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Processing of Pseudorabies Virus Glycoprotein gII

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The glycoprotein complex gII of pseudorabies virus was isolated by immunoprecipitation with the monoclonal antibody M5, which was covalently linked to protein A-Sepharose. After sodium dodecyl sulfate-polyarylamide gel electrophoresis under reducing conditions and blotting onto poly(vinylidene difluoride) membrane, its subunits, gIIa, gIIb, and gIIc, were subjected to N-terminal sequencing. gIIa and gIIb start at position 59 and gIIc starts at position 503 according to the amino acid sequence deduced from the gene, indicating that there is one major protein (gIIa) which is cleaved into the two protein fragments gIIb and gIIc. Protein labeling with ^{14}C-amino acids gave no indication that the three proteins (gIIa, gIIb, and gIIc) of the complex are present in equimolar ratios. It seems that gIIa is only a minor component of the complex, whereas gIIb and gIIc are contained in equimolar amounts.

Pseudorabies virus (PRV) causes Aujeszky's disease in swine. It is a member of the herpesvirus group. In its membrane it contains, besides other glycoproteins and non-glycosylated proteins (1), the glycoprotein complex gII, which was first described by Hampl et al. (1) and Lukàcs et al. (6). It consists of three glycoproteins, called gIIa, gIIb, and gIIc (1), with molecular masses of 110 to 120, 68, and 55 kilodaltons (kDa), respectively, which are covalently linked by disulfide bonds (1, 6). It was shown that gIIb and gIIc are cleavage products of gIIa (1, 6). First, a precursor protein of 100 kDa was made and subsequently glycosylated and processed to gIIa, gIIb, and gIIc (7). The gII gene was identified and sequenced by Robbins et al. (8). It shows strong homology to herpes simplex virus glycoprotein gB (8) and homologies of various extents to related proteins of other herpesviruses (9, 13).

It was predicted by Robbins et al. (8) that the mature gII protein (gIIa) should start with amino acid 54 (proline). They predicted further that gIIa is cleaved somewhere between amino acids 498 and 502 with the sequence Arg-Arg-Ala-Arg-Arg. This sequence is not present in herpes simplex virus glycoprotein gB, leaving this protein uncleaved. The corresponding proteins of other herpesviruses which are cleaved show the common sequence Arg-Arg-Ser (13), which are the amino acids of positions 501 to 503 in PRV gII. It was shown by N-terminal sequencing of protein gpII of varicella-zoster virus and of protein gB of cytomegalovirus that these proteins are cleaved between the second arginine and the serine (4, 11).

To get information on the correlation between gIIa, gIIb, gIIc, and the precursor protein of the complex, the three gII proteins were isolated and subsequently the N-terminal region was sequenced. In addition, labeling experiments were performed to investigate the stoichiometry of the individual proteins in the gII complex.

RK13 cells were grown in Eagle medium containing 10% fetal calf serum. PRV strain Ka (Kaplan and Vatter [3]) was grown on RK13 cells in 10-cm petri dishes. Cells were infected with a multiplicity of infection of 0.3, and virus was collected after complete cytopathic effect, which was reached 48 h postinfection. After low-speed centrifugation (10 min, 2,200 × *g*) of pooled, harvested, PRV-infected RK13 cells, the cell-free supernatant and cell debris were used for extraction of viral proteins. From supernatants, virus was concentrated and roughly purified by centrifuging for 1 h at 22,000 × *g* (13,500 rpm in an SW27Ti rotor; Beckman Instruments, Inc.). The pelleted virus was suspended by incubation for 20 min at 45°C in 1% Triton X-100 in phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2). Subsequently, undissolved material was removed by spinning for 1 h at 15,000 × *g*.

To extract virus proteins from the cell debris, the same steps for suspending (1% Triton X-100, 45°C) were used. The suspension was clarified by two-step centrifugation, first for 10 min at 3,000 × *g* and then for 1 h at 15,000 × *g*.

For immunoprecipitation of gII, we used the monoclonal antibody M5 (Hampl et al. [1]), which recognizes the gII complex as well as the gII precursor. This antibody was linked covalently to protein A-Sepharose (Schneider et al. [10], modified). M5 ascites fluid, containing about 3 mg of antibodies per ml, was mixed with protein A-Sepharose in 180 mM Tris-borate buffer (pH 8.2) (TBB) and incubated for 40 min at room temperature with gentle shaking. The suspension was washed two times with TBB and two times with 0.2 M triethanolamine (pH 8.2).

The antibody-protein A-Sepharose complex was cross-linked by incubation for 45 min with dimethyl pimelimidate dihydrochloride in 0.2 M triethanolamine (pH 8.2) at room temperature with gentle shaking. The reaction was stopped by pelleting the Sepharose and incubating for 5 min in 20 mM ethanolamine in triethanolamine (pH 8.2). Thereafter, the Sepharose was washed once with TBB, once with 0.05 M diethylamine at pH 11.5 containing 0.5% sodium deoxycholate, and three times with TBB. The antibodies coupled to protein A-Sepharose were stored in TBB containing 0.02% NaN_3.

Immunoprecipitation was done as follows. The M5-protein A-Sepharose was washed once with phosphate-buffered saline. The pelleted Sepharose was then incubated for 90 min at 4°C with solubilized PRV protein prepared either from pelleted PRV or from infected cell lysates. The Sepharose

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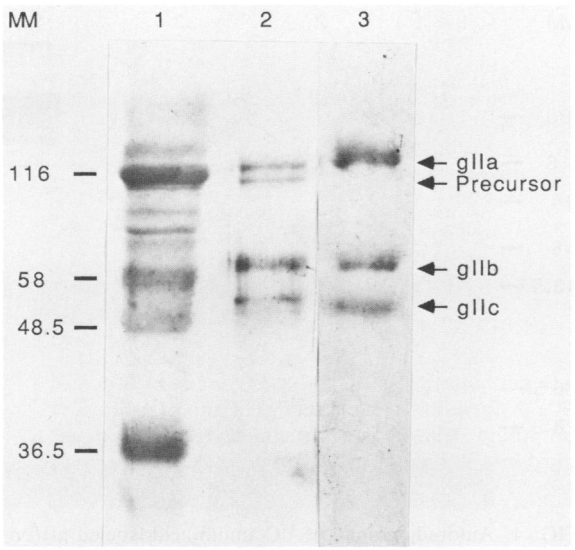

FIG. 1. Western blot (immunoblot) of immunoprecipitated and reduced gII complex stained with Pelikan India ink (2) for better visibility. Lanes: 1, molecular mass standards; 2, gII isolated from infected cell lysates; 3, gII isolated from purified virions. Numbers on the left side indicate the molecular masses (MM) of the standards in kilodaltons.

was washed seven times with NT buffer (50 mM Tris [pH 7.4], 150 mM NaCl, and 0.05% Triton X-100). Bound protein was eluted with 50 mM diethylamine at pH 11.5 containing 0.5% sodium deoxycholate. The eluate was instantly neutralized with 0.5 M NaH_2PO_4. The protein was precipitated with 20% trichloroacetic acid, pelleted for 15 min at 15,000 × *g*, washed once with acetone (−20°C), and centrifuged again for 15 min at 15,000 × *g*. The pellet was suspended in 1% sodium dodecyl sulfate (SDS) in 0.1 M Tris at pH 8.0 and mixed with sample buffer for electrophoresis. The sample buffer contained 5% β-mercaptoethanol for dissociating the gII complex into its subunits. The sample was boiled for 3 min and separated by 10% SDS-polyacrylamide gel electrophoresis (Laemmli [5]).

Electrophoresis and blotting were done as described by Walsh et al. (12) with slight modifications. The gel was prerun overnight at 1.5 V/cm with running buffer (27.5 mM Tris, 186 mM glycine, pH 8.4) containing 0.07% thioglycolic acid. Electrophoresis was carried out without thioglycolic acid at 60 mA at 18°C.

After the gel was equilibrated in blotting buffer (25 mM Tris [pH 8.4], 0.5 mM dithioerythritol, and 0.02% SDS), proteins were blotted onto a poly(vinylidene difluoride) transfer membrane (Millipore Corp.) for 1 h at 150 mA and for an additional 4 h at 650 mA in a transfer chamber (LKB Instruments) at 8°C. The blot was washed three times with water, stained for 2 min with 0.1% amido black in 50% methanol, and destained in 30% methanol (Fig. 1).

The blot was dried, and the bands corresponding to proteins gIIa, gIIb, and gIIc were excised and subjected to N-terminal sequencing in a pulsed-liquid-phase sequencer (model 477A; Applied Biosystems, Inc.) equipped with a model 120 phenylthiohydantoin-amino acid analyzer.

Sequencing results of blots from two independent blotting experiments are summarized in Fig. 2. The sequences can unambiguously be aligned to the deduced amino acid sequence of the gII gene (8).

The data show that proteins gIIa and gIIb have identical N termini. The N-terminal residue of proteins gIIa and gIIb is alanine 59 in the sequence of the precursor protein. The leader sequence is slightly longer than that predicted by Robbins et al. (8). Serine 503 is the N-terminal residue of protein gIIc, thus confirming the consensus cleavage site known from related proteins of other herpesviruses.

With respect to the data presented, one could postulate that after the precursor is processed to gIIa, this protein is cleaved between positions 502 and 503 to gIIb and gIIc. Thus, gIIa consists of 855 amino acids (positions 59 to 913), gIIb consists of 444 amino acids (positions 59 to 502), and gIIc consists of 411 amino acids (positions 503 to 913). From these data, the molecular masses of the protein subunits gIIa, gIIb, and gIIc can be calculated to be about 94, 48.8, and 45.2 kDa, respectively.

The differences from the apparent molecular masses on SDS gels are caused mainly by glycosylation of the proteins, which can be shown by deglycosylation of gII with *N*-glycosidase F (Fig. 3). RK13 cells were infected with PRV for 30 min at a multiplicity of infection of 3. Labeling was performed by supplying the infected cells with 25 μCi of ^{35}S-methionine per ml of Eagle medium without methionine containing 3.5% fetal calf serum. PRV and subsequently gII were isolated as described above. gII was incubated for 24 h at 37°C in phosphate-buffered saline containing 0.5% Triton X-100, 10 mM EDTA, and 1% β-mercaptoethanol. For deglycosylation, 0.2 U of *N*-glycosidase F was added to the buffer during the incubation. After deglycosylation, the apparent molecular masses were about 100 kDa for gIIa, 55 kDa for gIIb, and 46 kDa for gIIc (Fig. 3, lane 2).

To evaluate the stoichiometry of the proteins in the gII complex, the following experiments were performed.

For production of ^{14}C-amino acid-labeled gII, RK13 cells were starved in Eagle medium without amino acids. The

Sequence from gene:													
Position:	59	60	61	62	63	64	65	66	67	68	69	70	71
Amino acid:	A	A	V	T	R	A	A	S	A	S	P	T	P
N-terminal sequencing:													
gIIa:	A	?	V	T	?	A	A	S	A	?	P	?	P
gIIb:	A	?	V	?	R	A	A	S	A	S	P	T	P
gIIc:	S	P	G	P	A	G	T	P	E	P	P	A	V
Sequence from gene:													
Amino acid:	S	P	G	P	A	G	T	P	E	P	P	A	V
Position:	503	504	505	506	507	508	509	510	511	512	513	514	515

FIG. 2. Comparison of amino acid sequences of purified PRV gII proteins.

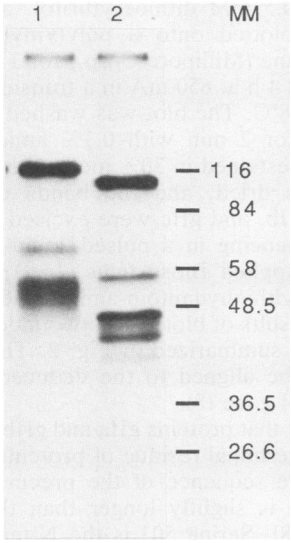

FIG. 3. Autoradiography of ^{35}S-methionine-labeled gII on SDS gel. Lanes: 1, gII, not deglycosylated; 2, gII, deglycosylated with *N*-glycosidase F. Numbers on the right side indicate molecular masses (MM) in kilodaltons.

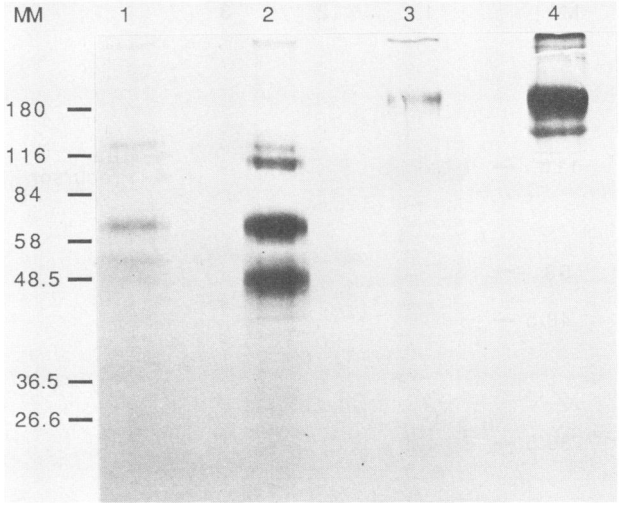

FIG. 4. Autoradiography of ^{14}C-amino acid-labeled gII on SDS gel. Lanes: 1 and 3, gII isolated from purified virions (reduced and unreduced, respectively); 2 and 4, gII isolated from infected cell lysates (reduced and unreduced, respectively). Numbers on the left side indicate molecular masses (MM) in kilodaltons.

cells were infected with PRV for 30 min at a multiplicity of infection of 3. PRV was removed, and cells were overlaid with Eagle medium without amino acids containing 3.5% dialyzed fetal calf serum and 2.5 μCi of ^{14}C-protein hydrolysate (purchased from Amersham Corp.) per ml. Cell debris and PRV were collected after complete cytopathic effect (24 h postinfection). Preparation of ^{14}C-PRV and ^{14}C-labeled infected cell lysates and the subsequent immunoprecipitation of ^{14}C-gII were done as described for unlabeled material (see above).

Precipitates of labeled gII were dissolved in sample buffer either containing β-mercaptoethanol (for dissociating the complex) or without β-mercaptoethanol (for maintaining the complex). The samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and subsequently fixed, washed, and dried. The gel was subjected to autoradiography (Fig. 4).

The separated protein bands of gIIa, gIIb, gIIc, the complex, and the precursor protein (Fig. 4) were cut out, and the radioactivity of the gel slices was determined in a liquid scintillation counter (Packard Instrument Co., Inc.) (Table 1).

According to their numbers of amino acid residues (see above), the relative amounts of gIIa, gIIb, and gIIc should be 2.08:1.08:1.00, if the complex consists of one molecule of each of the three. Assuming that the proteins are uniformly labeled (integration of ^{14}C-labeled material into carbohydrates is unlikely because of the low concentration of amino acids), the relative amounts of radioactivity should represent the protein portion of the glycoproteins. The sum of the radioactivity of gIIb plus gIIc should be equal to the radioactivity of gIIa. In two experiments, we found relative amounts of radioactivity of gIIa, gIIb, and gIIc of 0.36:1.10:1.00 and 0.14:1.16:1.00. In both experiments, the results were within the range of expectations for the quantities of gIIb and gIIc, indicating that the equimolar available proteins (gIIb and gIIc) derive from gIIa. In contrast to the assumption that gIIa, gIIb, and gIIc are equimolar in the complex, in our experiments an average of only 10% of the radioactivity of the complex could be defined as deriving from gIIa, whereas 48% derived from gIIb and 42% derived from gIIc.

In controls, the same amounts of ^{14}C-labeled gII complex were separated by SDS-polyacrylamide gel electrophoresis. The sum of the radioactivities of all three proteins should equal the radioactivity of the complex. As listed in Table 1, this is shown for gII extracted from cell debris (sum of counts of gIIa, gIIb, and gIIc [2,603 cpm] versus counts of the complex [2,601 cpm]). The data found for gII extracted from PRV show a difference (330 versus 202 cpm) which can be explained methodically (low counts in that specific experiment).

In summary, we conclude that the gII precursor is processed to gIIa, which subsequently is cleaved to gIIb and

TABLE 1. Relative amounts of gII subunits

gII (source)	Relative amt (cpm ± SD)[a] of:				
	Complex	Precursor	gIIa	gIIb	gIIc
Reduced (PRV)	–	27 ± 5	48 ± 6	148 ± 11	134 ± 10
Nonreduced (PRV)	202 ± 13	–	–	–	–
Reduced (infected cell lysates)	–	327 ± 16	163 ± 12	1,311 ± 32	1,129 ± 30
Nonreduced (infected cell lysates)	2,601 ± 46	436 ± 19	–	–	–

[a] Of ^{14}C-amino acid-labeled proteins. –, Not present under these conditions.

gIIc. The complex which is formed by these three proteins contains gIIb and gIIc in the same amounts but does not, in most cases, contain the gIIa protein. The complex appears on SDS gels as a band of 170 kDa (Fig. 4, lanes 3 and 4). With respect to the apparent molecular mass of the complex on SDS gels, the existence of three different complexes is possible: (i) two molecules of gIIb plus two molecules of gIIc; (ii) one molecule of gIIa, one molecule of gIIb, and one molecule of gIIc, and (iii) dimers of gIIa. Our results indicate that (ii) and (iii) represent only about 10% of the complex formed. Whether the complex forms higher oligomers that are not covalently linked cannot be decided on the basis of this experiment.

The process of complex formation itself is more likely to occur by dimerization of gIIa or possibly by dimerization of the precursor and following cleavage than by assembly of readily cleaved gIIb and gIIc. As preliminary data show, the precursor that we find on SDS gels carries high-mannose carbohydrate residues, as indicated by its reactivity to *Galanthus nivalis* agglutinin, a lectin that recognizes terminal mannose residues (data not shown). It probably does not carry the leader sequence any more. Dimerization may even occur on the level of unglycosylated protein that we could not identify on the gel.

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