JOURNAL OF VIROLOGY, Nov. 1984, p. 583–590
0022-538X/84/110583-08$02.00/0

Vol. 52, No. 2

Characterization of the Envelope Proteins of Pseudorabies Virus

HARTMUT HAMPL,[1,2]* TAMAR BEN-PORAT,[1] LORENTZ EHRLICHER,[2] KARL-OTTO HABERMEHL,[2] AND ALBERT S. KAPLAN[1]

Department of Microbiology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232,[1] and Institut für Klinische und Experimentelle Virologie, der Freien Universität, D-1000 Berlin 45, Federal Republic of Germany[2]

Received 2 April 1984/Accepted 18 July 1984

Previously we have reported that among the proteins of purified pseudorabies virions there are four major glycoproteins (T. Ben-Porat and A. S. Kaplan, Virology 41:265–273, 1970). Several minor glycoproteins can also be identified by two-dimensional gel electrophoresis. Removal of the viral envelope with Triton X-100 selectively removes from the virions all of the glycoproteins as well as several non-glycosylated proteins. Sedimentation analysis or chromatography of these proteins reveals that several are complexed with one another, some being covalently linked via disulfide bridges. Analysis of the proteins by immunoprecipitation with monoclonal antibodies reactive with the membrane proteins showed also that three of the four major virus glycoproteins (125K, 74K, and 58K; gIIa, gIIb, and gIIc, respectively) are linked covalently by disulfide bridges. Furthermore, all three share extensive sequence homology as indicated by the identity of their antigenic determinants and by partial peptide mapping; they probably originate from a single protein precursor. The fourth major glycoprotein (98K; gIII) is not complexed to any other protein. Three minor glycoproteins (130K [gI], 98K [gIV], and 62K [gV]), which form a noncovalently linked complex with a 115K nonglycosylated protein, have also been identified. Of the monoclonal antibodies used in this study, only those reactive with the major 98K glycoprotein (gIII) inhibit virus adsorption and neutralize virus infectivity in the absence of complement. However, all react with surface components of the virion, indicating that the proteins with which they react are exposed on the surface of the virions. A nomenclature for the pseudorabies virus glycoproteins is proposed.

Pseudorabies virus (herpes suis I) (PrV) is an important pathogen of swine. It causes Aujeszky's disease, which results in severe economic losses. PrV contains a linear, double-stranded DNA molecule approximately 92×10^6 daltons in size (1). The architecture of the virus is similar to that of other herpesviruses—an icosahedral capsid surrounded by a floppy envelope. The virus envelope that surrounds the icosahedral nucleocapsid contains four major glycoproteins (3).

Removal of the virion envelope with Triton X-100 allows the separation of the nucleocapsid fraction, which contains all of the DNA and approximately 50% of the virus protein. The envelope fraction contains the remainder of the proteins, including all of the glycoproteins (6). However, the organization and the functions of the envelope proteins have not been elucidated.

Because the envelope proteins are on the surface of the virion, they probably play an important role in eliciting the immune response of the host. Consequently, it is important to ascertain the organization of the envelope protein and to determine which of these proteins plays a role in the development of immunity. This information is essential for the development of vaccines effective in the control of Aujeszky's disease.

The experiments described in this paper represent part of an attempt to obtain some information about these questions.

(Preliminary reports of this work were presented at the Seventh and Eighth International Workshops on Herpesviruses at Cold Spring Harbor, N.Y., 1982, and Oxford, England, 1983, respectively.)

* Corresponding author.

MATERIALS AND METHODS

Virus and cell culture. The properties of standard PrV and cultivation of rabbit kidney (RK) cells have been described previously (7).

Media and solutions. The following media and solutions were used: Earle saline; EDS, Eagle synthetic medium plus 3% dialyzed bovine serum; PBS, 0.136 M NaCl–2.6 mM KCl–8 mM Na_2HPO_4–1 mM KH_2PO_4–20 mM $MgCl_2$–1.8 mM $CaCl_2$ (pH 7.0); TBS, same as PBS, but the phosphate is replaced by 0.01 M Tris-hydrochloride (pH 7.5); TBSA, TBS plus 1% crystalline bovine albumin.

Radiochemicals. [^{3}H]Leucine (specific activity, 55 Ci/mmol) was purchased from Schwarz/Mann, [^{3}H]glucosamine (specific activity, 31 Ci/mol) was from New England Nuclear Corp., and [^{35}S]methionine (1,300 Ci/mmol) was from Amersham Corp.

Labeling and purification of virions. RK cells were infected with 1 PFU/cell and incubated for 24 h (from the time of infection) in Eagle synthetic medium without amino acids and containing 3% dialyzed calf serum, 0.6 mM arginine and 50 μCi of either [^{35}S]methionine, [^{3}H]glucosamine, or [^{3}H]leucine per ml. Virions were purified as described previously (2). The virions were washed once with TBS and stored at −70°C in PBS.

Removal of virion envelope. The virion envelope was removed essentially as described previously (6). Triton X-100 (final concentration, 1%) was added to purified virions (in PBS), which were incubated at 45°C for 20 min with occasional shaking and spun for 1 h in an Eppendorf centrifuge. The supernatant (envelope fraction) was collected; the pellet (nucleocapsid fraction) was washed once with PBS.

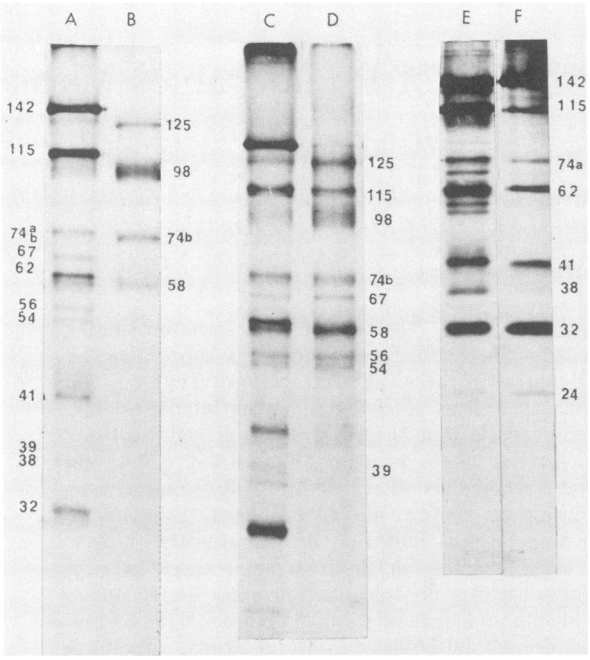

FIG. 1. PAGE of the structural proteins of PrV. Purified PrV, labeled with either [^{3}H]leucine or [^{3}H]glucosamine, and the nucleocapsid and solubilized envelope fractions obtained after the removal of the envelope from [^{3}H]leucine-labeled virus were analyzed by PAGE. Lanes: A, C, and E, [^{3}H]leucine-labeled virions; B, [^{3}H]glucosamine-labeled virions; D, [^{3}H]leucine-labeled solubilized envelope fraction; F, [^{3}H]leucine-labeled nucleocapsids without envelopes. Lanes A, B, C, and D are 8% polyacrylamide gels; lanes E and F are 10% polyacrylamide gels.

Fractionation of envelope proteins in gradients. The envelope proteins (with or without prior treatment with 0.5 M urea–0.1% sodium dodecyl sulfate [SDS]–0.01 M dithioerythritol [DTE] for 30 min at 37°C) were layered onto a 5 to 15% sucrose gradient in PBS containing 0.1% Triton X-100 and centrifuged for 20 h at 32,000 rpm in a Beckman SW40 rotor. Fractions (500 μl) were collected from the bottom of the tube. The distribution of radioactive proteins in the gradient was determined by counting a portion of each fraction in a spectrometer. The proteins in the samples were concentrated by acetone precipitation (3 volumes of acetone for 1 h at −5°C) and analyzed by polyacrylamide gel electrophoresis (PAGE).

Fractionation of envelope proteins on Sephacryl-200 columns. The envelope proteins (treated as described above) were loaded on a column (1 m long, 5 mm in diameter) of Sephacryl-200, suspended in PBS–0.1% SDS–0.1% Triton X-100 and eluted with the same buffer at a flow rate of approximately 20 ml/h. The distribution of radioactive proteins in the eluate was determined, and the proteins in the fractions were acetone precipitated and analyzed by PAGE.

Immunoprecipitation. Immunoprecipitation was performed essentially as described by Kessler (8). In brief, DTE-treated or untreated envelope fraction proteins in PBS containing 0.05% Triton X-100 were incubated for 90 min on ice with the appropriate antiserum or monoclonal antibody at a final concentration of 1:500. An excess of freshly washed, Formalin-treated *Staphylococcus aureus* Cowan strain was added, and the sample was kept on ice for 30 min. The samples were centrifuged for 1 min in an Eppendorf centrifuge (or for 10 min at 2,000 × *g*). The pellets were washed six times with 150 mM NaCl–50 mM Tris–0.05% Triton X-100, suspended in 6 M urea–2% SDS, boiled for 3 min, and centrifuged for 1 min in an Eppendorf centrifuge, and the supernatant was collected and analyzed by PAGE.

PAGE. Protein samples were brought to a final concentration of between 1 and 3 M urea (depending on the experiment) and 2% SDS. Unless otherwise stated, they were reduced by incubation with DTE (final concentration, 0.01 M) at 37°C for 30 min. The proteins were then electrophoresed on acrylamide gels as described previously (9).

Preparation of hybrid cell lines producing monoclonal antibodies against PrV structural proteins. The procedures used for the preparation of hybrid cell lines producing monoclonal antibodies against PrV structural proteins were essentially those of Nowinski et al. (11). TK$^-$ mouse myeloma cells, Sp 2.0 Ag14 (obtained from M. Cohn), were fused with spleen cells of BALB/c mice immunized with acetone-precipitated, purified PrV proteins. After selection in HAT medium, positive clones were identified by the ^{125}I-labeled *S. aureus* protein A method (11) and were recloned twice. Ascites fluids were produced as described by McKearn (10).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed as described by O'Farrell (12). Gels containing a mixture of 1.6% (pH 5 to 7) and 0.4% (pH 3 to 10) ampholines were used. Electrophoresis was for 12 h at 400 V and then for 1 h at 800 V. The second dimension was on a 10% SDS-polyacrylamide gel as described previously (9).

Partial peptide mapping. The partial peptide mapping method of Cleveland et al. (5) was used.

RESULTS

Identification of the envelope proteins of PrV. The minimal number of major polypeptides present in purified PrV was estimated originally to be 10; the number of glycoproteins was estimated to be 4 (3, 4, 6). Subsequently, using techniques that allowed better resolution, Stevely (14) showed that at least 20 polypeptides can be resolved when preparations of purified virions are analyzed by SDS-PAGE. Indeed, with gels of various concentrations, 27 different virus protein bands can be detected by one-dimensional PAGE and at least 40 spots that appear to be unrelated can be resolved on two-dimensional gels (unpublished results). Figure 1 shows autoradiograms obtained when [^{3}H]leucine-labeled virions (tracks A, C, and E) or [^{3}H]glucosamine-labeled virions (track B) are analyzed by SDS-PAGE. The glycoproteins appear as shadows on the autoradiograms of the [^{3}H]leucine-labeled virion proteins, but can be more clearly resolved when the virions are labeled with glucosamine (Fig. 1, track B). Only four major glycoprotein bands can be detected, but some of the glycoprotein bands are sometimes resolved as doublets. Several minor bands can also be detected upon longer exposure of the autoradiograms (data not shown).

Two-dimensional gel electrophoresis resolves four major and at least eight minor species of glycoproteins (Fig. 2). Polypeptides occupying the same position in the gels are not observed when extracts of uninfected cells are electrophoresed; these polypeptides therefore probably do not represent cellular contaminants. Some of them could, however, be breakdown products of the larger virus glycoproteins.

The envelope of mature PrV can be removed by treatment with nonionic detergent (6). The nucleocapsids without envelopes sediment more slowly on sucrose gradients than virions and contain a full complement of DNA. They have

lost 50% of the proteins originally present in the virions and do not retain any of the glycoproteins (6). Electron microscopic examination of the virions without envelopes shows that they retain the typical morphology of the nucleocapsids, but that the floppy envelope normally surrounding the virions is lost (6). These data indicate that treatment with Triton X-100 leaves the nucleocapsids intact and solubilizes the envelopes and the proteins associated with the envelopes. We have analyzed this envelope fraction in greater detail.

In Fig. 1 the protein compositions of intact virions (tracks A, C, and E), the nucleocapsid fraction (track F), and the fractions containing the solubilized proteins (track D) are compared. Eight major and at least two minor protein bands appear to be specifically solubilized by treatment of the virions with Triton X-100. Thus, in addition to all of the major glycoproteins, several nonglycosylated proteins are also solubilized by treatment with Triton X-100, as is the case for herpes simplex virus (13).

On the basis of their distribution between the nucleocapsid and envelope fractions, most of the proteins can be clearly assigned to one or the other fraction. However, some protein bands (for instance, 115K) seem to be almost evenly distributed between the envelope and the nucleocapsid fractions. Whether these proteins are identical in the two fractions or whether the envelope and nucleocapsid proteins only comigrate in the gels remains to be ascertained.

Fractionation of proteins in the envelope fraction. In an attempt to isolate and study the individual proteins (or protein complexes) in the envelope fraction, the soluble protein fractions obtained after removal of the envelopes of purified virions with Triton X-100 were sedimented in sucrose gradients or chromatographed on Sephacryl G-200. Figure 3 shows the distribution of the labeled proteins in a sucrose gradient. The proteins migrated as what appeared to be three peaks (two distinct peaks [I and III] and a shoulder on peak III [peak II]). Figure 4 illustrates the PAGE profile of the proteins in the different fractions obtained from the gradient.

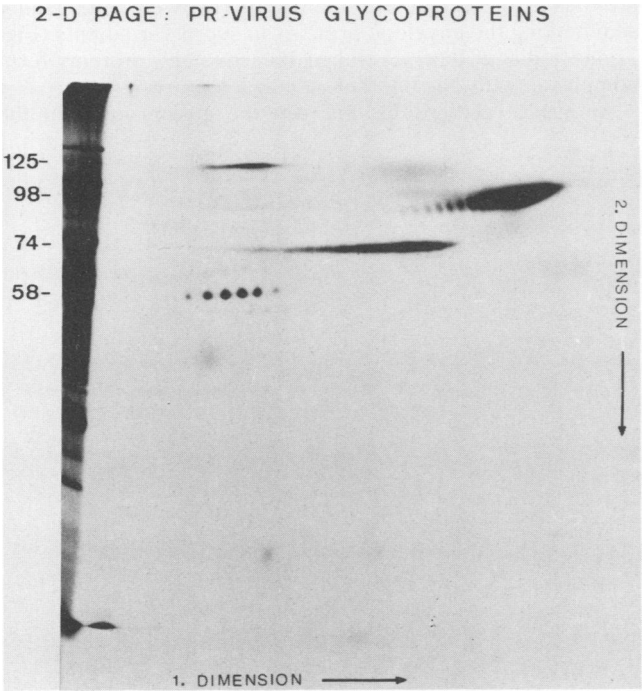

FIG. 2. Two-dimensional PAGE of PrV glycoproteins. Purified [^{3}H]glucosamine-labeled PrV was analyzed on two-dimensional gels as described in the text. The pH gradient was 4.6 to 7.5. The acidic end of the gel is on the left.

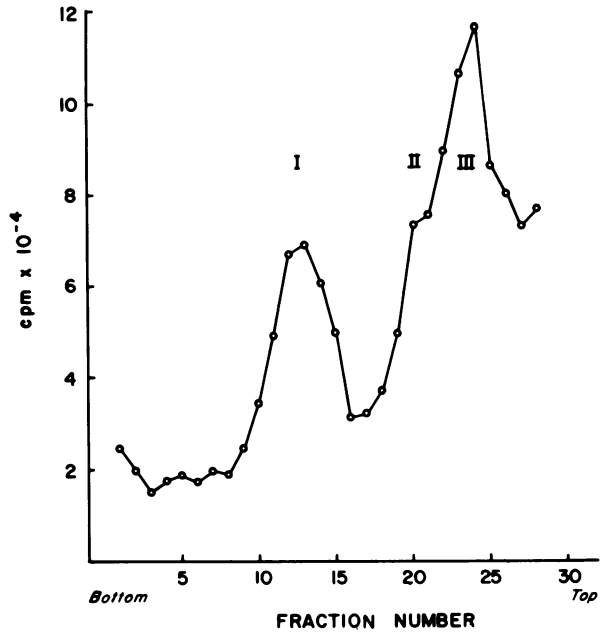

FIG. 3. Sedimentation analysis of the proteins that were solubilized after treatment of virions with Triton X-100. A soluble envelope fraction was prepared from [^{3}H]leucine-labeled PrV as described in the text, layered on a 5 to 15% TBS-sucrose gradient containing 0.1% Triton X-100, and sedimented at 4°C for 20 h at 32,000 rpm in an SW40 rotor. Fractions (~500 μl) were collected and assayed for radioactivity.

Inspection of the autoradiogram reveals that of the four major glycoproteins (125K, 98K, 74K, and 58K), three (125K, 74K, and 58K) cosedimented in the gradient (fractions 10 through 14; Fig. 3, peak I) and cochromatographed on Sephacryl gels (data not shown), indicating that these proteins may be complexed to one another. The other major glycoprotein (98K) appeared mainly to be uncomplexed with any other protein (fractions 20 through 23), although some appeared to cosediment with a protein with a molecular weight of 115K and with a protein with an approximate molecular weight of 130K (which was not resolved from the 125K on one-dimensional gels) (fractions 17 through 19; Fig. 3, peak II). That these proteins are indeed complexed to each other is discussed below in greater detail. Similar complexes were also observed when the envelope fractions obtained from virions labeled with [^{3}H]glucosamine were analyzed (data not shown).

When an experiment similar to the one described in Fig. 4 was performed, but the envelope fraction was treated with SDS, urea, and DTE before sedimentation in sucrose gradients or chromatography on sephacryl columns, the complexes were disrupted, as expected, and the proteins migrated according to their molecular weights (Fig. 5). Treatment of the proteins with SDS and urea without DTE did not disrupt the 125K–74K–58K complex, but did disrupt the 130K–115K–98K complex (data not shown, see Fig. 7, lane J).

We conclude tentatively from these results that several of the glycoproteins present in the virus envelope form complexes, some of which are linked by disulfide bridges.

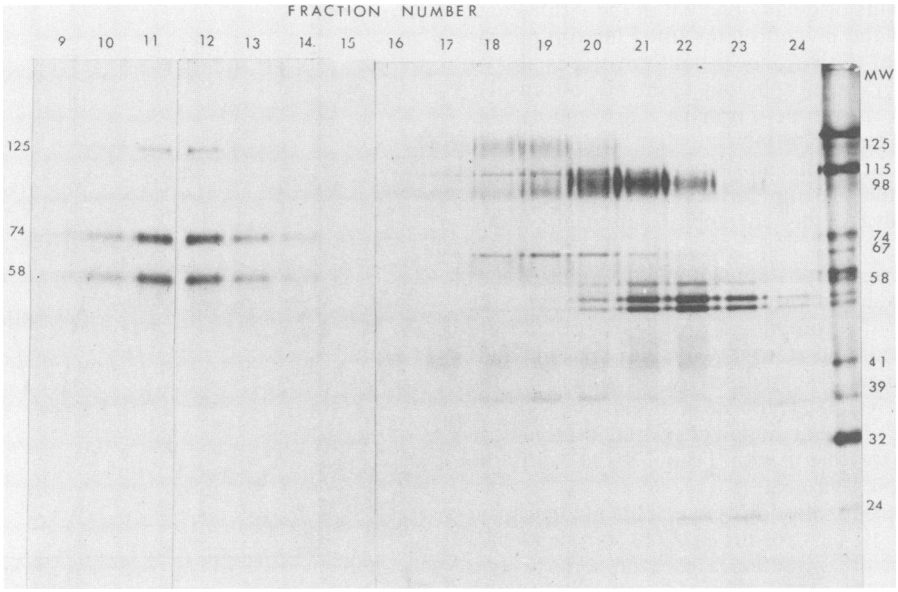

FIG. 4. PAGE of solubilized membrane proteins fractionated on sucrose gradients. The fractions obtained from the experiment in Fig. 3 were acetone precipitated and analyzed by PAGE. The last lane on the gel represents the [^{3}H]leucine-labeled proteins of purified PrV.

Reactivity of the virus envelope proteins with monoclonal antibodies. Monoclonal antibodies against purified PrV proteins were obtained as described above; several were found to be reactive with the virus glycoproteins. These monoclonal antibodies could be divided into three types on the basis of their reactivity, as determined by indirect immune precipitation. One type precipitated the 98K major virus glycoprotein (Fig. 6; M1, M7, and M4). Another type coprecipitated the other three major glycoproteins (Fig. 6, M2, M8, and M3; Fig. 7, M5). Finally, one monoclonal antibody, M9 (Fig. 6), coprecipitated 130K, 98K, and 62K glycoproteins. (A glycoprotein with a molecular weight of 62K appears as a shadow on one- as well as two-dimensional gels. However, it is more easily detected when the virion glycoproteins are analyzed by PAGE under nonreducing conditions [Fig. 7, lane J], i.e., under conditions in which it is not obscured by the major 58K glycoprotein.)

When the solubilized proteins obtained from virions labeled with [^{3}H]leucine were similarly immunoprecipitated with the monoclonal antibodies, the results were similar (Fig. 6). However, in addition to the 130K, 98K, and 62K glycoproteins, M9 precipitated another nonglycosylated protein (115K). The results obtained by immunoprecipitation are thus in agreement with the results that were obtained by sedimenting the envelope proteins in sucrose gradients (Fig. 3) and indicate that several of the envelope proteins form complexes with one another.

Antigenic relationships between the glycoproteins in the

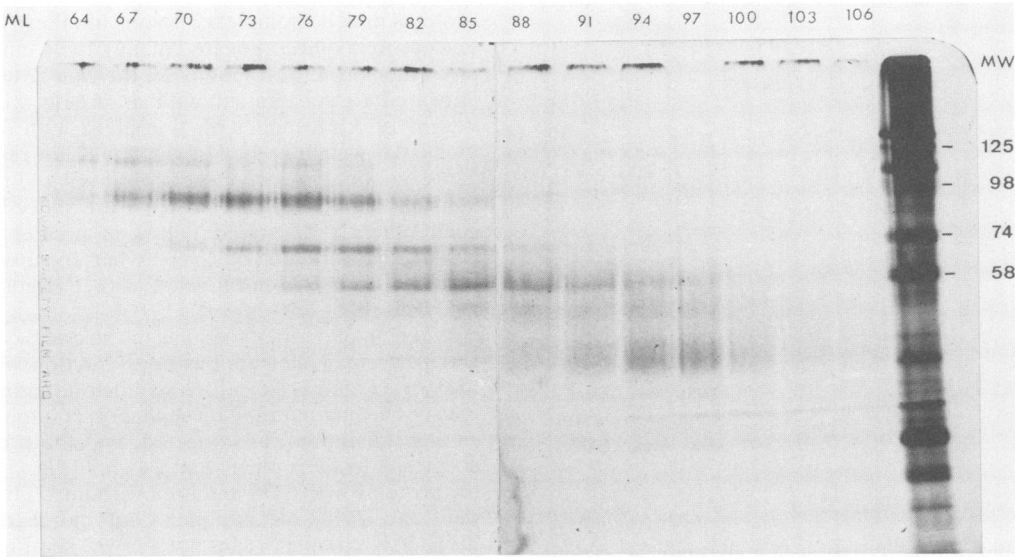

FIG. 5. Disruption of the glycoprotein complexes. The soluble envelope fraction from [^{3}H]glucosamine-labeled, purified PrV was treated with SDS (0.1%), DTE (0.01 M), and urea (0.5 M) and chromatographed on Sephacryl G-200 columns as described in the text. Fractions were acetone precipitated and analyzed by PAGE. The last lane on the gel represents the [^{3}H]leucine-labeled proteins of purified PrV.

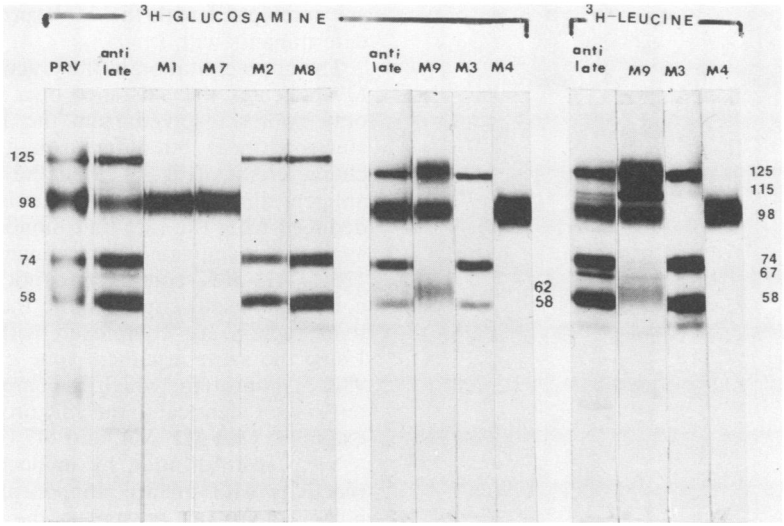

FIG. 6. Immunoprecipitation of [^{3}H]glucosamine- and [^{3}H]leucine-labeled, solubilized envelope proteins by monoclonal antibodies. [^{3}H]glucosamine- or [^{3}H]leucine-labeled, solubilized envelope proteins were immunoprecipitated with different monoclonal antibodies against PrV envelope proteins as described in the text or with polyvalent serum directed against lysates of cells 7 h postinfection (anti-late). The immunoprecipitates were analyzed by PAGE. The proteins of purified PrV labeled with [^{3}H]glucosamine are illustrated in the first lane (PRV).

complexes. The experiments described above show that several of the envelope proteins are complexed with one another and that these complexes are disrupted by treatment with agents that break hydrogen bonds or disulfide bridges. Several different approaches were used to ascertain whether antigenic relationships also exist between the proteins in the complexes.

(i) 125K–74K–58K complex. To determine whether the proteins within the complexes are antigenically related, the samples were treated with DTE (0.01 M) and SDS (0.1%) before immunoprecipitation, and the reactivity of the individual proteins with the various monoclonal antibodies was determined. Figure 7 shows that despite the fact that the proteins in the complexes had been dissociated from one another before immunoprecipitation (as indicated by their migration in polyacrylamide gels without further DTE treatment [data not shown]), the monoclonal antibodies that coprecipitated the 125K–74K–58K complex, as for example, monoclonal antibodies M3 and M5 (which behave in a manner similar to M2 and M8; Fig. 6), still coprecipitated the 125K and 74K proteins. A total of five different monoclonal antibodies, which were isolated independently (and cloned twice), were tested and found to behave in a similar manner. These results show that at least two of the three glycopro-

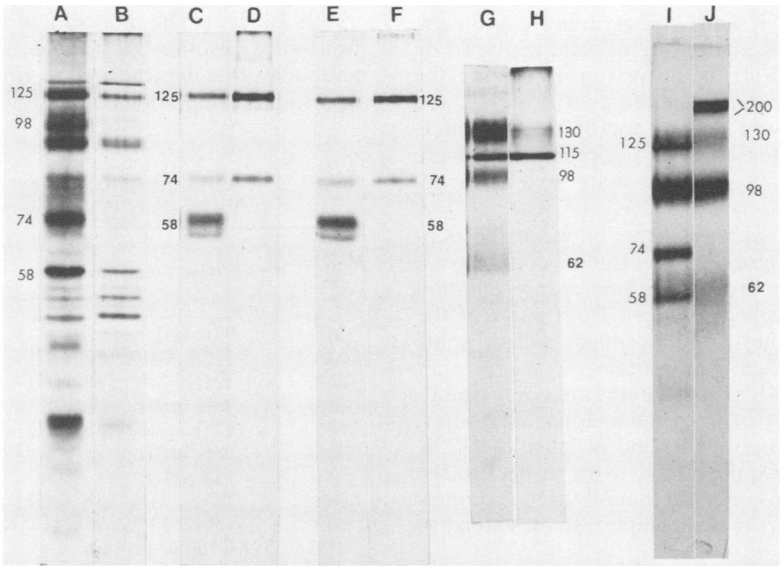

FIG. 7. Immunoprecipitation of envelope proteins under reducing conditions. Proteins solubilized from purified [^{3}H]leucine-labeled virions were either treated with SDS (0.1%) and DTE (0.01 M) (lanes D, F, and H) or left untreated (lanes C, E, and G) and immunoprecipitated either by M3 (lanes C and D), M5 (lanes E and F), or M9 (lanes G and H). As a control, DTE- and SDS-treated (lane B) and untreated (lane A) [^{3}H]leucine-labeled proteins from infected cells were immunoprecipitated with polyvalent (see legend to Fig. 6) serum against PrV. Panels I and J show the electrophoretic behavior of [^{3}H]glucosamine-labeled virion protein separated by PAGE under reducing (DTE treatment; lane I) or nonreducing (lane J) conditions.

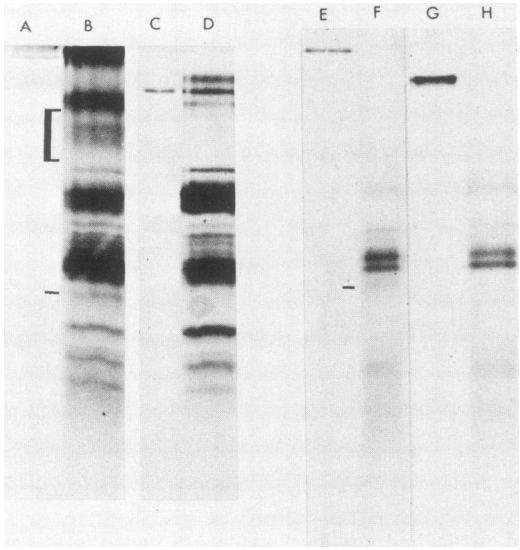

FIG. 8. Partial peptide mapping of 125K and 58K glycoproteins. [^{35}S]methionine-labeled, PrV-solubilized envelope proteins were immunoprecipitated with monoclonal antibody M5 and separated on an 8% polyacrylamide gel. The protein bands, identified by autoradiography, were cut out of the gel. The proteins were treated with 0.3 U of trypsin per sample (lanes B and D) or 0.6 U of trypsin per sample (lanes F and H) or left untreated (lanes A, C, E, and G) and incubated for 1 h at room temperature. The samples were analyzed on a 15% acrylamide gel. Lanes A, B, E, and F show the 125K glycoproteins; lanes C, D, G, and H show the 58K glycoproteins.

teins in the complex share several antigenic determinants. The third protein (58K) appears to be rendered antigenically inactive by treatment with SDS and DTE, as indicated by the fact that after this treatment it was not precipitated by polyvalent sera against PrV (Fig. 7, track B).

Because it was impossible to ascertain from the above experiments whether the 58K glycoprotein shares antigenic determinants with the 125K and 74K glycoproteins in the complex, the 125K and 58K glycoproteins were analyzed by partial peptide mapping (Fig. 8). Surprisingly, despite the twofold difference in size between the two proteins, the peptides generated were very similar, and only a few differences (which are indicated in the autoradiogram) were observed. Thus, these two proteins share extensive sequence homology.

The greater than expected similarities between the peptides generated by digestion of the two proteins could have a trivial explanation, such as the relative underrepresentation of methionine in the peptides produced from parts of the 125K protein. On the other hand, it might reflect some interesting structural feature of the protein.

Because the 125K and 74K glycoproteins are coprecipitated by several independently isolated monoclonal antibodies, even after DTE and SDS treatment, and because the 125K and 58K glycoproteins are quite similar with respect to the peptides they generate, we conclude that all three glycoproteins (125K, 74K, and 58K) in the complex share extensive homologies.

(ii) 130K–115K–98K–62K complex. As mentioned above, the 130K–115K–98K–62K complex is disrupted by treatment with SDS. Immunoprecipitation of the proteins with M9 after SDS (and DTE) treatment precipitated the 115K nonglycosylated protein only (Fig. 7, lane H). No evidence was obtained that the 130K, 98K, or 62K glycoproteins, which are complexed with the 115K protein, share any antigenic determinants with it.

The coprecipitation of the glycoproteins with the 115K by M9 indicates the existence of a 130K glycoprotein that is only poorly resolved from the 125K glycoprotein by gel electrophoresis, but differs from it somewhat in its migration characteristics (Fig. 6). That the 130K glycoprotein is also antigenically distinct from the 125K glycoprotein can be deduced from the fact that monoclonal antibodies directed against the antigenic determinants of the proteins in the 125K–74K–58K complex do not (under the experimental conditions used) coprecipitate the 115K, 98K, or 62K proteins, which are complexed with the 130K glycoprotein. Using the same argument, one can also conclude that the 98K glycoprotein, which is complexed with the 115K protein, is not identical to the 98K protein, which is reactive, for example, with M1, M4, and M7 (Fig. 6).

Virus neutralization by monoclonal antibodies and their reactivity with surface components of PrV. Since the PrV glycoproteins are present in the virus envelope, it was of interest to determine (i) which glycoprotein is a target for neutralization and (ii) which of the virus envelope proteins are at least partially exposed on the surface of the virions.

Table 1 shows the results of a neutralization test performed with PrV and some of the monoclonal antibodies that we have isolated. The results show the following. (i) All of the monoclonal antibodies directed against the major 98K glycoprotein neutralize virus infectivity in the absence of complement. (ii) In the absence of complement, none of the other monoclonal antibodies, i.e., those directed against the 125K–74K–58K complex or against the 115K protein, neutralized the virus. In the presence of complement, all of these monoclonal antibodies also had some neutralizing activity, although neutralization was not pronounced (at the most, 80% of the virus was neutralized under the experimental conditions used). The results in Table 1 were obtained with a 1:1,000 dilution of monoclonal antibodies. Increasing the concentration of all the monoclonal antibodies 50-fold did not change the results.

The effects of the monoclonal antibodies on virus adsorption were also tested (Table 2). All of the monoclonal antibodies that neutralized the virus also inhibited its adsorption to the cells; none of the others did.

TABLE 1. Virus neutralization by some monoclonal antibodies against PrV envelope proteins[a]

Monoclonal cell line	Reactive with:	Virus titer (PFU/ml)	
		Minus complement	Plus complement
M1	98K (gIII)	2.2×10^3	1.6×10^3
M2	125K, 74K, 58K (gII)	4.2×10^5	9.8×10^4
M3	125K, 74K, 58K (gII)	3.2×10^5	6.6×10^4
M4	98K (gIII)	3.7×10^3	2.1×10^3
M5	125K, 74K, 58K (gII)	3.7×10^5	1.9×10^5
M6	98K (gIII)	8.0×10^2	8.3×10^2
M7	98K (gIII)	8.2×10^2	7.2×10^2
M8	125K, 74K, 58K (gII)	4.0×10^5	1.6×10^5
M9	130K, 115K, 98K (115K)	4.6×10^5	2.0×10^5
1W	Cellular protein	3.9×10^5	2.6×10^5
TBSA		4.2×10^5	

[a] Duplicate samples containing 5×10^5 PFU of PrV per ml were incubated in Eagle synthetic medium containing 3% dialyzed calf serum and the various monoclonal antibodies (final dilution of each, 1:1,000). To one set of the samples, reconstituted guinea pig complement (50 μl) was added. The samples were incubated at 37°C for 1 h; the titer of the virus was determined by plaque assay.

To determine whether the proteins against which the monoclonal antibodies devoid of neutralizing activity are directed are accessible for interaction, i.e., are on the surface of the virions, the experiment in Table 3 was performed. In this experiment, labeled virions were first mixed with each of the various monoclonal antibodies. They were then reacted with Formalin-treated *S. aureus* (Cowan A strain), with the expectation that if the monoclonal antibody had reacted with a surface component of the virus, the virus would cosediment with the antibody-*S. aureus* protein A complex that would form. Table 3 shows that in every case in which a monoclonal antibody directed against envelope proteins was used, the virus cosedimented with the bacteria; when an unrelated monoclonal antibody was used, the virus remained in the supernatant. We conclude therefore that all of the proteins that react with the monoclonal antibody used in this study are exposed (at least partially) on the surface of the virions and are accessible to interaction with these antibodies. Only the monoclonal antibodies directed against the major 98K glycoprotein, however, have virus-neutralizing activity in the absence of complement.

DISCUSSION

The experiments described in this paper are part of an attempt to determine the organization of the envelope of PrV and to ascertain the functions of the various envelope proteins. Analysis of the virion glycoproteins by SDS-PAGE or two-dimensional gels indicates the presence of four major and several minor glycoprotein bands.

The envelope proteins can be solubilized by treatment of the virions with Triton X-100. Some of these proteins are complexed to one another, as indicated by their sedimentation behavior in sucrose gradients as well as by their chromatographic behavior on Sephacryl G-200. One complex, composed of three of the four major glycoproteins (125K–74K–58K), is disrupted by a combination of SDS and DTE treatments, indicating that these proteins are covalently linked to one another by disulfide bridges. Several independently isolated (twice cloned) hybridomas have been identified that secrete immunoglobulins that coprecipitate at least two of the three proteins in the complex, even after disruption of the complex by treatment with SDS and DTE, indicating that these two proteins share antigenic determinants. The third protein in the complex loses its antigenic determinants after treatment with SDS and DTE but is closely related to the largest glycoprotein in the complex, as determined by partial peptide mapping. Thus, three of the four major glycoproteins appear to share extensive sequence homology. This is also indicated by the fact that only a single precursor protein to all three glycoproteins has been detected (H. Hampl, manuscript in preparation). The processes involved in the generation of the three glycoproteins from this precursor are currently being investigated.

One monoclonal antibody (M9) precipitates a complex of three glycoproteins and one nonglycosylated protein (115K). This monoclonal antibody is directed against antigenic determinants on the 115K protein, as indicated by the results obtained by immunoprecipitation after dissociation of the complex. No evidence that the three glycoproteins (130K, 98K, and 62K) that coprecipitate with it share any antigenic determinants with the 115K protein or with each other has as yet been obtained.

The 130K glycoprotein, which is complexed with the 115K, 98K, and 62K proteins, is distinguishable from the major 125K glycoprotein by its slower migration in PAGE and more diffuse appearance. Indeed, inspection of the two-dimensional gels of the glycoproteins (Fig. 2) reveals that two glycoprotein bands with an approximate molecular weight of 125K are present. The major protein of the two, which is more acidic and less diffuse than the other, probably represents the 125K glycoprotein that is complexed with the 74K and 58K glycoproteins. The fact that monoclonal antibodies against the 125K–74K–58K glycoprotein complex do not (under the appropriate pH and salt conditions) coprecipitate the 115K protein also indicates that the 130K glycoprotein associated with the 115K protein is distinct from the 125K glycoprotein. These observations thus indicate that two different PrV glycoproteins with approximately the same migration characteristics, one being obscured by the other, are present in the 125K band observed in one-dimensional gels.

The minor 130K and 62K glycoproteins, which are complexed with the 115K and 98K proteins, are normally obscured on one-dimensional gels by the major 125K and 58K glycoproteins and are only faintly seen on two-dimensional gels. However, when the viral glycoproteins are analyzed by PAGE in the absence of reducing conditions (i.e., under conditions under which the covalently linked major 125K, 74K, and 58K glycoproteins are complexed with one another

TABLE 2. Effect of monoclonal antibodies on virus adsorption[a]

Monoclonal cell line	Reactive with:	Virus adsorbed (cpm × 10^{-3})	
		Expt 1	Expt 2
TBSA		242	146
M1	98K (gIII)	38	22
M2	125K, 74K, 58K (gII)	260	119
M3	125K, 74K, 58K (gII)	258	107
M4	98K (gIII)	48	17
M5	125K, 74K, 58K (gII)	195	97
M6	98K (gIII)	35	17
M7	98K (gIII)	41	14
M8	125K, 74K, 58K (gII)	185	120
M9	130K, 115K, 98K (115K)	165	163
1W	Cellular protein	215	140

[a] Purified [^{3}H]thymidine-labeled PrV in 1 ml of TBSA were incubated with the appropriate antibodies (final concentration of each, 1:100) for 30 min at 37°C. The PrV was then allowed to adsorb to monolayers of RK cells for 45 min at 37°. The cells were extensively washed and lifted from the glass surface, and the amount of radioactivity associated with the cells was determined.

TABLE 3. Reactivity of monoclonal antibodies with virion surface proteins[a]

Monoclonal cell line	Reactive with:	Virus in supernatant (cpm × 10^{-3})
M1	98K (gIII)	15
M2	125K, 74K, 58K (gII)	11
M4	98K (gIII)	14
M8	125K, 74K, 58K (gII)	12
M9	130K, 115K, 98K (115K)	12
1W	Cellular protein	365
Anti-PrV serum	Several virion proteins	12
TBSA		373

[a] Purified [^{3}H]thymidine labeled PrV (0.1 ml in TBSA) was incubated with the appropriate antibody (final concentration, 1:100) at 37°C for 30 min. Prewashed, Formalin-treated *S. aureus* A (Cowan strain) was then added, and the samples were further incubated at 37°C for 30 min. The samples were set in ice for 30 min and then centrifuged at 8,000 × *g* for 5 min. The amount of radioactivity remaining in the supernatant was determined.

TABLE 4. Tentative classification of the structural PrV glycoproteins[a]

Glycoprotein	Apparent molecular weight	Properties
gI	130K	In complex with 115K nonglycosylated protein, 98K (gIV) and 62K (gV)
gIIa gIIb gIIc	125K 74K 58K	These three glycoproteins share extensive homologies and are complexed to one another by disulfide bonds
gIII	98K	In the form of a monomer
gIV	98K	Complexed with 130 K (gI), 62K (gV) and 115K nonglycosylated protein
gV	62K	Complexed with 130K (gI), 98K (gIV), and 115K nonglycosylated protein

[a] The glycoproteins of PrV have been classified into five apparently unrelated groups (gI, gII, gIII, gIV, and gV). However, the available evidence does not exclude the possibility that gI, gIV, and gV are related.

[Fig. 7, lane J]), the presence of the 130K and 62K glycoproteins is revealed.

Because the monoclonal antibodies against the major 98K glycoprotein do not coprecipitate the 130K, 115K, and 62K proteins (which are complexed with the 98K glycoprotein), it appears that two different 98K glycoproteins also are present in the virus envelope and that the free 98K glycoproteins against which the monoclonal antibodies that we have isolated are reactive and the 98K protein that is complexed with the 130K, 115K, and 62K proteins differ from each other. Because four independently isolated monoclonal antibodies recognize the free 98K glycoprotein, but not the 98K glycoprotein that is in the complex, it is unlikely that two forms of the same 98K protein exist in the cells, one having been partially modified antigenically as a result of being complexed with the 130K, 115K, and 62K protein. However, the evidence for the existence of two different 98K glycoproteins needs to be reinforced by partial peptide mapping.

We have tentatively classified the structural glycoproteins of PrV (Table 4). Because the molecular weight determination by PAGE may be misleading (the values that we have obtained vary somewhat since different markers [commercial protein markers, structural proteins of adenovirus and of herpes simplex virus type 1] as well as when different types of acrylamide gels were used) and to avoid confusion with the glycoproteins of herpes simplex virus that are identified by capital letters (functional analogies between the glycoproteins of herpes simplex virus and PrV remain to be clarified), we have identified the major glycoproteins of PrV by Roman numerals.

The three major glycoproteins, designated as gIIa, gIIb, and gIIc, are all related (this paper) and are probably all derived from a single precursor protein (H. Hampl, manuscript in preparation). We have not yet obtained any evidence concerning the possible relationships among the other glycoproteins.

Of the monoclonal antibodies that we have used in this study, only those reactive with 98K glycoprotein (gIII) neutralize the virus in the absence of complement and inhibit virus adsorption, despite the fact that the sites of the glycoproteins reactive with the other monoclonal antibodies are exposed on the surface of the virions. However, we have studied only a relatively small number of monoclonal antibodies, and it is possible, therefore, that monoclonal antibodies against other virus glycoproteins, including the gII family, may also neutralize the virus.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-10947 from the National Institutes of Health. H.H. was supported by a fellowship from Deutsche Forschungsgemeinschaft.

LITERATURE CITED

1. **Ben-Porat, T.** 1982. Organization and replication of herpesvirus DNA, p. 147–172. *In* A. S. Kaplan (ed.), Organization and replication of viral DNA. CRC Press, Inc., Boca Raton, Fla.
2. **Ben-Porat, T., J. M. DeMarchi, and A. S. Kaplan.** 1974. Characterization of defective, interfering viral particles present in a population of pseudorabies virions. Virology **61**:29–37.
3. **Ben-Porat, T., and A. S. Kaplan.** 1970. Synthesis of proteins in cells infected with herpesviruses. V. Viral glycoproteins. Virology **41**:265–273.
4. **Ben-Porat, T., H. Shimono, and A. S. Kaplan.** 1970. Synthesis of proteins in cells infected with herpesvirus. IV. Analysis of the proteins in viral particles isolated from the cytoplasm and the nucleus. Virology **41**:256–264.
5. **Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli.** 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. **252**:1102–1106.
6. **Kaplan, A. S., and T. Ben-Porat.** 1970. Synthesis of proteins in cells infected with herpesvirus. VI. Characterization of the proteins of the viral membrane. Proc. Natl. Acad. Sci. U.S.A. **66**:799–806.
7. **Kaplan, A. S., and A. E. Vatter.** 1959. A comparison of herpes simplex and pseudorabies viruses. Virology **7**:394–407.
8. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **119**:1617–1624.
9. **Ladin, B. F., S. Ihara, H. Hampl, and T. Ben-Porat.** 1982. Pathway of assembly of herpesvirus capsids: an analysis using DNA^+ temperature-sensitive mutants of pseudorabies virus. Virology **116**:544–561.
10. **McKearn, T. J.** 1980. Method for growing hybridomas in rats or mice, p. 403–404. *In* R. H. Rennett, T. J. McKearn, and K. B. Bechtol (ed.), Monoclonal antibodies. Plenum Publishing Corp., New York.
11. **Nowinski, R. C., M.-E. Lostrom, M. R. Tam, M. R. Stone, and W. N. Burnette.** 1979. The isolation of hybrid cell lines producing monoclonal antibodies against the p15(E) proteins of murine leukemia virus. Virology **93**:111–126.
12. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.
13. **Spear, P. G., and B. Roizman.** 1972. Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpes virion. J. Virol. **19**:143–159.
14. **Stevely, W. S.** 1975. Virus-induced proteins in pseudorabies-infected cells. II. Proteins of the virion and nucleocapsid. J. Virol. **16**:944–950.