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Demonstration of Three Major Species of Pseudorabies Virus Glycoproteins and Identification of a Disulfide-Linked Glycoprotein Complex

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The glycoproteins of pseudorabies virus (PRV) Phylaxia were characterized with monoclonal antibodies as specific reagents. Three major structural glycoproteins with molecular weights of 155,000 (155K) (gC), 122K (gA), and 90K (gB) could be identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions. We investigated the processing of glycoproteins gA, gB, and gC by in vitro translation, pulse-chase experiments, and in the presence of the ionophore monensin which inhibits glycosylation. gA and gB were found to compose a single polypeptide, whereas gC was found to be a disulfide-linked glycoprotein complex. Immunoprecipitates formed with the aid of anti-gC monoclonal antibodies gave rise to three glycoprotein bands (gC_0 [120K], gC_1 [67K], and gC_2 [58K]) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions. Limited proteolysis of gC_0, gC_1, and gC_2 resulted in peptide maps of gC_0 related to those of both gC_1 and gC_2. No common peptide bands between gC_1 and gC_2, however, were seen. We suggest that (i) gC_1 and gC_2 arise by proteolytic cleavage from the same precursor molecule and stay joined via disulfide bridges and (ii) gC_0 is an uncleaved precursor.

The pseudorabies virus (PRV) (*Herpesvirus suid* 1) is a swine herpesvirus which causes severe losses among piglets and leads to latent infection in older animals. For the related herpes simplex viruses (HSVs) of humans, the glycoproteins, their precursors, processing, and role in inducing cellular and humoral immune responses are well characterized (for a review, see reference 23). In brief, in HSV-infected cells, four antigenically distinct structural glycoproteins are synthesized, designated gB, gC, gD, and gE. gB is essential for virus penetration into infected cells, presumably by promoting virus-cell fusion (27). gC is not essential for virus infectivity, but it has been proposed that gC might influence infectivity and virulence by the negative modulation of gB promoted fusion between host cell and virion membranes (8) and between cells (22). The physiological role of gD and gE is not known. Each of the four HSV glycoproteins can induce the formation of virus neutralizing antibodies, although only anti-gE sera require the presence of complement for neutralization in vitro (23, 25).

In contrast to HSV, very little is known about the glycoproteins of PRV. The only published information (1, 16, 18) about them is that there are four major species, the estimates of the molecular weights (MWs) of which fall within the ranges 100,000 (100K) to 128K, 72K to 98K, 60K to 74K, and 45K to 58K, respectively.

Our goal in this study has thus been the characterization of PRV glycoproteins and the production of monospecific reagents against them, as a prerequisite for further functional studies. We investigated the glycoproteins of PRV Phylaxia with monoclonal antibodies in radioimmune precipitation assays. On sodium dodecyl sulfate (SDS)-gels in the presence of mercaptoethanol, we found four major glycoprotein bands with apparent MWs of 122K (gA), 90K (gB), 67K (gC_1), and 58K (gC_2), in good agreement with the literature cited above (1, 16, 18). However, under nonreducing conditions only three glycoproteins were seen at 155K (gC), 122K (gA), and 90K (gB), which could be independently precipitated with monoclonal antibodies. We examined the processing of these three glycoproteins by immunoprecipitation of in vitro-translated or pulse-chase-labeled antigens and also after radioactive labeling in the presence of monensin.

MATERIALS AND METHODS

Production of monoclonal antibodies to PRV structural proteins. BALB/c mice were immunized with gradient-purified, heat-inactivated (60°C for 1h) PRV. Protein (50 μg) in complete Freund adjuvant was injected intraperitoneally (i.p.) for the first inoculation, followed 2 weeks later by i.p. and 4 weeks later by intravenous injection of the same amount of antigen without adjuvant. At 3 days after the last immunization, spleen cells were fused with Sp2/O–Ag14 or P3-NS1/Ag4 mouse plasmacytoma cells (17, 29) in a 3:1 ratio (24). Antibody secreting hybridoma cultures were selected by screening in enzyme-linked immunosorbent assay (ELISA) (6) on plates coated with 300 ng of protein per well of purified and sonicated virions, by overnight incubation at 4°C. Plates coated with sonicated, noninfected cells served as control. Bound antibody was detected with peroxidase-labeled F(ab')2 fragment of goat anti-mouse antibody (Tago Inc., Burlingame, Calif.). Positive hybridomas were cloned twice by limiting dilution.

Virus and cell culture. The PRV strain Phylaxia was grown in Madin-Darby bovine kidney cells (MDBK; ATCC CCL 221) or in normal rat kidney cells (NRK; obtained from T. Graf, European Molecular Biology Laboratories, Heidelberg, Federal Republic of Germany). For virus purification, the cells were infected with 1 to 5 PFU per cell and maintained in Eagle minimal essential medium (MDBK cells) or in Dulbecco modified Eagle minimal essential medium (NRK cells) for 24 to 36 h.

Virions were isolated from the supernatant and from infected cells after disruption with a Dounce homogenizer. Cell debris was removed by centrifugation at 10,000 × *g* for

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15 min, and virions were pelleted in an R19 rotor (Beckman) at 18,000 rpm for 90 min. The sediment was suspended in TEN buffer (20 mM Tris-hydrochloride [pH 7.5]–1 mM EDTA–150 mM NaCl), sonicated for three 5-s periods, and centrifuged at a low speed to remove large aggregates. The virions were further purified by centrifugation through a 35% (wt/vol) sorbitol cushion (SW27 Beckman rotor at 22,000 rpm for 60 min) and by rate velocity sedimentation in 12 to 52% (wt/vol) sucrose gradients (SW27 rotor at 22,000 rpm for 90 min). Banded virions were carefully aspirated, diluted with TEN buffer, and concentrated by being pelleted by using a SW27 rotor at 25,000 rpm for 1 h.

Radiolabeling of virus proteins. In vitro labeling of purified PRV with $Na^{125}I$ (Amersham Buchler, Braunschweig, Federal Republic of Germany) was performed by the chloramine T method (9).

For in vivo labeling, monolayer cell cultures were infected at a multiplicity of 20 PFU per cell. After 1 h, nonadsorbed virus was removed by washing. Unless otherwise indicated, cells were labeled at between 4 and 8 h postinfection with 40 to 100 μCi of [^{35}S]methionine per ml in methionine-free medium or by adding D-[6-^{3}H]glucosamine at a concentration of 0.5 mCi/ml in glucose-free medium.

Immunoprecipitation. Radiolabeled infected monolayer cells were washed with phosphate-buffered saline (PBS) and frozen at −20°C until use. Cells (10^7) were lysed in 2 ml of lysis buffer (PBS, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 2 mg of ovalbumin per ml, 0.2% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 mM methionine, and 2.5 mM KI). The lysates were clarified by centrifugation in 50Ti rotor at 39,000 rpm for 1 h and preadsorbed with 200 μl of a 10% suspension of fixed *Staphylococcus aureus* cells (15). Lysates containing 10^6 cpm were mixed with 100 μl of hybridoma supernatant or 5 μl of antiserum. After incubation for 90 min on ice, 100 μl of *S. aureus* cells were added, and the samples were further incubated for 30 min. The immunoprecipitates were washed four times with lysis buffer and eluted with 60 μl of sample buffer (0.12 M Tris-HCl [pH 6.8]–4% SDS–20% glycerol, with or without 10% 2-mercaptoethanol) by heating at 95°C for 2 min.

RNA isolation. Whole-cell RNA was isolated from infected and mock-infected cells, essentially as described (32). Briefly, the trypsinized cells were lysed in 5.8 M guanidine hydrochloride–0.05 M lithium chloride, 0.1 M 2-mercaptoethanol–0.5% (wt/vol) Sarkosyl. The lysate was centrifuged through a CsCl cushion (5.7 M in 20 mM Tris [pH 7.0]–100 mM EDTA) in a SW41 rotor at 32,000 rpm for 22 to 24 h at 20°C. After decanting the supernatant, the RNA pellet was suspended in TESR (20 mM Tris-hydrochloride [pH 7.0], 1 mM EDTA, 0.1% SDS) and precipitated with ethanol in the presence of 0.3 M potassium acetate. Finally, the RNA pellet was dried under vacuum, suspended in sterile bidistilled water, and stored at −70°C.

In vitro translation. In vitro translation of cytoplasmic RNA isolated late in infection was performed in a rabbit reticulocyte lysate system (New England Nuclear, Dreieich, Federal Republic of Germany) by using the recommendations of the manufacturer. Before immunoprecipitation, the translation reaction mixtures (25 μl) were diluted with 300 μl of lysis buffer, preadsorbed with 50 μl of the *S. aureus* suspension, and immunoprecipitated as described above.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of viral proteins was carried out by the method of Laemmli (19) in separating gels containing 10% acrylamide monomer cross-linked with bisacrylamide. The acrylamide-to-bisacrylamide ratio was 30:0.8, except for in the pulse-chase experiment where a ratio of 30:1 was used.

When the samples were labeled with ^{3}H or ^{35}S, En^{3}Hance (New England Nuclear) was used to decrease the exposure time to XAR5 film (Eastman Kodak Co., Rochester, N.Y.) (3). Iodinated samples were autoradiographed by using Curix MR 600 (Agfa-Gevaert, Leverkusen, Federal Republic of Germany) screens (20).

Labeling of viral glycoproteins in polyacrylamide gels. Iodination of concanavalin A (ConA) and labeling of glycoproteins with [^{125}I]ConA in the gels were carried out as described by Burridge (4) with some modifications.

Two identical sets of purified virions and reference protein samples were separated by SDS-PAGE (19). The gels were stained with Coomassie brillant blue, photographed, and then equilibrated with ConA buffer, consisting of 50 mM Tris-hydrochloride (pH 7.5), 0.15 M NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MnCl_2$, and 0.1% NaN_3. [^{125}I]ConA was diluted to 10^6 cpm/ml with ConA buffer supplemented with 1 mg of horse hemoglobin per ml. The gel was divided into two identical sets of lanes, both sets were sealed in a separate plastic bag and incubated over night with [^{125}I]ConA or [^{125}I]ConA plus 40 mg of α-methyl-D-mannoside per ml, respectively, using an amount of ConA solution equal to the gel volume. Unbound [^{125}I]ConA was washed out with ConA buffer in the absence (probes) or presence (control) of 20 mg of α-methyl-D-mannoside per ml. The radioactive bands were visualized by autoradiography (20).

Peptide mapping by limited proteolysis in an SDS-polyacrylamide gel. ^{125}I-labeled PRV was immunoprecipitated with monoclonal antibody 5/14. After elution of the antigen, a preparative 8.75% SDS-PAGE was run, fixed for 20 min, and exposed overnight at room temperature. The three antigen bands were located with the aid of the autoradiograph and cut out. The gel slices were placed onto a 15% polyacrylamide gel (acrylamide-to-bisacrylamide ratio, 40:1) containing a 4.5-cm stacking gel. The slices were overlaid with 1 or 5 μg of *S. aureus* V8 protease and electrophoresed as described previously (5).

Western blots. PRV proteins from gradient-purified virions (applied at a rate of 0.10 to 0.15 μg of protein per mm^2 of gel surface [2]) were separated by SDS-PAGE. The gel was renaturated (31), and proteins were transferred electrophoretically to nitrocellulose sheets (pore size, 0.2 μm; Schleicher and Schüll, Dassel, Federal Republic of Germany), with a Bio-Rad destainer (Bio-Rad Laboratories, Richmond, Calif.) as power supply. Free binding sites on the nitrocellulose membrane were blocked by incubation with 1% (wt/vol) bovine serum albumin, and the strips were incubated overnight with 5 to 10 ml of hybridoma supernatant at room temperature. A wash with PBS, containing 0.1% Triton X-100, was followed by an incubation with peroxidase-conjugated F(ab')2 fragment of goat anti-mouse immunoglobulin G (Tago Inc.) for 2 h at room temperature. Bound enzyme was located with chloronaphtol (0.3 mg/ml) and H_2O_2 (0.015%).

RESULTS

Glycoproteins of PRV. The structural glycoproteins of PRV Phylaxia were identified in SDS-polyacrylamide slab gels on the basis of [^{125}I]ConA binding (Fig. 1a). PRV was isolated from either MDBK or NRK cells. Four major glycopolypeptides were detected under reducing conditions with MWs of 122K, 90K, 67K, and 58K (Fig. 1a, lane 5, and 6); the same set of glycopolypeptides was found after in vivo [^{3}H]glucosamine labeling of PRV or periodic acid-Schiff's

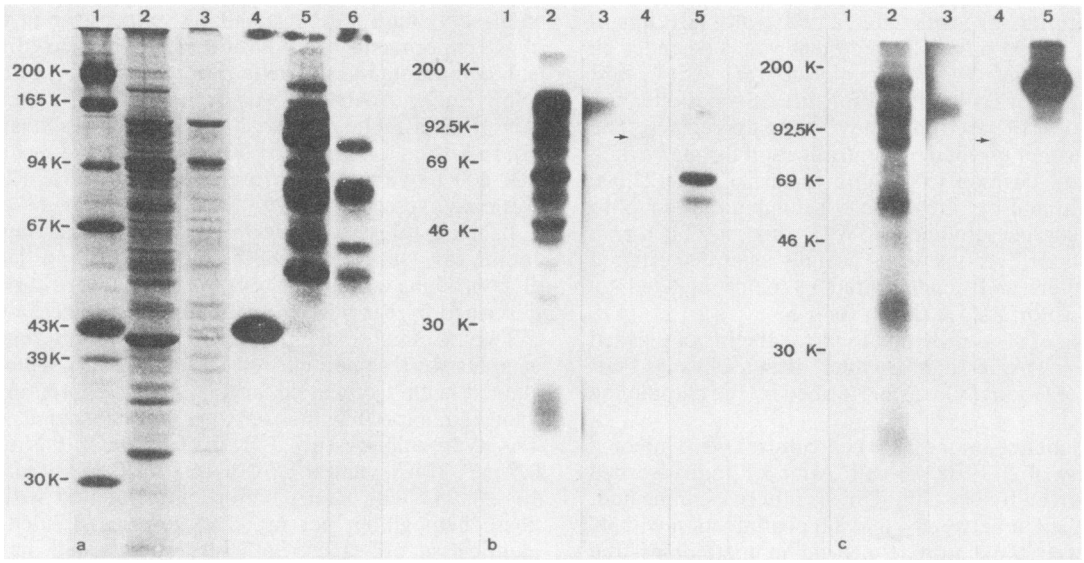

FIG. 1. Major glycoproteins of PRV. (a) Virus proteins and glycoproteins. Protein standards (lanes 1 and 4) and proteins of purified virions isolated from MDBK (lanes 2 and 5) or NRK (lanes 3 and 6) cells were separated in 10% SDS-PAGE under reducing conditions. The photograph of Coomassie blue stained gel (lanes 1 to 3) and the autoradiogram of the same gel after incubation with [^{125}I]ConA (lanes 4 to 6) is shown. MWs of protein standards are indicated on the left. (b) Autoradiogram of glycoproteins after immunoprecipitation and electrophoresis under reducing conditions. Purified virions were iodinated, lysed, and immunoprecipitated with pig normal serum (lane 1), pig hyperimmune serum against PRV (lane 2), and with the anti-glycoprotein monoclonal antibodies 3/6 (lane 3), 3/15 (lane 4), and 5/14 (lane 5). (c) Autoradiogram of glycoproteins after immunoprecipitation and electrophoresis under nonreducing conditions. Lanes 1 to 5 correspond exactly to those in (b), except that electrophoresis was carried out in the absence of 2-mercaptoethanol.

staining in SDS slab gels (not shown). In lane 5, a number of bands in addition to those of the four major glycoproteins are visible. The apparent difference between lane 5 (PRV isolated from MDBK cells) and lane 6 (PRV isolated from NRK cells) in Fig. 1a is mainly due to the different protein concentration of probes (compare with corresponding Coomassie-stained lanes 2 and 3). After prolonged exposure, almost the same pattern of minor bands could be seen in lane 6. It is not yet clear whether the minor glycoprotein bands represent additional viral glycoprotein species, contaminating host proteins, partially glycosylated viral products, or dimer formation. The control for nonspecific binding of [^{125}I]ConA (see above) was completely negative (not shown).

To further characterize the PRV glycoproteins, we produced a panel of monoclonal antibodies against them. The antigen specificity of the antibodies was determined by immune precipitation and by Western blotting. A total of 18 hybridoma lines were cloned, which could be divided into three groups according to specificity: group I (12 hybridoma lines), group II (1 line), and group III (5 lines). One antibody from each group was selected for the immunoprecipitation experiments in this paper: 3/6 from group I, 3/15 from group II, and 5/14 from group III. Our selection criteria for the 3/6 and 5/14 antibodies were good activity in immunoprecipitation and, in the case of 3/6 antibody, ability to recognize the unprocessed precursor after in vitro translation. Although the immunoprecipitation activity of the 3/15 antibody was much poorer than that of the other two antibodies, it was used because it was the only member of that group. An antibody designated P11 ([28] a kind gift of P. Symmons) was used as a negative control; it reacts with a *Drosophila* nuclear protein.

All four major glycopolypeptides described above could be found in the immune precipitates formed with our monoclonal antibodies. Antibodies 3/6 and 3/15 precipitated the 122K and 90K glycopeptides, respectively (Fig. 1b, lanes 3 and 4). The composition of the 5/14 immunoprecipitate was more complex: it comprised the two remaining major glycopolypeptides of 67K and 58K and, in addition an antigen with an apparent MW of 120K (Fig. 1b, lane 5). When electrophoresis was carried out under nonreducing conditions, the 122K and 90K antigens remained essentially unaltered (Fig. 1c). However, the 67K and 58K bands disappeared, and a new glycopolypeptide emerged at 155K (Fig. 1c, lane 5), indicating that the antigen recognized by the 5/14 monoclonal antibody is a disulfide-linked glycoprotein complex. In addition to the intense 155K glycopolypeptide, a weaker antigen band near 120K was also present.

We designated the major structural glycoproteins of PRV gA (122K), gB (90K), and gC (MW under nonreducing conditions, 155K), and the three gC glycopolypeptides seen under reducing conditions as gC_0 (120K), gC_1 (67K) and gC_2 (58K). We suppose gC_0 to represent a glycosylated precursor of the fully processed gC protein. Below, we present evidence to support this hypothesis.

Pulse-chase experiments. To establish precursor-product relationships for each of the three PRV glycoproteins, infected cells were pulse-labeled, lysed after different chase times, immunoprecipitated with monoclonal antibodies, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The results shown in Fig. 2 were obtained after 30 min of pulse-labeling. After pulse-labeling for 15 min, the protein bands at 122K for gA and at 120K, 67K, and 58K for gC were less intense, but the results were otherwise identical.

The earliest form of gA identifiable in vivo was an 86K protein, slightly larger than the nonglycosylated pgA precursor precipitated after in vitro translation (82K) (Fig. 2, in vitro translation). Fully glycosylated gA at 122K could already be seen after 30 min of pulse-labeling (Fig. 2, lanes 2).

No precursor(s) for gB could be identified in pulse-chase experiments, maybe because the very low affinity of the

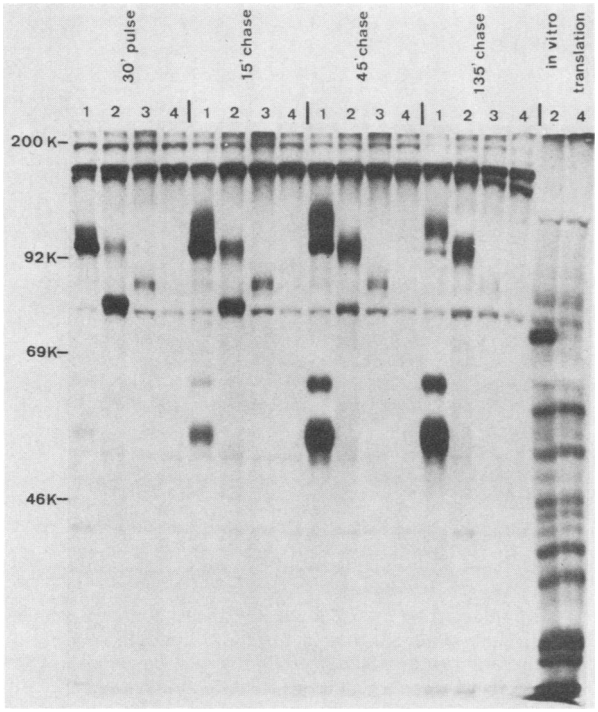

FIG. 2. Precursor forms of the major PRV glycoproteins, identified by immunoprecipitation after pulse labeling. NRK cells were infected with PRV (20 PFU per cell) and pulse labeled with 100 μCi [^{35}S]methionine per ml at 2 h p.i. Infected cells were lysed after 30 min of pulse-labeling or after 30 min of pulse-labeling followed by a 15-, 45-, and 135-min chase. Immunoprecipitation was carried out with the 5/14 anti-gC (lanes 1), 3/6 anti-gA (lanes 2), 3/15 anti-gB (lanes 3) or P11 control (lanes 4) monoclonal antibodies. Each panel shows an identical set of immunoprecipitates. The fluorogram of immunoprecipitated pgA after in vitro translation is shown on the right.

antibody prevented successful immune precipitation (Fig. 2, lane 3).

We found only one precursor for the gC glycoprotein complex, (MW, 110K) which was processed, most probably by glycosylation, to 120K at the same time as the gC_1 (67K) and gC_2 (58K) subunits began to appear (Fig. 2, lanes 1). At prolonged chase times, the intensity of the 110K and 120K bands decreased, and the 67K and 58K bands became more prominent. After an overnight chase, the 110K precursor was no longer detectable (data not shown).

Inhibition of glycoprotein processing with monensin. Monensin is an Na^+-selective carboxylic polyether ionophor (26) that induces disruption of cellular Na^+-K^+ gradients. Despite its pleiotropic action on cellular metabolism, monensin is often used in glycosylation studies, because it inhibits the transport of glycoproteins and viral particles through the Golgi complex and has an effect on the processing of some glycoproteins (12, 13, 34). Monensin interfered with the processing of the gA, gB, and gC PRV glycoproteins (Fig. 3). The polypeptides precipitated by the 3/6 and 3/15 antibodies from monensin-treated cells migrated more rapidly in SDS-PAGE than those from untreated cells (Fig. 3, lanes 3 and 4). These proteins are presumably differently glycosylated from the mature glycoproteins; they might represent precursors, underglycosylated or abortively glycosylated forms. For convenience, we designated them with the prefix "p". pgA had a MW of 108K and pgB had a MW of 79K. The underglycosylated form of gB (79K) was also found in gradient-purified virus preparations from uninhibited cells by Western blotting. Besides the gA and pgA bands at 122K and 108K, additional glycoprotein bands at 60K and 51K to 56K, respectively are present in lanes 3. These proteins may be disintegration products of gA and pgA, because they are only occasionally present in immunoprecipitates from cell lysates.

Monensin showed some differences in its effect on the glycosylation of gC in independent experiments (Fig. 3a and b and Fig. 4), however, two important features of its inhibition were common to all experiments: (i) the high-MW protein bands at 110K and 115K were strongly enhanced, and (ii) the amount of low-MW pgC_1 and pgC_2 subunits was significantly reduced. A typical experiment is demonstrated in lanes 5 of Fig. 3. In the control, three major glycopolypeptides appeared with similar intensity: gC_0 at 120K, gC_1 at 67K and gC_2 at 58K, and a weak band at 110K. After monensin treatment, the dominant glycoprotein migrated in 110K to 115K region, and only very faint pgC_1 and pgC_2 bands were seen, moving slightly faster than fully glycosylated gC_1 and gC_2. In some experiments the pgC_1 and pgC_2 subunits were more pronounced, and two additional bands at 50K and 20K emerged in monensin inhibited and in control samples as well (Fig. 4 [with 2-mercaptoethanol], lane 2). It is not yet clear whether the 50K or 20K polypeptides or both represent underglycosylated proteins relevant in vivo or whether they simply originate from an elevated proteolytic activity of host cells in vivo or in vitro. Under nonreducing conditions, the most strongly represented gC species was a glycoprotein at 115K in monensin inhibited cells. A continuous distribution of glycosylated products with MWs between 110K and 144K was observed with discrete bands at 144K and, occasionally at 132K (Fig. 4 (without 2-mercaptoethanol).

Antigenic relationship of the gC_0, gC_1, and gC_2 glycopolypeptides. The results of the pulse-chase and monensin inhibition experiments allow different models for the composition of the gC complex depending upon whether an antigenic relationship between gC_0 and gC_1-gC_2 exists or whether the monoclonal antibody used recognizes an antigenic determinant on gC_0 only. To decide which alternative was correct, we used Western blots to investigate which of the three gC polypeptides reacted with the 5/14 and three other anti-gC monoclonal antibodies. As shown in Fig. 5 (lanes 2 and 4), the N12 and 2/22 antibodies were directed against antigenic determinants present on gC_1 and on gC_0 as well. A further antibody (N3) reacted with gC_2 and gC_0 (lane 3). The 5/14 antibody, which was used for the immunoprecipitations shown in the previous sections, gave a weak reaction only with gC_1 (lane 5). In all experiments the amount of antibody bound by gC_0 was clearly less than that bound by gC_1 and gC_2. This is presumed to be the result of poor transfer of this high-MW component in the Western blots and the underrepresentation of gC_0 in comparison to gC_1 and gC_2 in purified virions (Fig. 1). The finding that all three monoclonal antibodies reacted both with gC_0 and either gC_1 or gC_2 indicates an antigenic relationship between gC_0 and gC_1-gC_2, and taken together with the previous results suggests that gC_0 might be a precursor of gC_1 and gC_2.

Peptide mapping of gC_0, gC_1, and gC_2 glycopolypeptides. To elucidate structural relationships between the gC_0, gC_1, and gC_2 glycopolypeptides peptide maps were made by limited proteolysis with *S. aureus* V8 protease in SDS-polyacrylamide gels (5). Two protease concentrations (1 and 5 μg) were used to minimize the error arising from coincidental comigration of structurally unrelated peptides. The re-

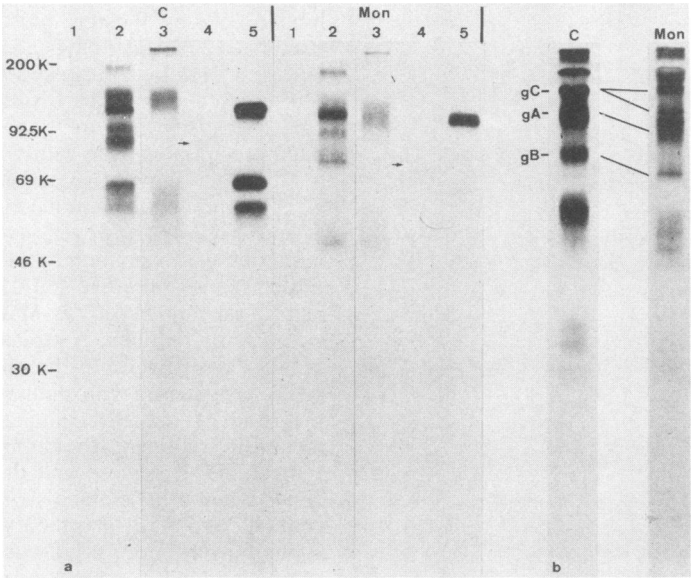

FIG. 3. Inhibition of posttranslational processing by monensin. NRK cells were infected with PRV at 20 PFU per cell and preincubated in glucose-free DMEM from 1 to 4 h p.i. Infected cells were labeled with D-[6-^{3}H]glucosamine at a concentration of 0.5 mCi per ml in the absence (C) or presence (Mon) of 1 μM monensin from 4 to 8 h p.i. Immunoprecipitation from labeled cell lysates was carried out with pig control serum (lane 1), pig hyperimmune serum (lane 2), 3/6 anti-gA 3), 3/15 anti-gB (lane 4), or 5/14 anti-gC (lane 5) monoclonal antibodies. (a) Fluorogram of immunoprecipitates after electrophoresis in 10% SDS-PAGE under reducing conditions. (b) Glycoprotein pattern after immunoprecipitation with pig hyperimmune serum and electrophoresis under nonreducing conditions.

sults are shown in Fig. 6. At the lower enzyme concentration three major and at least one minor peptides were common to gC_0 and gC_2 (lanes 1 and 3), in addition, gC_0 also shared a characteristic double band with gC_1 (lanes 1 and 2). The same double band was also present after digesting both the gC_0 and the gC_1 samples with 5 μg of V8 protease (lanes 4 and 5). The peptide pattern of gC_2 changed more strikingly than that of gC_1, but one of its dominant and two of its minor bands corresponded to gC_0 peptides (lanes 4 and 6). No common cleavage products of gC_1 and gC_2 could be identified at either protease concentration. These results suggest, that both gC_1 and gC_2 share homologous sequences with gC_0, but that they are not related structurally to each other.

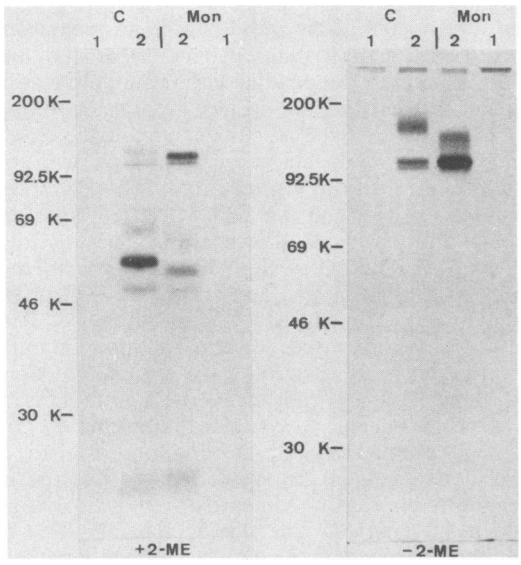

FIG 4. Influence of monensin on glycosylation and cleavage of gC. MDBK cells infected with PRV at 20 PFU per cell, were labeled with [^{35}S]methionine (100 μCi/ml) between 4 and 8 h p.i. in the absence (C) or in the presence (Mon) of 1 μM monensin. Fluorograms of immunoprecipitates obtained from lysates of infected cells by incubation either with P11 control monoclonal antibody (lanes 1) or 5/14 anti-gC monoclonal antibody (lanes 2) are shown after electrophoresis under reducing (with 2-mercaptoethanol [+2-ME]) or under nonreducing (without 2-mercaptoethanol [−2-ME]) conditions. The MWs of standard protein markers are indicated on the left.

DISCUSSION

The results presented here demonstrate the presence of at least three serologically different glycoproteins in pseudorabies virions, gA, gB, and gC, identified by their specific reaction with monoclonal antibodies. One of the glycoproteins, gC, is a disulfide-linked protein complex. In immune precipitates from purified virions we find two gC species, designated gC_1+gC_2 (MW, 155K) and gC_0 (MW, 120K) after electrophoresis under nonreducing conditions. In the presence of 2-mercaptoethanol, the 155K polypeptide dissociates into subunits gC_1 (67K) and gC_2 (58K). Thus, under reducing conditions, gA, gB, and gC resolve into four glycoprotein bands in SDS-PAGE with apparent MWs of 122K (gA), 90K (gB), 76K (gC_1), and 58K (gC_2); gC_0 migrates to almost exactly the same position as gA and is therefore not visible. These MWs correspond to those of the four major glycoprotein bands of purified virions (Fig. 1; references 1, 16, 18); therefore, we conclude that the antigens gA, gB, and gC are the major glycoproteins of pseudorabies virus.

Monoclonal antibodies were used to gain information about the biosynthesis of the major PRV glycoproteins and to characterize the gC complex. In pulse-chase experiments a gA precursor with a MW of 86K and a gC precursor with a MW of 110K (reducing conditions) was shown. In the presence of the Na^+-selective ionophore monensin, reduced glycosylation of all three major glycoproteins was observed. Monensin caused greatly increased formation of undergly-

cosylated gA and gB species with apparent MWs of 108K and 79K, respectively. Furthermore, it led to the accumulation of the 110 to 115K protein forms of gC and to depletion of the gC_1 and gC_2 subunits. Monensin has been shown to influence glycosylation by inhibiting transport through the Golgi complex (12). Although the sites glycosylation and of the budding process for herpes viruses have not been unequivocally identified, several authors have reported the inhibition of glycosylation and of virus release in herpes simplex virus infected cells by monensin and suggested that the Golgi complex plays a role in these processes (13, 30, 34). Our results suggest that such a scheme is also used in PRV-infected cells. In addition, if our hypothesis that gC_1 and gC_2 arise from a common precursor by proteolytic cleavage (see below) is correct, the Golgi complex may also be involved in the proteolytic cleavage of pgC.

One purpose for which we produced a set of monoclonal antibodies against the individual glycoproteins, was to use these specific reagents to identify the regions of the PRV genome coding for their precursors. The frequency of monoclonal antibodies which react with such precursors in immunoprecipitation seems to be rather low, as in the case of other herpes viruses (21). After in vitro translation we were able to precipitate a nonglycosylated precursor for gA (MW, 82K); the precursor was recognized by 2 of our 12 anti-gA monoclonal antibodies. It is not yet clear, whether the lack of immunoprecipitation after in vitro translation by both anti-gB and anti-gC antibodies was caused by the underrepresentation of pgB and pgC polypeptides in comparison to pgA in the translation system, or whether the respective antigenic determinants were not present or were inaccessible on the nonprocessed polypeptides. The existence of the pgA precursor-specific reagent has allowed us to localize the pgA-gene in the pseudorabies virus genome and to characterize the pgA polypeptide in different PRV strains (22a).

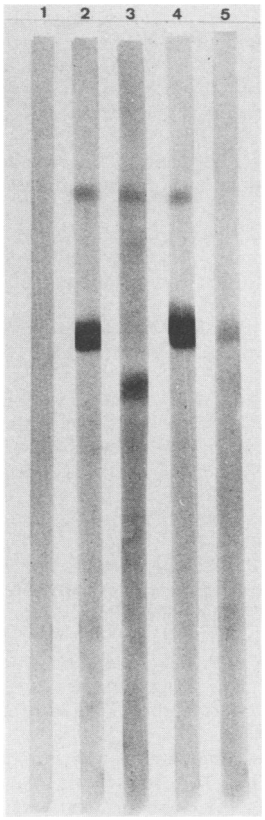

FIG. 5. Reaction pattern of four different anti-gC monoclonal antibodies on Western blots. Proteins of gradient-purified PRV were separated in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose sheets. Nitrocellulose strips were incubated overnight with 10 ml supernatant of control (1), N12 (2), N3 (3), 2/22 (4) or 5/14 (5) hybridoma lines, respectively. Bound antibody was visualized as described in the text.

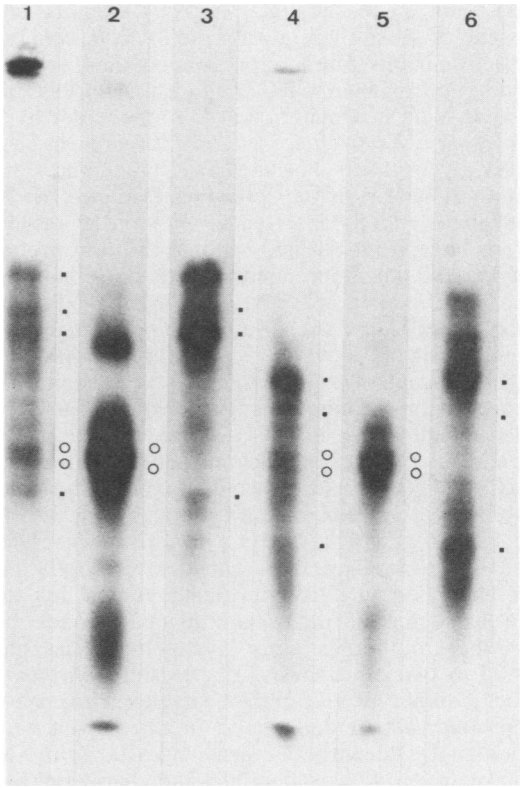

FIG. 6. Peptide mapping of the gC_0, gC_1 and gC_2 glycopolypeptides by limited proteolysis in SDS-polyacrylamide gel. gC was precipitated from ^{125}I-labeled PRV by the 5/14 antibody and the three antigen bands separated in 8.75% SDS-PAGE. gC_0, gC_1, and gC_2 were cut out, placed onto a 15% polyacrylamide gel containing a 4.5-cm stacking gel, overlaid with *S. aureus* V8 protease and electrophoresed as described by Cleveland et al. (5). The proteins were digested with 1 μg (lanes 1 to 3) or 5 μg (lanes 4 to 6) of V8 protease. The peptide pattern of gC_0 is shown in lanes 1 and 4, of gC_1 in lanes 2 and 5, of gC_2 in lanes 3 and 6. Peptides shared between gC_0 and gC_1 (○) and between gC_0 and gC_2 (■) are indicated. Lanes 1 and 5 were exposed for 7 days, the remaining lanes for 3 days. There is a difference in intensity between lanes containing gC_0 (lanes 1 and 4) and those containing either gC_1 (lanes 2 and 5) or gC_2 (lanes 3 and 6), because no attempt was made to compensate for the lower yield of gC_0 (compared with that of gC_1 or gC_2) by radioimmunoprecipitation before protease treatment.

The structure of the gC glycoprotein and the relationship of its subunits was investigated in detail because of the more complex pattern, which it produced in immunoprecipitates. As described above, two antigens were precipitated by monoclonal antibodies: gC_0 (120K) and gC_1+gC_2 (155K), the latter glycoprotein dissociated into subunits gC_1 (67K) and gC_2 (58K) in the presence of 2-mercaptoethanol. These results might indicate either that the native gC complex

consist of three, i.e., gC_0, gC_1, and gC_2 subunits, or simply that some of these three glycoproteins have common antigenic determinants. Our interpretation of these results is that gC_1 and gC_2 are linked together by disulfide bridges and that they arise from a common high-MW precursor by proteolytic cleavage. Accordingly, gC_0 might represent an at least partially glycosylated, but uncleaved precursor. This interpretation is based on the following evidence. (i) Using a monoclonal antibody that reacts with the gC_1 subunit on Western blots from purified virions, the first product that could be radioimmunoprecipitated after pulse-labeling was a protein with an apparent MW of 110K in SDS-PAGE under reducing conditions. (ii) After inhibition of glycosylation with monensin an elevated amount of high-MW (>110K) antigens (presumably related to gC_0) was precipitated by the same monoclonal antibody, even in experiments where the smaller subunits (gC_1 and gC_2) were hardly detectable. (iii) Independently isolated monoclonal antibodies reacted either with gC_0 and gC_1 or with gC_0 and gC_2 on Western blots. No cross-reaction between gC_1 and gC_2 was seen. (iv) Limited proteolysis of gC_0, gC_1, and gC_2 gave rise to peptide bands shared between gC_0 and gC_1 and between gC_0 and gC_2. These findings strongly support our precursor hypothesis.

The existence of interchain disulfide bonds between homodimers of herpes virus glycopolypeptides has been reported in two other cases. Eberle and Courtney (7) described a dimer of the herpes simplex virus type 2 gC glycoprotein, which dissociated in the presence of mercaptoethanol. Recently a varicella-zoster virus specific glycoprotein, gp140 was identified and characterized. gp140 was cleaved into a gp66 under reducing conditions, however, no other cleavage products could be shown (10). To our knowledge, among herpes virus glycoproteins, the gC complex of PRV with its structurally and antigenically different subunits is the first disulfide-linked glycoprotein heterodimer to be described.

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ADDENDUM IN PROOF

After submission of this manuscript, two groups (14, 32) reported on glycoproteins of PRV. In addition, Hampl et al. (11) have suggested a different nomenclature for PRV structural glycoproteins: gI instead of gA, and gII instead of gC complex.

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