecular weights (×1,000 [k]).

cules that were metabolically as opposed to extrinsically labeled required analysis with monoclonal rather than polyclonal reagents. To serve this purpose, we made hybridoma antibodies from spleens of mice that had been immunized with virus pelleted from spent culture medium of infected cells. An ELISA was used for preliminary screening of the hybridomas. Antibodies that reacted with infected but not uninfected lysates were then tested for the ability to react with the surfaces of infected but not uninfected cells in indirect immunofluorescence assays. The molecules recognized by antibodies positive by both these screening protocols were determined by immunoprecipitation of proteins labeled with [³⁵S]methionine, [³H]glucosamine, and ¹²⁵I. Fifteen antibodies obtained in this initial study immunoprecipitated virus-induced proteins from infected cells. Anti-

bodies with three types of immunoprecipitation pattern were found and compared with the immunoprecipitation patterns previously seen with polyclonal sera (Fig. 2, 3, and 4). Type 1 and type 2 antibodies, exemplified by antibodies 14B6 and 11C3, immunoprecipitated proteins of 145 and 44 kDa, respectively, that labeled with [³⁵S]methionine and [³H]glucosamine but not with ¹²⁵I (lanes 2 and 4 of each figure). The failure to label either molecule with ¹²⁵I was somewhat surprising since the antibodies were selected for their ability to react with cell membranes in immunofluorescence assays, and this may mean that neither molecule is present on the surface of the virion. Type 3 antibodies, exemplified by antibody 12B5, immunoprecipitated a complex of five proteins of 115, 110, 105, 78, and 48 kDa from [³⁵S]methionine- or [³H]glucosamine-labeled cells (the gp115 complex), two of which, those of 78 and 48 kDa, labeled with ¹²⁵I (lane 3 of each figure). The 78-kDa molecule did not label as intensely with [³⁵H]methionine as did the other members of the complex but labeled strongly with [³H]glucosamine. Spent culture medium from each hybridoma antibody were tested for the ability to neutralize virus infectivity; two antibodies, from hybridomas 12B5 and 31B3, that recognized the gp115 complex reduced plaque numbers by more than 50%. Neutralizing activity of both antibodies was retained after purification from culture supernatants or ascites fluid (Table 2). Although both antibodies were of the immunoglobulin G1 subclass and contained kappa light chains, the kinetics of neutralization were slightly different, suggesting that they recognized distinct epitopes. The antibodies neutralized both the 1982 isolate of AHV-1 and the prototype isolate WC11 (data not shown). Because of our interest in functionally important proteins that may serve as important targets of the immune response, the complex of

TABLE 1. AHV-1-induced proteins immunoprecipitated from virus and virus-infected cells by polyclonal rabbit antibody

Mol wt of virus-induced proteins	Proteins labeled with:		
	[³⁵ S]methionine	[³ H]glucosamine	¹²⁵ I
145,000	+	+	—
130,000	+	+	+
115,000	+	+	—
110,000	+	+	+
105,000	+	+	+
95,000	+	+	+
78,000	+	+	+
72,000	—	—	+
62,000	—	+	—
48,000	+	+	+
46,000	+?	+	+

FIG. 4. SDS-PAGE in 9% polyacrylamide of proteins immunoprecipitated from ^{125}I -labeled AHV-1 virions by rabbit anti-AHV-1 antibody (lane 1) or monoclonal antibodies 14B6 (lane 2), 12B5 (lane 3), and 11C3 (lane 4). Arrows on the right indicate virus-induced proteins specifically immunoprecipitated by monoclonal antibodies and their molecular weights ($\times 1,000$ [k]).

proteins recognized by the neutralizing antibodies was studied in further detail. All experiments reported were done with antibody 12B5; similar results could, however, be obtained with antibody 31B3.

The gp115 complex. Envelope glycoproteins of several herpesvirus include disulfide-linked complexes (5, 7, 8, 12).

TABLE 2. Effect of monoclonal antibodies 31B3 and 12B5 on infectivity of AHV-1 strain 1982

Antibody concn ($\mu\text{g/ml}$)	% Inhibition ^a with:	
	31B3	12B5
1,000	92	90
330	82	63
100	69	46
33	42	30
10	40	4
3	12	0
1	8	0

^a % Inhibition = $1 - \frac{\text{PFU in absence of antibody}}{\text{PFU in presence of antibody}} \times 100$.

To this point, all analyses of AHV-1 proteins were done by SDS-PAGE under reducing conditions. Analysis of proteins immunoprecipitated by antibody 12B5 was then first repeated under nonreducing conditions to determine whether any of the molecules of the gp115 complex were coprecipitated because of linkages that are susceptible to reducing agents. Under nonreducing conditions, the number of [^{35}S] methionine-labeled molecules visualized by autoradiography fell from five (Fig. 5, lanes 1) to three (Fig. 5, lane 2). The 78- and 48-kDa species became undetectable, and the 115-kDa species became more abundant. The 78- and 48-kDa proteins precipitated from ^{125}I -labeled virions (Fig. 5, lane 4) likewise became undetectable under nonreducing conditions and were replaced by a single protein of 115 kDa (Fig. 5, lane 3). This suggested that the mature glycoprotein present in the virion was a disulfide-linked dimer composed of a 78- and 48-kDa species and was consistent with the possibility that the remaining molecules that labeled with [^{35}S]methionine were processing intermediates. The relatedness of the molecules immunoprecipitated by antibody was further examined by Western blotting of infected and uninfected cell lysates.

FIG. 5. SDS-PAGE in 9% acrylamide of proteins immunoprecipitated by antibody 12B5 from AHV-1-infected cells labeled with [^{35}S] methionine (lanes 1 and 2) and from AHV-1 virus extrinsically labeled with ^{125}I (lanes 3 and 4). Proteins were electrophoresed under reducing (lanes 1 and 4) or nonreducing (lanes 2 and 3) conditions. Arrows indicate the positions and molecular weights ($\times 1,000$ [K]) of proteins specifically precipitated by antibody.

FIG. 6. Reactivity of antibody 12B5 (lanes 2 and 4) and control antibody E8D2 (lanes 1 and 3) with uninfected (lanes 1 and 2) and AHV-1-infected (lanes 3 and 4) cell lysates separated by electrophoresis in 9% acrylamide under reducing conditions and blotted onto nitrocellulose. Numbers on right show molecular weight ($\times 1,000$).

Antibody 12B5 recognized four proteins in infected cells with molecular masses corresponding to those of the 115-, 110-, 105-, and 78-kDa species (Fig. 6, lane 4). Some slight reactivity was also seen with two bands of uncertain origin of about 58 kDa, but no reactivity was seen with a protein of 48 kDa. This again is consistent with the hypothesis that the molecules of the complex are related by a processing event which includes cleavage into a fragment that bears the 12B5 epitope (the 78-kDa species) and one that does not (the 48-kDa species). The presumed precursor-product relationships between the molecules of the complex were therefore examined in pulse-chase experiments. At 36 h postinfection, AHV-1-infected cells were pulsed with [35 S]methionine for 15 min and then chased with cold methionine for 15, 30, 45, 60, or 120 min or 24 h. Samples of cells harvested at the end of each pulse or chase period were immunoprecipitated with antibody 12B5 and analyzed by SDS-PAGE under both reducing and nonreducing conditions. Irrespective of the presence or absence of 2-mercaptoethanol, only one protein of 110 kDa was specifically immunoprecipitated by 12B5 from the 15-min pulse (Fig. 7A and B, lanes 1). During the 15-min chase (lanes 2), two proteins were visible under both reducing and nonreducing conditions, the heavily labeled 110-kDa protein and a lighter band representing a protein at 105 kDa. After a 30-min chase (lanes 3), an additional molecule of 115 kDa could be seen. The 45-min chase (lanes 4) was the first time interval at which the protein patterns were clearly different under reducing and nonreducing conditions. During this chase period, the 115-kDa protein seen under nonreducing conditions (Fig. 7A, lanes 4 to 7) was a rather heavy band. In contrast, when run under reducing conditions (Fig. 7B, lanes 4 to 7), it was barely noticeable. Instead, it appeared to be replaced by two proteins, one at 78 kDa and one at 48 kDa.

Reduction-alkylation. All the data so far were consistent with the hypothesis that the 115-kDa molecule was actually a heterodimer composed of the 78- and 48-kDa molecules. To test this hypothesis, virus proteins immunoprecipitated

FIG. 7. SDS-PAGE in 9% acrylamide of proteins immunoprecipitated from AHV-1-infected cells by monoclonal antibody 12B5. Lanes show infected cells with [35 S]methionine for 15 min at 24 h postinfection (lane 1), [35 S]methionine for 15 min at 24 h postinfection followed by chase for 15, 30, 45, 60, and 120 min and 24 h (lanes 2 to 7, respectively), in nonradioactive medium containing cycloheximide and 100 $\times$ cold methionine. Arrows indicate the positions and molecular weights ($\times 1,000$ [k]) of proteins specifically immunoprecipitated with antibody 12B5. (A) Electrophoresis under nonreducing conditions; (B) electrophoresis under reducing conditions.

FIG. 8. Reelectrophoresis in 9% acrylamide under reducing conditions of proteins immunoprecipitated from [35 S]methionine-labeled cells infected with AHV-1 by antibody 12B5 and electrophoresed under nonreducing conditions. Proteins with molecular weights of 110,000 (lane 1) and 115,000 (lane 2) were electroeluted from gels run under nonreducing conditions and subjected to reduction and carboxymethylation of cysteine residues before reanalysis. Arrows indicate molecular weights of proteins ($\times 1,000$).

with antibody 12B5 were electrophoresed under nonreducing conditions and the 115- and 110-kDa proteins were electroeluted from gel slices and subjected to reduction and carboxymethylation of cysteine residues. The reduced and carboxymethylated proteins were then analyzed by SDS-PAGE. A portion of the 115-kDa protein was thereby reduced to a 78- and a 48-kDa protein, although some of the molecule remained intact (Fig. 8, lane 2). When the 110-kDa molecule was subjected to this same protocol, relatively more of the protein retained its original size and the breakdown products were 48 and 66 kDa (Fig. 8, lane 1). This experiment provided additional evidence in favor of generation of the 78- and 48-kDa molecules via a proteolytic cleavage event and suggested that cleavage occurred predominantly after posttranslational processing of the 110-kDa precursor into the 115-kDa form.

N-Glycanase. Herpesvirus glycoproteins have been identified which contain both N- and O-linked sugars. To determine whether the processing of the 110-kDa precursor to the 115-kDa molecule resulted solely from the modification of N-linked sugars or from the addition of O-linked residues, the gp115 complex was immunoprecipitated and digested with *N*-glycanase under reducing conditions. This procedure resulted in a loss of the readily identifiable 115- and 110-kDa proteins (Fig. 9, lane 1) and the appearance of a new protein at approximately 100 kDa (Fig. 9, lane 2); the breadth of label in this new molecule made it impossible to determine the fate of the 105-kDa protein. The poorly labeled 78-kDa

protein was no longer visible in the digested sample, label in the 48-kDa molecule was reduced in intensity, and two new species of approximately 45 and 60 kDa became apparent.

DISCUSSION

The herpesviruses are large complex enveloped viruses that typically incorporate three or more glycoproteins into their outer membranes. Our experiments, reported here, indicated that AHV-1 is no exception. Preliminary studies in which polyclonal sera were used to immunoprecipitate extrinsically iodinated virus suggested that a least eight proteins could be found in the virion envelope. Whether or not all of them were unique species or included both mature proteins and their breakdown products is not clear. It also remains uncertain how many of them were glycosylated. At least one of the iodinated proteins, the 72-kDa molecule, could not be visualized with either 35 S or 3 H label, presumably because it contained few methionine residues and little or no glucosamine. Molecules with mobilities similar to those of the remaining iodinated proteins were identified in immunoprecipitates of infected cells that had been labeled with 3 H, but in the absence of monoclonal or monospecific antibodies to each protein, it was impossible to know whether different isotopes were labeling the same protein or two proteins which comigrate in one-dimensional gels. In fact, studies with our first panel of monoclonal antibodies suggested that at least two virus-induced proteins comigrate at both 110 and 105 kDa, since glycoproteins of these

FIG. 9. SDS-PAGE analysis of *N*-glycanase susceptibility of [35 S]methionine-labeled proteins immunoprecipitated from AHV-1-infected cells by antibody 12B5. Immunoprecipitates were mock digested (lane 1) or digested with *N*-glycanase (lane 2) before electrophoresis in 9% acrylamide. Arrows indicate molecular weights ($\times 1,000$ [k]) of proteins.

molecular masses that form part of the gp115 complex are not expressed on the virus surface and cannot be labeled with ^{125}I . The total number of proteins readily identifiable in [^{35}S]methionine-labeled infected cell extracts was surprisingly small. A herpesvirus with a genome as large as that of AHV-1 would be expected to encode at least 60 to 80 proteins. There are several possible explanations for this. First, we have been deliberately conservative in our interpretation of autoradiographs of [^{35}S]methionine-labeled proteins and only pointed out those molecules that we are confident can be unequivocally attributed to infection with virus; the background of labeled cell proteins is greater with a label such as [^{35}S]methionine than [^3H]glucosamine and complicates analysis. Second, as described above, more than one protein may migrate to the same position in a one-dimensional gel. Third, we assume that many proteins may be made in amounts that are undetectable when precipitated with polyclonal sera. The 44-kDa protein immunoprecipitated by monoclonal antibody 11C3 provides an example of such a protein. In this respect, it is not surprising that the majority of [^{35}S]methionine-labeled proteins detected in infected cells would seem to be structural proteins or their precursor molecules, since such species are perhaps likely to be among the most abundant.

A start has been made on the task of making monoclonal antibodies to individual molecules so that some of these confounding problems can be better avoided. Of those antibodies made to date, the most interesting have been those that recognize the gp115 complex. Two of the seven antibodies made to this complex reduced virus infectivity in the absence of complement. This indicates that the members of the complex may be important immunologic targets, although it does not rule out the possibility that, like certain envelope proteins of several herpesviruses, they may not be essential for replication *in vitro* (13, 18, 24).

Proteins of the gp115 complex labeled with both [^{35}S]methionine and [^3H]glucosamine, suggesting that they are relatively abundant molecules. Four of the five molecules, gp115, -110, -105, and -78, that were immunoprecipitated with antibody 12B5 reacted with the same antibody in Western blots, indicating that they are antigenically related. Pulse-chase analyses done to elucidate the processing pathways of the related molecules were disappointing in that no clear-cut flow of label was apparent from the 110-kDa protein, the only member of the complex seen in the pulse period and thus presumably the precursor molecule, to the 115-kDa protein, which appeared later and was the species found in the outer virion envelope. This may in part reflect the difficulty of obtaining quantitative information from experiments that require that proteins be immunoprecipitated before analysis. The temporal appearance of the different species and the fact that the 110- and 115-kDa molecules are antigenically related was, however, at least suggestive of a biosynthetic pathway in which a primary translation product of 110 kDa, to which N-linked sugars had been added cotranslationally, was processed by trimming and further addition of sugar to a 115-kDa product. Whether or not the 105-kDa molecule was an intermediate in this process or simply an alternate form of the protein is unclear. The 115-kDa glycoprotein first appeared in the 30-min chase period, and in samples analyzed under nonreducing conditions, label continued to accumulate in the molecule for the duration of the experiments. When the pulse-chase samples were run under reducing conditions, most of the 115-kDa molecule was replaced by a 78- and a 48-kDa molecule. Analysis of immunoprecipitated lysates of ^{125}I -labeled viri-

ons under reducing and nonreducing conditions confirmed the provenance of the 78- and 48-kDa species. When the 115- and the 110-kDa proteins were excised from preparative gels run under nonreducing conditions, degradation of the 115-kDa protein into 48- and 78-kDa fragments was pronounced. Breakdown of the 110-kDa protein also occurred, resulting in a 48-kDa species similar to that resolved from the 115-kDa molecule and a second fragment of 66 kDa. Proportionally more of the 110-kDa molecule than the 115-kDa molecule remained intact. These results suggested that the cleavage takes place principally after processing of gp110 to gp115 and also that the majority of the posttranslational modification of sugars takes place on the 78-kDa fragment of the mature dimer. Since digestion of the 115-kDa complex with *N*-glycanase replaced the 115- and 110-kDa proteins with a single molecule of approximately 100 kDa, it would appear that the difference between the two molecules is the result of modifications only in N-linked sugars and that the mature 115-kDa protein contains little or no O-linked sugar.

Posttranslational modification involving processing of sugars and proteolytic cleavage of a larger polypeptide into a disulfide-linked complex of virion envelope proteins has been described for several members of the herpesvirus family (5, 7, 8, 12). It is quite possible that processing intermediates have been missed in our analyses because they are transient, because they cannot be resolved with the techniques used, or because they cannot be precipitated with our monoclonal antibodies. Our data suggest, however, that the biosynthetic pathway of the gp115 complex involves processing of the sugars of a primary translation product, gp110, possibly via a 105-kDa intermediate, to a protein of 115 kDa. This 115-kDa molecule then appears to be cleaved into two proteins of 78 and 48 kDa which are linked by disulfide bonds and inserted into the virion envelope. Preliminary data derived in a competitive ELISA with sera taken from 102 cattle indicated that naturally infected cattle processing neutralizing antibody to AHV-1 also have antibodies that will compete with the neutralizing monoclonal antibodies for binding to virus-infected cell lysate (S. Adams, C. Metz, and L. Hutt-Fletcher, unpublished data). This suggests that the epitope(s) is highly conserved and immunogenic in nature. Further study of the gp115 complex could thus be of importance to understanding the biology of AHV-1, to immunodiagnosis, and, potentially, to prophylaxis of disease.

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