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Bovine Herpesvirus 1 U_S Open Reading Frame 4 Encodes a Glycoproteoglycan

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Sequence analysis of the short unique (U_S) segment of the bovine herpesvirus 1 (BHV-1) genome predicted that the U_S open reading frame (ORF) 4 encodes a protein with homology to glycoprotein G (gG) of other alphaherpesviruses (P. Leung-Tack, J.-C. Audonnet, and M. Riviere, Virology 199:409–421, 1994). RNA analysis showed that the U_S ORF4 is contained within two transcripts of 3.5 and 1.8 kb. The 3.5 kb RNA represents a structurally bicistronic RNA which encompasses the U_S ORF3 and U_S ORF4, whereas the 1.8-kb RNA constitutes the monocistronic U_S ORF4 mRNA. To identify the predicted BHV-1 gG, recombinant vaccinia virus expressing the U_S ORF4 was used to raise specific antibodies in rabbits. The antiserum recognized a 65-kDa polypeptide and a very diffusely migrating species of proteins with an apparent molecular mass of between 90 and greater than 240 kDa in supernatants of BHV-1-infected cells which was also precipitated together with 61- and 70-kDa polypeptides from cell-associated proteins. The specificity of the reaction was demonstrated by the absence of these proteins from the supernatant of cells infected with the U_S ORF4 deletion mutant BHV-1/gp1-8. Treatment of the immunoprecipitated proteins with glycosidases and chondroitinase AC showed that the 65-kDa protein constitutes gG, which contains both N- and O-linked carbohydrates, and that the high-molecular-mass proteins contain glycosaminoglycans linked to a 65-kDa glycoprotein that is antigenically related to gG. These molecules were therefore named glycoproteoglycan G (gpgG). Pulse chase experiments indicated that gG and gpgG were processed from a common precursor molecule with an apparent molecular mass of 61 kDa via a 70-kDa intermediate. Both gG and gpgG could not be found associated with purified virions. In summary, our results identify the BHV-1 gG protein and demonstrate the presence of a form of posttranslational modification, glycosamino-glycosylation, that has not yet been described for a herpesvirus-encoded protein.

Bovine herpesvirus 1 (BHV-1) is an alphaherpesvirus of cattle which can cause severe clinical symptoms including respiratory and genital tract infections, conjunctivitis, and abortions (for reviews, see references 16 and 44). Within the *Alphaherpesvirinae*, it is grouped into the genus *Varicellovirus* together with varicella-zoster virus, equine herpesviruses 1 and 4 (EHV-1 and EHV-4), and pseudorabies virus (PRV). The 140-kbp viral genomic DNA has the typical arrangement of group D herpesvirus genomes. It consists of a 106-kbp unique long segment (U_L) and a 10-kbp unique short region (U_S) which is flanked by inverted repeats of 11 kbp. Two isomeric forms of the genome, which are created by inversion of the U_S relative to the U_L, exist (28). BHV-1 expresses at least seven glycoproteins, of which only gB, gC, gD, and gH have been unequivocally assigned to their respective genes (3, 15, 41, 43).

In a recent report, Leung-Tack et al. (22) described the complete sequence of the U_S of BHV-1 strain ST. Sequence analysis revealed open reading frames (ORFs) whose deduced translation products exhibited homology to herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) glycoproteins gD, gE, gG, and gI. In contrast to BHV-1 gD, which has been analyzed in more detail (6, 13, 40), the nature and function of the gene products of BHV-1 U_S ORF4, U_S ORF6, and U_S ORF7, which are predicted to encode gG, gI, and gE (22), had not been described previously. Positional homologs of BHV-1 U_S ORF4 have been sequenced for several alphaherpesviruses, including asinine herpesvirus 3 (14), BHV-5 (19), EHV-1 (39), EHV-4 (8), HSV-1 (29), HSV-2 (30), PRV (32), simian herpes B virus (36), and simian herpesvirus SA8 (10). The respective proteins were found to be nonessential for replication of BHV-1 (18), HSV-1 (25), HSV-2 (17), and PRV (39). Whereas the BHV-1 U_S ORF4 deletion mutant exhibited an attenuated phenotype in cattle (18), gG-negative PRV is fully virulent in pigs (20).

To date, gG homologous proteins have been identified and characterized only for EHV-4 (8, 9), HSV-1 (1, 33), HSV-2 (2, 27, 30, 34, 37, 38), and PRV (4, 5, 32, 40). The last, which was originally named gX, is now also designated gG. With the exception of HSV-1 gG, and a 108-kDa cleavage product of HSV-2 gG, herpesvirus gG homologs are secreted into the medium of infected cells. Secretion of EHV-4 and PRV gG correlated with an increase in the mobilities of the respective proteins which was probably due to removal of a putative membrane anchor domain at the carboxy terminus by a host cell-specific protease (5, 8). Neither the secreted 34-kDa HSV-2 gG component nor PRV gG was found associated with purified virions. A hitherto unique feature of PRV gG is the addition of sulfate groups (5, 12, 31), which was hypothesized to occur at tyrosine residues of the protein backbone (5). In this report, we identify transcripts encompassing the BHV-1 U_S ORF4 and identify and characterize BHV-1 U_S ORF4 gene products.

MATERIALS AND METHODS

Cells and viruses. BHV-1 strain Schönböken was obtained from O. C. Straub (Tübingen, Germany) and was propagated on Madin-Darby bovine kidney cell clone Bu100 (MDBK-Bu100; kindly provided by W. Lawrence and L. Bello, Philadelphia, Pa.). Cells were grown in Dulbecco's minimum essential medium supplemented with 5% fetal calf serum, 100 U of penicillin, 100 μg of streptomycin, and 0.35 mg of L-glutamine per ml. CV-1 cells used for the propagation

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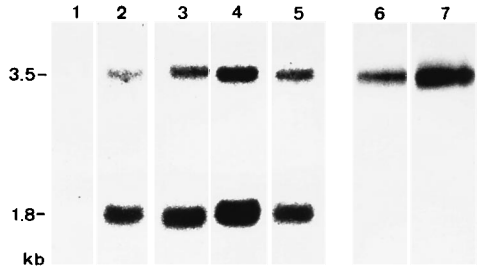

FIG. 1. Identification of transcripts encompassing the predicted U_S ORF4. Cytoplasmic RNA was isolated at 4 (lanes 1 and 2), 6 (lanes 3 and 6), and 12 (lanes 4, 5, and 7) h p.i. of MDBK-Bu100 cells with BHV-1 and transferred to nitrocellulose after agarose gel electrophoresis. Filters were hybridized to ^{32}P-labeled probes from U_S ORF4 (lanes 1 to 5) or U_S ORF3 (lanes 6 and 7). Bound radioactivity was visualized by autoradiography. RNAs isolated from cells infected in the presence of cycloheximide or PAA were assayed in lanes 1 and 5, respectively. The sizes of transcripts are indicated.

of vaccinia viruses were maintained in the same medium containing 10% fetal calf serum. The U_S ORF4 deletion mutant BHV-1/gp1-8 will be described in detail elsewhere. Briefly, in this mutant the sequences from 25 nucleotides upstream the U_S 4 TATA box until 173 nucleotides downstream the U_S 4 poly(A) signal (nucleotides 3143 to 4831 in reference 22) were deleted by site-specific recombination (19).

Construction of U_S ORF4-expressing vaccinia virus. All cloning procedures followed established methods (26). The plasmid for the generation of recombinant vaccinia viruses expressing BHV-1 U_S ORF4 was obtained by insertion of a 2.9-kbp *Sma*I fragment derived from the genomic *Hin*dIII L fragment (13) into the *Sma*I-cleaved vaccinia virus recombination vector pCS43. The resulting plasmid contains the complete U_S ORF4 downstream of the vaccinia virus p7.5 promoter. It was integrated into the genome of vaccinia virus strain WR according to established procedures. The recombinant vaccinia virus was named VacgG. Antisera against U_S ORF4 encoded proteins expressed by VacgG were collected 4 weeks after infection of rabbits with 5×10^7 PFU of the vaccinia virus recombinant.

Immunoprecipitation and glycosidase digestions. Confluent monolayers of MDBK cells grown in 6-well tissue culture dishes (Greiner, Frickenhausen, Germany) were infected with a multiplicity of infection of 10. After 1 h of adsorption, the inoculum was replaced by 500 μl of Dulbecco's minimum essential medium lacking methionine and cysteine. After the addition of [^{35}S]methionine (500 μCi/ml) and [^{35}S]cysteine (250 μCi/ml), cells were incubated for the times indicated, and purification of released virus particles and immunoprecipitation of proteins from cell lysates, cell culture supernatants, or purified virions were carried out as described elsewhere (13). Labeled proteins were visualized after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by fluorography (13). To remove N- or O-linked carbohydrates, immunoprecipitated glycoproteins were incubated overnight at 37°C with 0.4 U of N-glycosidase F (Boehringer, Mannheim, Germany) or 1.5 mU of O-glycosidase (Boehringer). For removal of high-mannose N-glycans, immunoprecipitates were digested with endo-β-*N*-acetylglucosaminidase (Boehringer) overnight at 37°C. All deglycosylation reactions were performed under conditions recommended by the supplier. Heparinase (Sigma, Munich, Germany) and chondroitinase AC (Sigma) digestions of immunoprecipitated proteins were performed in phosphate-buffered saline (PBS) for 5 h at 37°C. Reaction mixtures contained heparinase or chondroitinase AC at final concentrations of 25 and 2.5 U/ml, respectively.

Northern (RNA) blot hybridization. Isolation of cytoplasmic RNA, gel electrophoresis, and transfer to nitrocellulose were done as described previously (26). DNA probes used for hybridization were labeled with [α-^{32}P]dCTP by using a random-primed DNA labeling kit (Boehringer).

RESULTS

Transcript analyses. Northern blot hybridizations were performed to determine size and steady-state levels of mRNAs encompassing the U_S4 ORF predicted to encode gG of BHV-1 (22). Cytoplasmic RNA was isolated at different times after infection and in the absence or presence of cycloheximide (100 μg/ml) or phosphonoacetic acid (PAA; 250 μg/ml) and was probed with a 902-bp *Sal*I-*Nru*I DNA fragment containing codons 95 to 395 of the U_S ORF4. In RNA isolated at 4 h postinfection (p.i.), the probe hybridized to a 1.8-kb transcript and a 3.5-kb RNA of lesser abundance (Fig. 1, lane 2). Both transcripts were also detected in RNA isolated at 6 and 12 h p.i. (Fig. 1, lanes 3 and 4) and in RNA from cells infected in the presence of PAA and purified at 12 h p.i. (Fig. 1, lane 5). Hybridization to RNA from cells infected in the presence of cycloheximide was not observed (Fig. 1, lane 1). Thus, the 1.8- and the 3.5-kb RNAs are transcribed in the early phase of infection and remain present during the late phase. The reduction in intensities of the hybridization signals obtained with RNA synthesized in the presence of PAA at 12 h p.i. indicates that both transcripts originate from delayed-early regulated promoters. Single-stranded cRNA complementary to the U_S ORF4 also hybridized to the 1.8- and 3.5-kb RNAs, whereas no specific signal was obtained with cRNA transcribed in the sense direction (not shown). Hybridization of the U_S ORF4 probe to two mRNAs was not unexpected, because no consensus sequence for polyadenylation is found immediately downstream from the predicted U_S ORF3 of BHV-1 strains ST (22) and Schönböken (19). To support the assumption that the 3.5-kb RNA represents a structurally bicistronic transcript encompassing the U_S ORF3 and U_S ORF4, Northern blots were hybridized to ^{32}P-labeled DNA extending from codon 279 to 4 nucleotides downstream of the stop codon of the U_S ORF3. The result shown in Fig. 1, lanes 6 and 7, demonstrated that this probe recognized only the 3.5-kb RNA, leading to the conclusion that the 1.8-kb transcript constitutes the monocistronic U_S ORF4 mRNA.

Reactivity of the anti-VacgG serum with secreted proteins from cells infected with VacgG and BHV-1. To identify the U_S ORF4-encoded protein, a monospecific antiserum was raised in rabbits against a recombinant vaccinia virus expressing the U_S ORF4 (VacgG). The anti-VacgG serum and the corresponding preimmune serum were incubated with [^{35}S]methionine-cysteine-labeled proteins from the culture supernatant of VacgG (Fig. 2, lanes 1 and 2) and BHV-1-infected MDBK cells (Fig. 2, lanes 5 to 7). Immunoprecipitated proteins were visualized by fluorography after SDS-PAGE. As a control, proteins secreted from cells infected with vaccinia virus expressing BHV-1 glycoprotein D (VacgD) were also immunoprecipitated (Fig. 2, lanes 3 and 4). From the supernatant of VacgG-infected cells, the antiserum precipitated a 65-kDa protein (Fig. 2, lane 1) that was not recognized by the preimmune serum (Fig. 2, lane 2) and was not detected in supernatants from the VacgD-infected cells (Fig. 2, lanes 3 and 4). From the supernatant of BHV-1-infected cells, the anti-VacgG serum

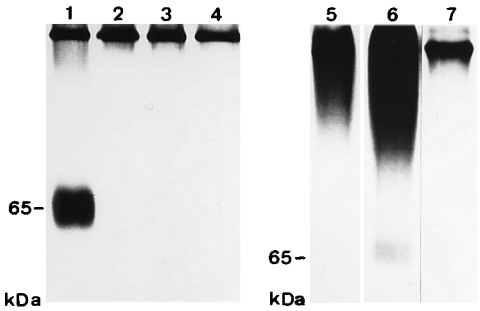

FIG. 2. Reactivity of anti-VacgG serum with proteins secreted from MDBK cells infected with vaccinia virus recombinants or BHV-1. MDBK cells were infected with vaccinia virus recombinants VacgG (lanes 1 and 2) or VacgD (lanes 3 and 4) or with BHV-1 (lanes 5 to 7), and were incubated from 2 to 10 h p.i. in the presence of [^{35}S]methionine-cysteine. Proteins from cell culture supernatants were precipitated with the anti-VacgG serum (lanes 1, 3, 5, and 6) or the corresponding preimmune serum (lanes 2, 4, and 7). Precipitated proteins were analyzed by SDS-PAGE and fluorography. Exposure times were 14 days for lanes 1 to 4, 3 days for lane 5, and 21 days for lanes 6 and 7. Lane 6 is a longer exposure of lane 5.

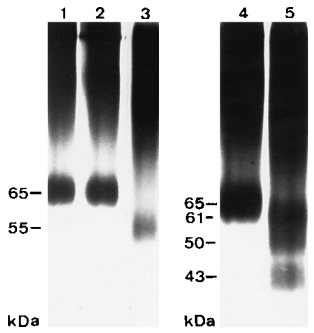

FIG. 3. Analysis of carbohydrates in gG. [^{35}S]methionine-cysteine-labeled proteins from BHV-1-infected cell culture supernatants were immunoprecipitated with the anti-VacgG serum and were separated on SDS-10% polyacrylamide gels after digestion with endo-β-*N*-acetylglucosaminidase H (lane 2), neuraminidase and O-glycosidase (lane 3), and N-glycosidase F (lane 5) or after incubation in PBS only (lanes 1 and 4). Labeled proteins were visualized by fluorography. Apparent molecular masses are indicated in kilodaltons.

precipitated proteins which migrated as a diffuse band ranging in size from approximately 90 to more than 240 kDa (Fig. 2, lane 5). After extended exposure of the gel, a weak protein band at 65 kDa was also observed (Fig. 2, lane 6). Both proteins were not detected after precipitation with the preimmune serum (Fig. 2, lane 7). The band visible in all lanes is generated by a high-molecular-mass protein that nonspecifically binds to the *Staphylococcus aureus* used for immunoprecipitation (see also Fig. 4). These results indicated that a 65-kDa protein precipitated from supernatants of VacgG-infected cells by the antiserum is expressed from U_S ORF4. However, in supernatants from BHV-1-infected cells, a significantly different form of the U_S ORF4 gene product appears to be present.

Analysis of carbohydrates in gG. Sequence analysis of the U_S ORF4 of BHV-1 strains Schönböken and ST predicted the presence of three consensus sequences for addition of N-glycans in the deduced gG protein. To test whether the 65-kDa protein contains carbohydrates, proteins immunoprecipitated from the supernatants of BHV-1-infected cells were incubated with endoglycosidase H, N-glycosidase F, or neuraminidase and O-glycanase. After digestion with endoglycosidase H, the electrophoretic mobilities of the 65-kDa protein and the high-molecular-mass proteins appeared unchanged in comparison to those of the PBS-treated control (Fig. 3, lanes 1 and 2). In contrast, incubation with N-glycosidase F resulted in the disappearance of the 65-kDa protein and the appearance of proteins ranging in size from 43 to 61 kDa (Fig. 3, lane 5). Since the high-molecular-mass proteins also migrated faster after N-glycosidase F digestion, it is unclear which of the smaller proteins resulted from de-N-glycosylation of the 65-kDa polypeptide. Nevertheless, these results demonstrate that the 65-kDa protein contains N-linked carbohydrates of the complex type. In addition, digestion with O-glycanase after treatment with neuraminidase results in a slight increase in mobility of the high-molecular-mass proteins and generation of a discrete 55-kDa protein (Fig. 3, lane 3). Because treatment with neuraminidase alone resulted in an only slight increase in the electrophoretic mobility of the 65 kDa protein (data not shown), this result demonstrates that the 65-kDa protein also contains O-glycans. Therefore, the 65-kDa protein precipitated by the anti-VacgG serum was designated glycoprotein G.

The diffuse migration patterns of the gG related high-molecular-mass proteins resembled the migration behavior of proteoglycans. Therefore, immunoprecipitates from supernatants of BHV-1-infected cells were incubated with heparinase or chondroitinase AC and were either analyzed directly by SDS-PAGE (Fig. 4, lanes 1 to 3) or were again immunoprecipitated with the anti-VacgG serum. For that purpose, the *S. aureus* antibody-antigen complexes were disintegrated by incubation at 95°C for 5 min and then by low-speed centrifugation. Disruption was controlled by SDS-PAGE, which proved that the high-molecular-mass proteins were released into the supernatant, whereas the nonspecifically precipitated protein remained associated with the bacteria in the pellet (Fig. 4, lanes 7 and 8). Digestion with heparinase, which might have contained inherent chondroitinase ABC activity, only slightly increased the mobilities of the high-molecular-mass proteins (Fig. 4, lanes 2 and 5), whereas chondroitinase AC treatment converted them into a polypeptide with an apparent molecular mass of 65 kDa (Fig. 4, lanes 3 and 6). This protein, as well as the untreated high-molecular-weight protein and the heparinase reaction products, was recognized by the anti-VacgG serum after reprecipitation (Fig. 4, lanes 4 to 6). This demonstrates that the high-molecular-mass protein, the 65-kDa protein generated by chondroitinase AC treatment, and gG are antigenically related, which led us to conclude that the BHV-1 U_S ORF4 gene product is secreted predominantly as a proteoglycan which contains chondroitin sulfate.

This conclusion was confirmed by incubation of infected cells with $Na_2{}^{35}SO_4$ and immunoprecipitation of secreted proteins with the anti-VacgG serum and then by chondroitinase AC digestion and SDS-PAGE. The results are shown in Fig. 5. From the medium of $Na_2{}^{35}SO_4$-labeled cells, the anti-VacgG serum precipitated the U_S ORF4 proteoglycan (Fig. 5, lane 3), which, after incubation with chondroitinase AC, was no longer detectable (Fig. 5, lane 4). A 65-kDa protein which is released by chondroitinase AC from the [^{35}S]methionine-cysteine-labeled U_S ORF4 proteoglycan (Fig. 5, lane 2) was also not detectable after chondroitinase AC digestion (Fig. 5, lane 4). This also indicates that the protein backbone of both the U_S ORF4 proteoglycan and gG is not significantly sulfated at tyrosine residues, which contrasts with the situation in gG of PRV (5, 12).

To clarify whether the BHV-1 U_S ORF4 gene product associates tightly with chondroitin sulfate chains or whether the glucosaminoglycan molecules are covalently attached to the 65-kDa protein, cells were infected in presence of methylumbelliferyl-β-D-xyloside at a final concentration of 3 mM. β-D-

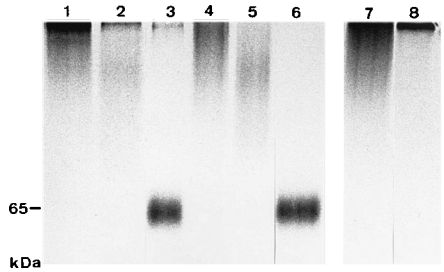

FIG. 4. U_S ORF4 encodes a proteoglycan. BHV-1-infected MDBK cells were incubated with [^{35}S]methionine-cysteine from 2 to 16 h p.i., and secreted proteins were precipitated with the anti-VacgG serum. The *S. aureus*-bound immunocomplexes were then incubated with PBS (lanes 1, 4, and 7), heparinase (lanes 2 and 5) or chondroitinase AC (lanes 3 and 6). After incubation, half of the reaction products were directly separated on SDS-10% polyacrylamide gels (lanes 1 to 3). The other half was incubated at 95°C for 5 min, and bacteria were removed by low-speed centrifugation. The supernatants were reprecipitated with the anti-VacgG serum and were analyzed by SDS-PAGE (lanes 4 to 6) and then by fluorography. In lane 7, supernatant from a PBS control assay was analyzed after incubation at 95°C. Lane 8 shows labeled proteins still bound to the bacteria after the heat treatment. The position of the 65 kDa protein is indicated.

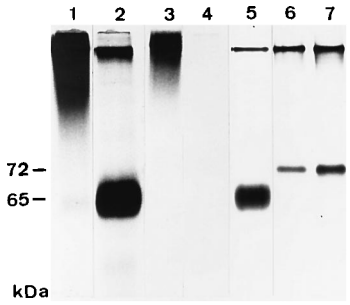

FIG. 5. The U_S ORF4 proteoglycan is sulfated. MDBK cells, infected in the presence of methylumbelliferyl-β-D-xyloside (lanes 5 and 7) or without inhibitor (lanes 1 to 4 and 6) were incubated from 2 to 16 h p.i. with [^{35}S]methionine-cysteine (lanes 1, 2, and 5 to 7) or $Na_2{}^{35}SO_4$ (lanes 3 and 4), and proteins from cell culture supernatants (lanes 1 to 5) or from lysed cells (lanes 6 and 7) were precipitated with the anti-VacgG serum (lanes 1 to 5) or a BHV-1 gD-specific polyclonal rabbit serum (lanes 6 and 7). The *S. aureus*-bound immunocomplexes were then either incubated with PBS (lanes 1 and 3) or chondroitinase AC (lanes 2 and 4), which was followed by SDS-PAGE and fluorography, or analyzed directly (lanes 5 to 8). The 65- and 72-kDa proteins are indicated.

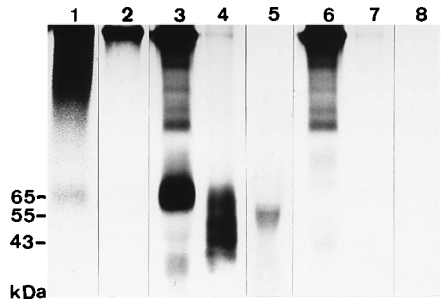

FIG. 6. The backbone of the U_S ORF4 proteoglycan is a glycoprotein. MDBK cells were infected with wild-type BHV-1 (lanes 1, 3, 4, and 5) or the U_S ORF4 deletion mutant BHV-1/gp1-8 (lanes 2, 6, 7, and 8) incubated with [^{35}S]methionine-cysteine from 2 to 16 h p.i. Secreted proteins were precipitated with the anti-VacgG serum. The *S. aureus*-bound immunocomplexes were then incubated with PBS (lanes 1 and 2) or chondroitinase AC (lanes 3 to 8) and then incubated with PBS (lanes 3 and 6), N-glycosidase F (lanes 4 and 7), or neuraminidase and O-glycosidase (lanes 5 and 8). Reaction products were analyzed by SDS-PAGE and fluorography. Molecular masses are indicated in kilodaltons.

xyloside inhibits the substitution of core protein serine residues with polymers of chondroitin sulfate but does not prevent synthesis of free chondroitin sulfate chains (35). As shown in Fig. 5, the addition of methylumbelliferyl β-D-xyloside to BHV-1-infected cells resulted in secretion of the 65-kDa protein, whereas the anti-VacgG serum did not detect the U_S ORF4 proteoglycan (Fig. 5, lane 5), demonstrating that its generation from the 65-kDa core protein requires covalently linked chondroitin sulfate molecules. Immunoprecipitations from infected cell lysates with a gD-specific rabbit antiserum demonstrated synthesis of the 72-kDa BHV-1 gD in treated and untreated cells (Fig. 5, lanes 6 and 7), indicating that β-xyloside treatment did not interfere with N- and O-linked glycosylation.

To determine whether the 65-kDa protein released from the U_S ORF4 proteoglycan carries carbohydrates, [^{35}S]methionine-cysteine-labeled proteins from the supernatants of cells infected with wild-type BHV-1 and the U_S ORF4 deletion mutant BHV-1/gp1-8 were immunoprecipitated with the anti-VacgG serum, digested with chondroitinase AC, and then incubated with N-glycosidase F or neuraminidase and O-glycanase, which was followed by SDS-PAGE and fluorography. Chondroitinase AC digestion of the wild-type BHV-1-expressed U_S ORF4 proteoglycan released the 65-kDa protein (Fig. 6, lane 3), which was converted into faster migrating forms by N-glycosidase F (Fig. 6, lane 4) and neuraminidase and O-glycosidase treatment (Fig. 6, lane 5). These smaller polypeptides correspond to the ones detected after deglycosylation of 65-kDa gG (Fig. 3). No U_S ORF4 proteoglycan and consequently none of the glycosidase digestion products were precipitated from the supernatants of BHV-1/gp1-8-infected cells (Fig. 6, lanes 2, 7, and 8). Additional proteins seen in Fig. 6, lane 3, most probably represent degradation products of the nonspecifically precipitated polypeptide, since they were also detected in the chondroitinase AC-digested immunoprecipitates from the supernatants of BHV-1/gp1-8-infected cells (Fig. 6, lane 6). These results indicate that gG and the glycoprotein component of the U_S ORF4 proteoglycan are closely related. Therefore, we designated the high-molecular-mass form glycoproteoglycan G (gpgG).

Maturation of gG and gpgG. To analyze the synthesis and export of gG and gpgG, pulse-chase experiments were performed. MDBK cells were infected with BHV-1 and were labeled at 6 h p.i. with [^{35}S]methionine-cysteine for 30 min. Thereafter, radioactive medium was replaced by normal cell culture medium, and cells and cell culture supernatants from parallel cultures were harvested at 0, 15, 30, 60, 90, and 120 min after the pulse. Proteins from cell lysates (Fig. 7a) and supernatants (Fig. 7b) were immunoprecipitated with the anti-VacgG serum. The most abundant protein detected by the antiserum after the pulse is a 61-kDa polypeptide (Fig. 7a, lane 2) which is not precipitated by the preimmune serum (Fig. 7a, lane 1). Within 60 min after the pulse, most of the labeled 61-kDa polypeptide is converted to a 70-kDa protein, and, simultaneously, high-molecular-mass forms appear which migrate in a manner similar to that of gpgG and which both disappear at later chase times (Fig. 7a, lanes 3 to 7). Starting at 60 min after pulse, increasing amounts of labeled gG and gpgG could be precipitated from the cell culture media (Fig. 7b, lanes 2 to 7).

In summary, kinetic analyses demonstrate that gG and gpgG originate from a 61-kDa precursor molecule which is converted to a 70-kDa form. Our results also indicate that in parallel to the secretion of gG, the cell-associated 70-kDa form is processed to the extracellular 65-kDa gG. The results of the kinetics, thus, are in accordance with the assumption that the glycoprotein components of gpgG and gG are identical.

Glycosylation inhibition studies. MDBK-Bu100 cells were infected without inhibitors or in the presence of castanospermine, tunicamycin, or brefeldin A. Castanospermine inhibits

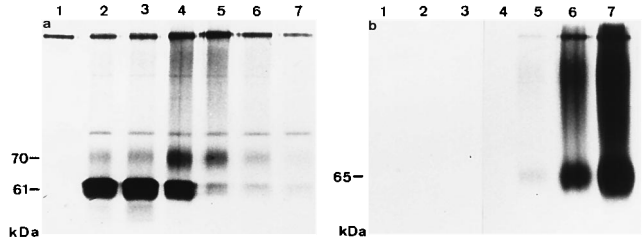

FIG. 7. Kinetics of maturation and secretion of gG. MDBK cells were infected with BHV-1, and proteins were labeled with [^{35}S]methionine-cysteine for 30 min at 6 h p.i. Thereafter, cells were washed with normal cell culture medium and were further incubated for 0 (lanes 1 and 2), 15 (lanes 3), 30 (lanes 4), 60 (lanes 5), 90 (lanes 6), and 120 min (lanes 7). Labeled proteins from lysed cells (a) or the cell culture supernatants (b) were immunoprecipitated with a rabbit anti-VacgG serum (lanes 2 to 7) or the corresponding preimmune serum (lanes 1). Precipitated proteins were separated on SDS-10% polyacrylamide gels and were visualized by fluorography. Apparent molecular masses are indicated in kilodaltons.

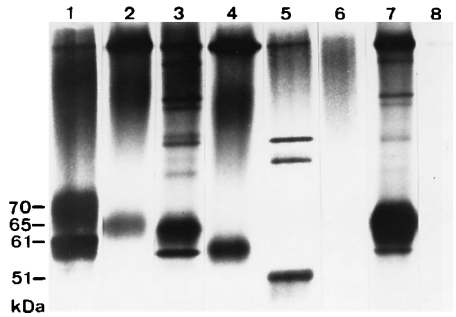

FIG. 8. Glycosylation inhibitor studies. Proteins in infected MDBK cells were labeled with [^{35}S]methionine-cysteine from 4 to 12 h p.i. without inhibitors (lanes 1 and 2) or in the presence of castanospermine (lanes 3 and 4), tunicamycin (lanes 5 and 6), or brefeldin A (lanes 7 and 8). Labeled proteins from lysed cells (lanes 1, 3, 5, and 7) or the cell culture supernatants (lanes 2, 4, 6, and 8) were immunoprecipitated with the anti-VacgG serum. Precipitated proteins were separated on an SDS-10% polyacrylamide gel and were visualized by fluorography. Apparent molecular masses are indicated in kilodaltons.

formation of complex N-linked oligosaccharides from mannose-rich precursor molecules (for a review, see reference 11). Tunicamycin inhibits the formation of dolichol-pyrophosphate-GlcNAc and thus blocks the addition of N-glycans to polypeptides within the endoplasmic reticulum (for a review, see reference 11). Brefeldin A inhibits the transport of newly synthesized membrane proteins into the Golgi apparatus and affects the maturation of herpesvirus membrane glycoproteins (7, 23, 24, 42). Proteins were labeled with [^{35}S]methionine-cysteine from 4 to 12 h p.i. and were precipitated from cell lysates and cell culture supernatants with the anti-VacgG serum. From cells infected without inhibitor, the gG-specific antiserum precipitated the gpgG, the 70-kDa cellular gG, and the 61-kDa precursor (Fig. 8, lane 1). From the cell culture supernatant, gG and gpgG was precipitated (Fig. 8, lane 2). Presence of castanospermine (20 μg/ml) during the infection slightly reduced the apparent molecular weight of both the cellular and secreted gpgG and resulted in an increase in the relative mobilities of the cell associated and extracellular gG which migrated with apparent molecular masses of 64 and 59 kDa, respectively (Fig. 8, lanes 3 and 4). The precursor protein now appeared with a relative mobility corresponding to 58 kDa (Fig. 8, lane 3). From cells infected in the presence of tunicamycin (2 μg/ml), the antiserum precipitated the high molecular weight gpgG and a 51-kDa polypeptide which could constitute the unglycosylated precursor of the U_S ORF4-encoded protein. The identity of the proteins with apparent molecular masses of 83 and 95 kDa (Fig. 8, lane 5) is unclear. We assume that they represent nonspecific coprecipitates. From the supernatant of the tunicamycin-treated cells the anti-VacgG serum precipitated only gpgG (Fig. 8, lane 6).

After infection in the presence of brefeldin A, no gG-related proteins could be detected in the supernatant after immunoprecipitation with the anti-VacgG serum (Fig. 8, lane 8). From the cell lysates, the cellular gpgG, a 65-kDa cellular gG, and a 59-kDa protein which probably represents the precursor polypeptide were precipitated (Fig. 8, lane 7).

These results show that conversion of mannose-rich N-glycans to the complex form adds 5 kDa to the apparent molecular masses of both intracellular and excreted gG. They further demonstrate that N-glycosylation is not required for formation and secretion of gpgG, which is, however, dependent on an intact Golgi apparatus, as is secretion of gG.

gG and gpgG are not associated with virions. To determine whether gG and/or gpgG are incorporated into virions, infected cells were labeled with [^{35}S]methionine-cysteine from 6 to 24 h p.i., and proteins were immunoprecipitated from purified virions and the cell culture supernatant. As shown in Fig. 9, the anti-VacgG serum precipitated gG and gpgG from the infected cell culture supernatant (Fig. 9, lane 3) but not from virions (Fig. 9, lane 2). As a control, a gD-specific rabbit antiserum precipitated gD from the virus particles (Fig. 9, lane 1). Thus, gG and gpgG are not (major) constituents of BHV-1 virions.

DISCUSSION

Of the glycoproteins predicted to be encoded by the BHV-1 U_S segment, only glycoprotein D has been unequivocally assigned to the U_S ORF5 (41). It was proposed to designate the predicted gene products from U_S ORF4, U_S ORF6, and U_S ORF7 BHV-1 gG, BHV-1 gI, and BHV-1 gE, respectively, because their deduced amino acid sequences have significant homology with those of HSV-2 gG, HSV-1 gI, and HSV-1 gE, respectively (22). To characterize the transcripts encompassing BHV-1 U_S ORF4, cytoplasmic RNA from BHV-1 infected cells was isolated at different times p.i. and hybridized to U_S ORF3- and U_S ORF4-specific probes after gel electrophoresis and transfer to nitrocellulose. As expected from the sequence analysis, U_S ORF4 is contained within two mRNAs of 3.5 and 1.8 kb. The 3.5-kb transcript probably constitutes a structurally bicistronic transcript which encompasses U_S ORF3 and U_S ORF4. From the hybridization data and the locations of transcriptional control sequences flanking the U_S ORF4 of BHV-1 strains Schönböken (19) and ST (22), it is reasonable to assume that the 1.8-kb transcript represents the monospecific U_S ORF4 mRNA. The 3.5- and the 1.8-kb RNAs were found at early and late times of infection. Although only steady-state levels were analyzed, comparison of the hybridization signals in RNA isolated 12 h p.i. from cells infected with or without PAA indicated that transcription of both RNAs is directed by delayed-early controlled promoters.

The U_S ORF4-encoded protein was identified by using a monospecific rabbit antiserum against a U_S ORF4-expressing recombinant vaccinia virus. From the supernatants of BHV-1-infected cells, this anti-VacgG serum precipitated a 65-kDa glycoprotein, designated gG, and high-molecular-mass proteins which proved to be glycoproteoglycans containing chondroitin sulfate. Interestingly, the 65-kDa glycoprotein backbone of these molecules is closely related or even identical to gG. Formation of the glycoproteoglycans could be inhibited by

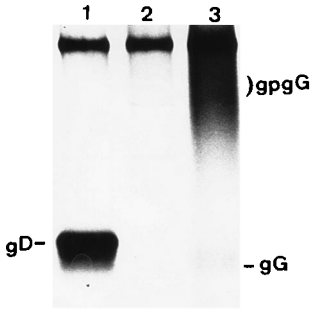

FIG. 9. BHV-1 gG and gpgG are not associated with virions. [^{35}S]methionine-cysteine-labeled proteins from infected cell culture supernatants at 24 h p.i. (lane 3) or from purified virions (lanes 1 and 2), isolated 24 h p.i., were immunoprecipitated with a BHV-1 gD-specific polyclonal rabbit antiserum (lane 1) or the anti-VacgG serum (lanes 2 and 3). Precipitated proteins were separated on an SDS-10% polyacrylamide gel and were visualized by fluorography. The positions of gD, gG, and gpgG are indicated.

β-xyloside treatment (35), demonstrating that chondroitin sulfate chains are covalently linked to the 65-kDa core glycoprotein and that the addition of chondroitin molecules is not a prerequisite for secretion of the U_S ORF4 gene product from BHV-1-infected cells. The high-molecular-mass proteins were therefore named glycoproteoglycan G (gpgG).

In proteoglycans, glucosaminoglycans are bound to serine residues in the core protein. No universal consensus sequence for their addition could be defined (21). Therefore, further studies are needed to identify the chondroitin sulfate substitution domains within the 65-kDa glycoprotein.

To our knowledge, this is the first time a herpesvirus proteoglycan has been described. Secretion of the BHV-1 gG gene products into the medium of infected cells is consistent with the properties of gG of EHV-1, EHV-4 (8, 9), and PRV (32) and the 34-kDa gG cleavage product of HSV-2 (38).

An obvious question arising from this result concerns why the vaccinia virus-expressed gG is not modified by the addition of glycosaminoglycans. This may be due to specifics of vaccinia virus replication or the requirement for another BHV-1 gene product(s).

The intracellular maturation of both gG and gpgG in BHV-1-infected cells was analyzed by pulse-chase experiments, glycosidase treatment, and synthesis in the presence of glycosylation inhibitors. The first U_S ORF4 gene product detected by the anti-VacgG serum was the 61-kDa protein precipitated after a 30-min [^{35}S]methionine-cysteine pulse-label of infected cell proteins. This 61-kDa protein was subsequently converted to a cell-associated 70-kDa protein and proteins migrating similarly to gpgG. Both processed forms were secreted into the culture medium, from which a 65-kDa gG was precipitated. The size difference of 5 kDa can by explained by proteolytic cleavage of the 70-kDa intracellular form to remove a potential membrane anchor domain at the carboxy terminus. This assumption was also made for gG of PRV (5) or EHV-4 (8). Proteolytic cleavage has been shown to occur with gG of HSV-2, which results in a 108-kDa membrane-bound glycoprotein and a 34-kDa secreted product (2, 38). However, the cleavage mechanisms appear to be different, since gG cleavage products are generated and further processed within HSV-2-infected cells. This is in contrast to the situation after infection with BHV-1, whereby the 65-kDa gG could be detected only in the supernatant. We hypothesize that if cleavage occurs, this reaction is temporally and spatially linked to the secretion process. Whether the cellular gpgG is also modified during export into the cell culture medium remains to be analyzed.

Glycosidase treatment of gG and the 65-kDa glycoprotein component of extracellular gpgG demonstrated that both contain N- and O-linked oligosaccharides. Digestion with neuraminidase and O-glycosidase reduced the apparent molecular masses of both proteins to 55 kDa (Fig. 3 and 6), indicating that they are very similar. Incubation of gG and the 65-kDa glycoprotein component of extracellular gpgG with N-glycosidase F resulted in an increase in their electrophoretic mobilities, and deglycosylation products of 61 to 43 kDa appeared. Since a comparable migration pattern of the digestion products was obtained with or without chondroitinase AC treatment of the anti-VacgG-precipitated proteins (Fig. 3 and 6), it is reasonable to assume that gG constitutes the 65-kDa glycoprotein part of gpgG. The reason why N-glycosidase F digestion of gG did not result in an uniform reaction product is currently not understood. Extension of the incubation time or increase of the enzyme concentration yielded similar results, as shown in Fig. 3 and 6, whereas after digestion of immunoprecipitated gD, only the expected 63-kDa deglycosylated protein was observed (not shown).

Infection of cells in the presence of castanospermine confirmed the conclusion that gG is N-glycosylated. Since castanospermine inhibits formation of complex N-linked oligosaccharides (for a review, see reference 11), glycoproteins containing immature N-glycans normally migrate faster in SDS-polyacrylamide gels. For BHV-1 gG, castanospermine led to a decrease in apparent molecular mass from 70 to 64 kDa for the cell-associated molecules and from 65 to 59 kDa for the excreted gG. Inhibition of N-glycosylation by tunicamycin demonstrated that both gpgG formation and secretion were not impeded, although the overall yield appeared to be reduced. However, this could be due to additional effects of the inhibitor on cellular processes or to weaker reactivity of the anti-VacgG serum with de-N-glycosylated gpgG. After tunicamycin treatment, a 51-kDa polypeptide was detected within the infected cells which might represent the nonglycosylated gG precursor molecule. However, it should be noted that the anti-VacgG serum does not react with gG or gpgG on immunoblots and that it did not bind to in vitro-synthesized and processed polypeptides translated from in vitro-transcribed gG mRNA (not shown).

Brefeldin A disrupts the Golgi apparatus and thereby inhibits transport and secretion of newly synthesized glycoproteins. For PRV, it has been proposed that brefeldin A prevents complete O-glycosylation (42), resulting in viral glycoproteins with reduced molecular masses. Since cellular gG is O-glycosylated, we assume that the 65-kDa intracellular gG synthesized in the presence of brefeldin A represents an incompletely modified form and not intracellularly retained, proteolytically cleaved gG. Brefeldin A does not prevent but only reduces the formation of cellular gpgG. Since in the presence of brefeldin A Golgi enzymes are redistributed to the endoplasmic reticulum in an active form (23), we hypothesize that addition of glycosaminoglycans to the intracellular precursor of gpgG most likely occurs in the Golgi apparatus and not in the *trans*-Golgi network. Moreover, the absence of gG and gpgG in the supernatant of brefeldin A-treated infected cells indicates that their secretion requires a functional Golgi apparatus.

Comparison of the ratios between gG and gpgG in the experiments shown in this report demonstrates that processing of the 61-kDa precursor can vary. In the pulse-chase experiment shown in Fig. 7, a relatively large amount of gG was precipitated from the cell culture supernatant. In contrast, in two other experiments, the kinetics of the intracellular processing and secretion of gpgG were comparable to the result shown in Fig. 7. However, 65-kDa gG was not detectable in the supernatants. The reason for this puzzling phenomenon is not clear.

In summary, we identified different forms of the BHV-1 gG protein. A precursor of 61 kDa is converted to an extracellular 65-kDa form which also serves as a backbone for the chondroitin sulfate-containing gG-glycosaminoglycan. To our knowledge, this is the first demonstration of a glycosaminoglycan-containing herpesvirus protein, and, therefore, represents the first identification of a herpesvirus proteoglycan.

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