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Role of N-Linked Glycans in Antigenicity, Processing, and Cell Surface Expression of Bovine Herpesvirus 1 Glycoprotein gIV†

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Glycoprotein gIV, a structural component of bovine herpesvirus type 1, stimulates high titers of virus-neutralizing antibody. The protein contains three potential sites for the addition of N-linked carbohydrates. Three mutants were constructed by oligonucleotide-directed mutagenesis, in each case changing one N-linked glycosylation site from Asn-X-Thr/Ser to Ser-X-Thr/Ser. A fourth mutant was altered at two sites. The altered forms of the gIV gene were cloned into a vaccinia virus transfer vector to generate recombinant vaccinia viruses expressing mutant proteins. Analysis of these mutants revealed that only two (residues 41 and 102) of the three (residues 41, 102, and 411) potential sites for the addition of N-linked glycans are actually utilized. Absence of glycans at residue 41 (gN1) showed no significant effect on the conformation of the protein or induction of a serum neutralizing antibody response. However, mutant proteins lacking glycans at residue 102 (gN2) or residues 41 and 102 (gN1N2) showed altered reactivity with conformation-dependent gIV-specific monoclonal antibodies. These mutants also induced significantly lower serum neutralizing antibody responses than wild-type gIV. Nonetheless, each of the mutant proteins were modified by the addition of O-glycans and transported to the cell surface. Our results demonstrate that absence of N-linked glycans at one (residue 102) or both (residues 41 and 102) utilized N-linked glycosylation sites alters the conformation but does not prevent processing and transport of gIV to the cell surface.

The carbohydrate moieties attached covalently to asparagine residues of the Asn-X-Ser/Thr (N-glycosylation acceptor sites) on the viral polypeptides have been implicated to be important for a variety of biological and immunological functions (8, 18). Evidence has also been presented for their role in the formation and maintenance of the native conformation of glycoproteins, prevention of intracellular aggregation, and protection from proteolytic degradation (7, 18). However, studies in which N-linked glycosylation of polypeptides has been blocked, either by interfering with synthesis of the lipid-linked precursor oligosaccharides (e.g., by the drug tunicamycin) or by mutagenesis of the glycosylation acceptor sites, have shown that some proteins which normally contain N-linked oligosaccharides are not affected (22), whereas others fail to reach their correct cellular destination or are degraded (14, 19).

Glycoprotein gIV of bovine herpesvirus 1 (BHV-1) is an integral component of the virion envelope and appears to be essential for virus replication (5). This glycoprotein elicits high titers of complement-independent neutralizing antibodies (1, 9) and has been implicated in virus adsorption (9, 13), penetration (5, 9), and cell fusion (23). The gIV gene encodes a polypeptide of 417 amino acids (23). The protein contains a signal sequence of 18 amino acids (23), which is cleaved during translocation of the polypeptide in the endoplasmic reticulum; a large amino-terminal extracellular domain of 343 amino acids; a transmembrane domain of 29 amino acids; a highly charged cytoplasmic domain of 29 amino acids (23), and both N- and O-linked oligosaccharides (24, 25).

Glycoprotein gIV contains three predicted sites for addition of N-linked oligosaccharides, two of which are located in the extracellular domain (residues 41 and 102), while the third is present in the cytoplasmic domain (residue 411) (23). Previous studies in which N-linked glycosylation was blocked by tunicamycin indicated that addition of N-linked oligosaccharides was critical for the intracellular transport of BHV-1 glycoproteins to the plasma membrane and the production of infectious virions (24). However, these studies could not distinguish between a generalized requirement for N-linked glycans and effects due to the loss of a particular glycan.

By using site-directed mutagenesis (31), the consensus sequences of N-linked glycosylation sites (10) were destroyed by changing the codon for the first amino acid in the sequence. The altered forms of the gIV gene were then inserted into vaccinia virus (VV), which directed the expression of mutant gIV proteins in infected cells. This allowed the determination of the role played by the individual glycans in the native conformation and intracellular transport of gIV in the absence of tunicamycin (16).

MATERIALS AND METHODS

Reagents and media. Cell culture media and fetal bovine serum were purchased from GIBCO/BRL, Burlington, Ontario, Canada. Reagents for DNA manipulations and protein A-Sepharose were purchased from Pharmacia, Dorval, Quebec, Canada, and used as recommended by the manufacturer. The enzyme neuraminidase, 5′-bromo-2′-deoxyuridine and other reagents for protein analysis were obtained

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from Sigma Chemical Co., St. Louis, Mo. The sugar-modifying enzymes *O*-glycanase and glycopeptidase F (PNGase F) were procured from Boehringer Mannheim, Dorval, Quebec, Canada. The reagents for immunoperoxidase staining were purchased from Dimension Laboratories, Mississuaga, Ontario, Canada, and used as recommended by the manufacturer. Radioisotopically labelled compounds and reagents for fluorography were purchased from ICN, Irvine, Calif.

Cells and viruses. BSC-1 cells and human thymidine kinase-negative (TK^-) 143 cells were grown as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum. $LMTK^-$ cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% fetal bovine serum. Wild-type VV (WR strain) was propagated in BSC-1 cells, while recombinant VV were cultivated in $LMTK^-$ cells (15).

Construction of glycosylation mutants. A 1.3-kb *Bgl*II fragment containing the entire gIV (Cooper strain) coding sequence was excised from the gIV expression vector pRSD neogIV (23) and subcloned into the *Bgl*II site of a modified pTZ18R (27). Site-directed mutagenesis was carried out as described previously (12). Three synthetic oligonucleotide primers were used, each having a single-base mismatch designed to change aspargine to serine at the three potential N-linked glycosylation sites. The sequences of the oligonucleotides used in the mutagenesis were as follows: site 1 (residue 41), ATGCCGCGATACAGCTACACTGAA; site 2 (residue 102), GCGCGCGCGTACAGCGCCACGGTC; and site 3 (residue 411), TTTGGCAACGTCAGCTACAGCGCG. In each case the mismatched base is underlined. Mutants were isolated, and their DNA sequences were verified by dideoxynucleotide sequencing (20). The double mutant was constructed by replacing the *Apa*I-*Sca*I fragment of the gN1 construct with the *Apa*I-*Sca*I fragment of the gN2 construct. The altered gIV gene was subcloned into the *Bgl*II site of the VV transfer vector pVVSL1 (11) in which the expression of the foreign gene was under the control of a synthetic promoter, the sequence of which is based on the VV late promoter consensus sequence (4).

Isolation of recombinant vaccinia viruses. The recombinant VVs were made by homologous recombination as previously described (15), except that the linearized plasmid DNA was electroporated into the wild-type VV-infected cells by using a Pharmacia Gene Pulser set at 200 V and 500 μF. The recombinant VVs were identified by screening TK^- plaques for the expression of recombinant proteins by immunocytochemistry (6) before plaque purification and making viral stocks.

Polyclonal and monoclonal antibodies. The production and characterization of the gIV-specific monoclonal antibodies (MAbs) used in this study have been described earlier (9, 29). Before use, MAb ascitic fluids were clarified and filtered. Monospecific polyclonal rabbit antisera that specifically recognize gIV have been previously described (9).

In vivo labelling and immunoprecipitation. $LMTK^-$ cells were infected with 5 PFU of recombinant VV per cell for 10 h in the presence or absence of tunicamycin (2 μg/ml). Cells were starved in methionine-cysteine-free DMEM for 2 h, labelled with 100 μCi of [^{35}S]methionine-cysteine for 4 to 8 h, and harvested. In pulse-chase experiments, cells were labelled at 10 h postinfection with 150 μCi of [^{35}S]methionine-cysteine for 15 min. The cells were harvested either immediately (pulse) or after the cells were washed and incubated in DMEM containing excess cold methionine for 15, 30, 60, and 90 min (chase). Proteins were immunoprecipitated from cell lysates and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as previously described (29).

Enzyme digestions. The immunoprecipitated proteins were eluted from protein A-Sepharose in 20 μl of 0.5% SDS by boiling for 3 to 5 min. The digestion of protein with PNGase F (removes high mannose and complex forms of N-linked sugars) was carried out in 0.1 M sodium phosphate buffer (pH 8.5) containing 0.15 M 2-mercaptoethanol, 15 mM EDTA, 1% Nonidet P40, 0.1% SDS, and 4 U of PNGase F for 18 h at 37°C. Neuraminidase (to remove sialic acid) digestion was performed in 0.02 M Tris-maleate (pH 6.4), 10 mM calcium acetate, 1% Nonidet P40, 0.1% SDS, 10 mM D-galactoso-γ-lactone, and 0.1 U of enzyme. After 2 h at 37°C, half of the digestion mixture was treated with 1.5 mU of *O*-glycan-peptide hydrolase (to remove O-linked oligosaccharides) and further incubated at 37°C for 16 h. Finally, the proteins were analyzed by SDS-PAGE as previously described (29).

Immunoperoxidase staining. $LMTK^-$ cells grown in 4-well Lab-Tek chamber slides were infected with recombinant VV at a multiplicity of infection of 5. Infected cells grown in the presence of tunicamycin (2 μg/ml) were exposed to the drug for 2 h before infection. At 16 h postinfection, the cells were fixed either with 3% paraformaldehyde for 15 min at 4°C for surface staining or methanol for 15 min at −20°C for internal staining. Finally, the cells were stained with immunoperoxidase as previously described (6).

Immunization of mice and antibody titer determination. C3H/HeJ mice were immunized intraperitoneally with 0.2 ml of 10^6 PFU of wild-type VV (WR strain) or recombinant VVs (SVLgIV, SVLgN1, SVLgN2, and SVLgN1N2) per ml at 10 and 12 weeks of age. Pooled sera were obtained at 14 weeks of age from groups of three identically immunized mice. BHV-1-specific total antibody responses were measured by enzyme-linked immunosorbent assay (ELISA) (2, 26, 29) with affinity-purified native gIV as an antigen at a concentration of 0.05 μg per well. Serum neutralizing antibody responses were determined as described previously (2, 29) by using 100 PFU of BHV-1. Titers were expressed as reciprocals of the highest antibody dilution that caused 50% reduction in number of plaques relative to the control.

RESULTS

Construction of recombinant VV expressing gIV N-glycosylation mutants. In order to determine the role of N-linked oligosaccharides in the antigenicity and transport of gIV, site-directed mutagenesis was used to alter each of the three potential N-glycosylation sites (23) by changing asparagine, the first amino acid residue of the acceptor site, to serine (Fig. 1a). While changing the asparagine to serine at amino acid 411, an additional silent G to A in the codon for amino acid 414 (alanine), GCG to GCA was introduced. This change did not affect the amino acid sequence of the gN3 protein. The wild-type and mutant genes were cloned in the VV transfer vector pVVSL1 (Fig. 1b) to generate recombinant VVs designated here as SVLgIV, expressing wild-type gIV; SVLgN1, expressing the mutant protein gN1; SVLgN2, expressing the mutant protein gN2; SVLgN3, expressing the mutant protein gN3; and SVLgN1N2, expressing protein gN1N2.

Analysis with monospecific, polyclonal, anti-gIV serum. To characterize each of the mutant proteins, $LMTK^-$ cells infected with recombinant VVs expressing mutant proteins were labelled for 4 to 8 h with [^{35}S]cysteine-methionine. The gIV protein expressed in SVLgIV-infected cells was also

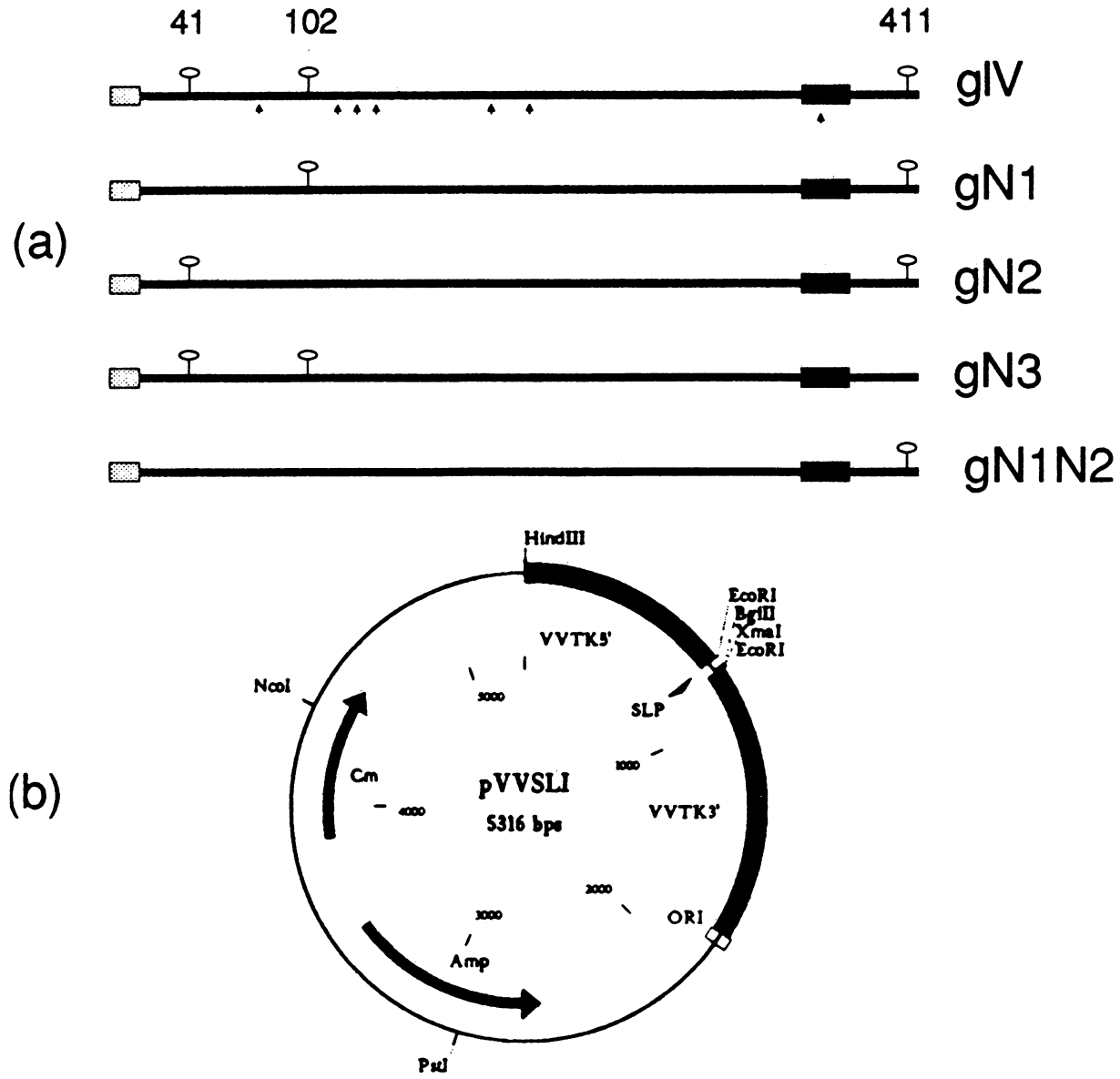

FIG. 1. (a) Schematic representation of gIV and N-glycosylation mutants. The important features of gIV protein, which include the 18-amino-acid signal sequence (▥), the 29-amino-acid transmembrane anchor domain (■), seven cysteine residues (↑), and three potential sites (residue 41, 102, and 411) for the addition of N-linked glycans (♆) are depicted. The name given to the wild type and each mutant protein is indicated on the right. (b) Diagram of VV transfer plasmid pVVSL1. The origin of DNA sequences included in the plasmid are as follows: pBR328, solid line; VV TK gene sequence, dark box; synthetic VV late promoter, arrowhead.

labelled in the presence and absence of tunicamycin. The labelled proteins were immunoprecipitated from cell lysates and analyzed by SDS-PAGE (Fig. 2). The gN1 (lane B) and gN2 (lane C) proteins migrated as two distinct bands, while wild-type gIV (lane A), gN3 (lane E), and gN1N2 (lane D) migrated as single bands. The gN1 (lane B) and gN2 (lane C) proteins were slightly smaller than wild-type gIV (lane A), and both migrated in the gel to a position consistent with the absence of one N-linked glycan. The migration of the gN1N2 protein (lane D) is consistent with the absence of two N-linked glycans. After PNGase F treatment (Fig. 2, lane G to L), wild-type gIV (lane K), gN1 (lane H), gN2 (lane I), gN3 (lane L), gN1N2 (lane J), and gIV synthesized in the presence of tunicamycin (lane G) migrated to an identical position, thus confirming that the altered mobilities observed above were due to the absence of N-linked glycans. This suggestion was further supported by the observation that gN1N2 migrated to the same position in the gel as the PNGase F-treated proteins (lane D versus lane G to L). In contrast, gN3 (lane E) migrated to a position in the gel similar to that of gIV (lane A).

Analysis with gIV-specific MAbs. The antigenic structure of the mutated gIV proteins was determined by using a panel of gIV-specific MAbs which recognized both continuous (MAbs 3D9S, 9D6, and 10C2) and discontinuous (MAbs 136, 3E7, 2C8, 3C1, and 4C1 [9, 29]) epitopes. The labelled gIV

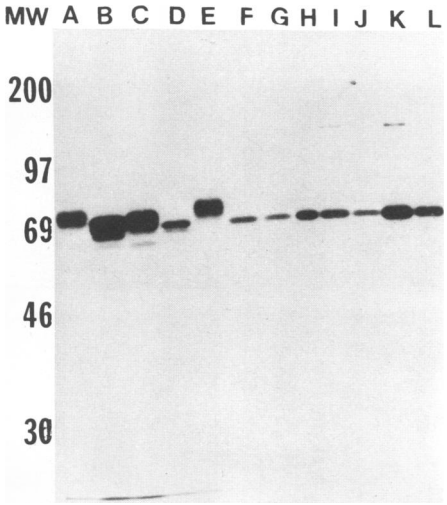

FIG. 2. Immunoprecipitation of gIV and glycosylation mutant proteins by polyclonal anti-gIV serum. Radiolabelled lysates prepared from recombinant VV-infected LMTK$^-$ cells with (lanes F and G) or without (lanes A to E and H to L) tunicamycin treatment were immunoprecipitated with polyclonal anti-gIV serum. The immunoprecipitated proteins were analyzed by SDS-PAGE under reducing conditions with (lanes G to L) or without (lanes A to F) PNGase F treatment and are as follows: wild-type gIV (lane A), gN1 (lane B), gN2 (lane C), gN1N2 (lane D), gN3 (lane E), tunicamycin-treated wild-type gIV with (lane G) or without (lane F) PNGase F treatment, PNGase F-treated gN1 (lane H), PNGase F-treated gN2 (lane I), PNGase F-treated gN1N2 (lane J), PNGase F-treated wild-type gIV (lane K), and PNGase F-treated gN3 (lane L). Molecular size markers (MW) in kilodaltons are indicated at the left.

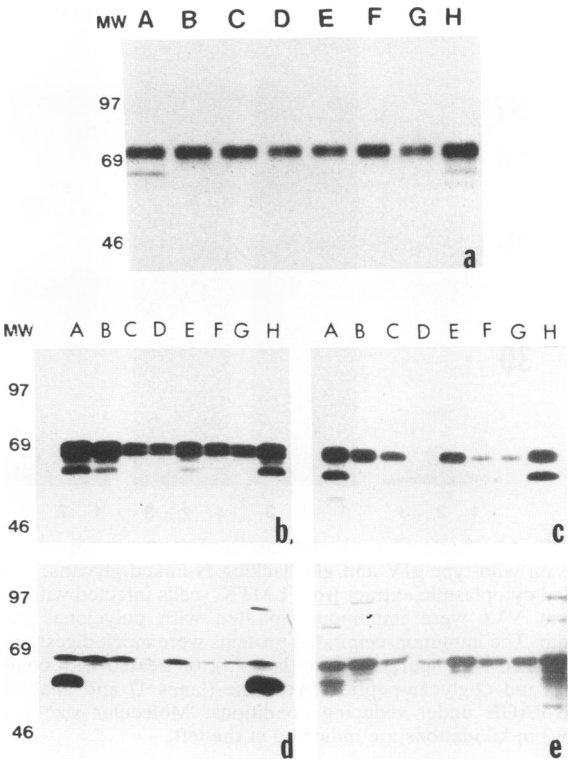

FIG. 3. Antigenic analysis of gIV and glycosylation mutant proteins. Radiolabelled extract of LMTK$^-$ cells infected with recombinant VVs expressing wild-type gIV in the presence (e) or absence (a) of tunicamycin and gN1 (b), gN2 (c) and gN1N2 (d) proteins were immunoprecipitated with MAb 9D6 (lanes A), 136 (lanes B), 3E7 (lanes C), 2C8 (lanes D), 3C1 (lanes E), 4C1 (lanes F), 10C2 (lanes G), 3D9S (lanes H) and analyzed by SDS-PAGE under reducing conditions. Molecular size markers in kilodaltons are indicated at the left.

proteins were immunoprecipitated with individual MAbs and analyzed by SDS-PAGE (Fig. 3; lane A, 9D6; lane B, 136; lane C, 3E7; lane D, 2C8; lane E, 3C1; lane F, 4C1; lane G, 10C2; lane H, 3D9S). All MAbs recognized gN1 (Fig. 3b) at a level comparable to wild-type gIV (Fig. 3a); however, the recognition of gN2 (Fig. 3c) and gN1N2 (Fig. 3d) by certain MAbs differed compared with that of wild-type gIV (Fig. 3a). In particular, MAbs 2C8 (epitope IIIc), 4C1 (epitope IIIb), and 10C2 (epitope IIIa) barely recognized gN2 and gN1N2. Wild-type gIV synthesized in the presence of tunicamycin was recognized by all MAbs (Fig. 3e), albeit at reduced levels compared with fully glycosylated wild-type gIV (Fig. 3a). In addition, all MAbs which recognize discontinuous epitopes reacted more strongly with the mature form of the protein (Fig. 3), while the MAb 9D6 (Fig. 3, lanes A) and 3D9S (Fig. 3, lanes H), which recognize continuous epitopes, reacted with both the precursor and the mature forms of the proteins in an equivalent manner. The ratio of the product to precursor was lower for the mutant proteins (Fig. 3b, c, and d) and gIV synthesized in the presence of tunicamycin (Fig. 3e) compared with wild-type gIV (Fig. 3a).

Processing of mutant proteins. Previously, pulse-chase analysis was used to examine the maturation of gIV from BHV-1-infected cells (25). To extend these studies and determine what effect removal of a specific N-linked glycosylation site had on the maturation of the glycoprotein, two approaches were used. First, recombinant VV-infected cells were pulse labelled for 15 min with [^{35}S]cysteine-methionine and either harvested immediately (0 min) or chased for 15, 30, 60, and 90 min (Fig. 4, lanes 1 to 5). The proteins were immunoprecipitated, from infected cell extracts, with monospecific, polyclonal, anti-gIV serum and analyzed by SDS-PAGE. The wild-type and mutant proteins were each processed from a faster migrating precursor form to the more slowly migrating product form (Fig. 4); however, the rate of processing of the mutant proteins was different from the wild-type molecule. Wild-type gIV processing was evident

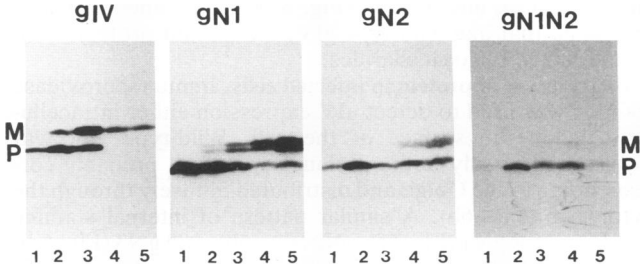

FIG. 4. Pulse-chase analysis of gIV and glycosylation mutant proteins. LMTK$^-$ cells infected with recombinant VVs were pulse-labeled with [^{35}S]cysteine-methionine for 15 min (lanes 1) and then chased for 15 min (lanes 2), 30 min (lanes 3), 60 min (lanes 4), and 90 min (lanes 5) in unlabeled medium. Cell extracts were immunoprecipitated with polyclonal anti-gIV serum and analyzed by SDS-PAGE under reducing conditions. Precursor (P) and mature (M) forms of the recombinant proteins are indicated.

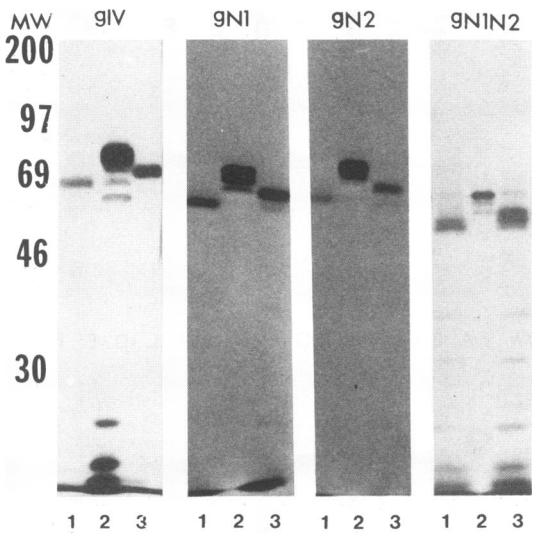

FIG. 5. Effects of neuraminidase and *O*-glycan-peptide hydrolase on wild-type gIV and gIV lacking N-linked glycans. Radiolabelled cytoplasmic extract from LMTK$^-$ cells infected with recombinant VVs were immunoprecipitated with polyclonal anti-gIV serum. The immunoprecipitated proteins were mock digested (lanes 2), treated with neuraminidase (lanes 3), or treated with neuraminidase and *O*-glycan-peptide hydrolase (lanes 1) and analyzed by SDS-PAGE under reducing conditions. Molecular size markers (MW) in kilodaltons are indicated at the left.

within 15 min and was nearly complete by 60 min. In contrast, depending on the mutant, the processing of altered gIV proteins was evident at 15 min (Fig. 4, gN1), 15 to 30 min (Fig. 4, gN1N2), and 30 to 60 min of pulse (Fig. 4, gN2) and was not complete by 90 min.

Since the acquisition of sensitivity to neuraminidase or *O*-glycanase is dependent on the transport of a glycoprotein from the endoplasmic reticulum to the Golgi apparatus, where O-linked oligosaccharides are added (10), the gIV proteins were treated with neuraminidase and *O*-glycanase to examine the processing and transport of the mutant proteins. Wild-type gIV expressed by the recombinant VV SVLgIV (Fig. 5, gIV, lane 2) contained O-linked glycans, since its mobility increased after digestion with neuraminidase (Fig. 5, gIV, lane 3) or neuraminidase and *O*-glycanase (Fig. 5, gIV, lane 1). Similar mobility increases were observed for gN1 (Fig. 5, gN1, lanes 1 and 3), gN2 (Fig. 5, gN2, lanes 1 and 3), and gN1N2 (Fig. 5, gN1N2, lanes 1 and 3), indicating that like wild-type gIV, the mutant proteins contain O-linked oligosaccharides.

Distribution of protein in infected cells. Immunoperoxidase staining was used to detect gIV expression either intracellularly or on the surface of the cell. Wild-type gIV was localized internally in the perinuclear region, probably corresponding to the Golgi, and distributed diffusely through the cytoplasm (Fig. 6b). A similar pattern of internal staining was found for gN1 (Fig. 6f), gN2 (Fig. 6h), and gN3 (Fig. 6d). The internal staining pattern of gN1N2 (Fig. 6j) and gIV synthesized in the presence of tunicamycin (Fig. 6l) differed from that of wild-type gIV (Fig. 6b) in that the mutant protein and gIV synthesized in the presence of tunicamycin appeared to be concentrated only in the Golgi. All forms of gIV except the one synthesized in the presence of tunicamycin (Fig. 6k) could be detected on the surface of the cell (Fig. 6a, c, e, g, and i).

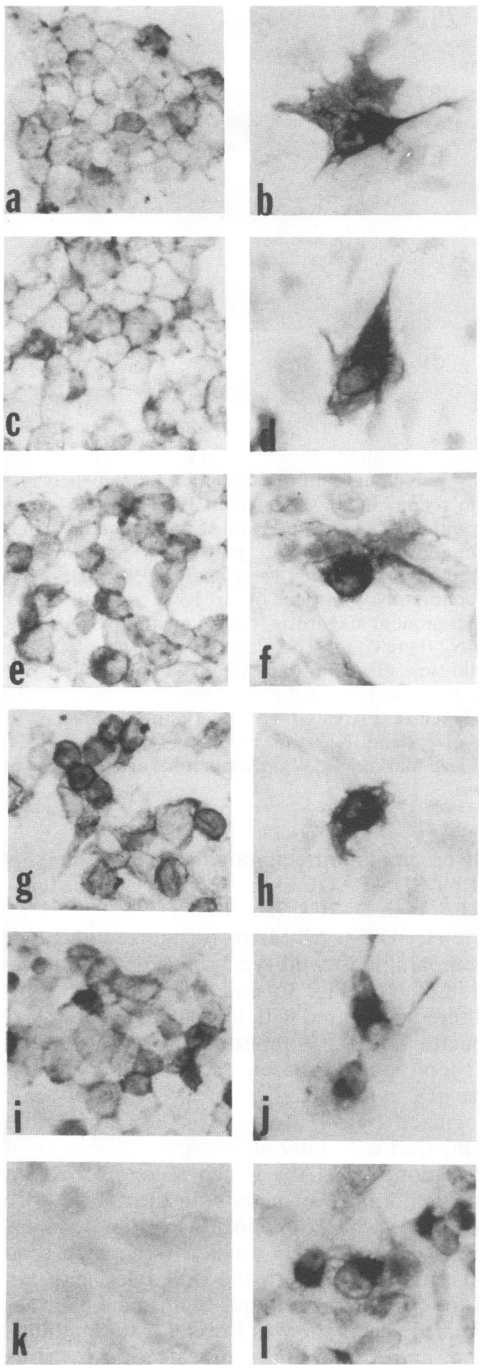

FIG. 6. Analysis of distribution of gIV and glycosylation mutant proteins. LMTK$^-$ cells infected with recombinant VVs were fixed either with 3% paraformaldehyde to detect protein on the cell surface (a, c, e, g, i, and k) or methanol to detect protein in the cell (b, d, f, h, j, and l). The fixed cells were treated with polyclonal anti-gIV serum and then subjected to immunoperoxidase staining as previously described (6). Wild-type gIV (a and b), gN1 (e and f), gN2 (g and h), gN3 (c and d), gN1N2 (i and j), and tunicamycin-treated wild-type gIV (k and l).

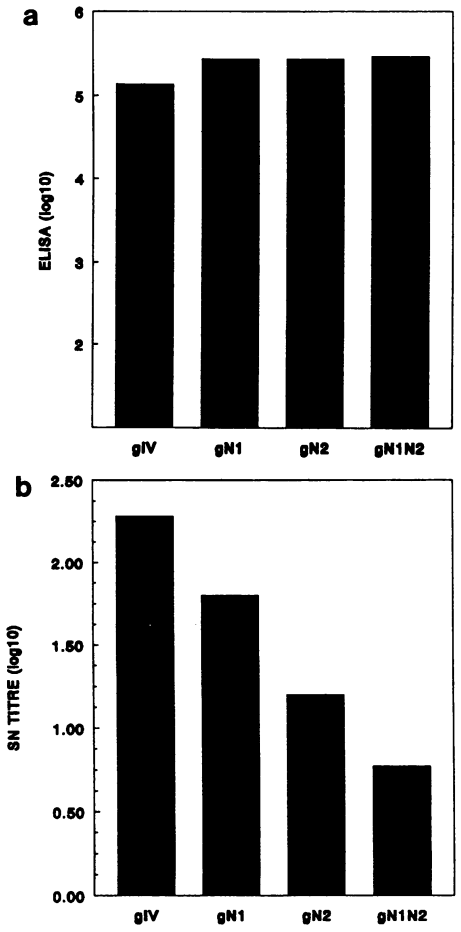

FIG. 7. Antibody response to N-linked glycosylation mutants and wild-type gIV. Mice were immunized with 0.2 ml of 10^6 PFU of wild-type or recombinant VVs at 10 and 12 weeks of age. Mice were bled at 14 weeks of age. (a) Total antibody response was determined by ELISA using affinity-purified native gIV as the antigen. (b) Serum neutralizing antibody responses to BHV-1. Titers are expressed as the reciprocal of the highest antibody dilution that caused a 50% reduction in the number of plaques relative to control. Titers shown are the averages of two experiments.

Immunogenicity of mutant proteins. In order to examine the effect of N-linked glycan removal on immunogenicity of gIV, the wild-type and mutant gIV proteins were tested for their abilities to elicit an antibody response. As shown in Fig. 7a, there was no significant difference in the total antibody response elicited by wild-type or mutant gIV proteins. However, mutant proteins elicited lower (3- to 32-fold) serum neutralizing antibody responses than wild-type gIV (Fig. 7b).

DISCUSSION

N-linked oligosaccharides are thought to be important for a variety of structural and functional properties of a glycoprotein; however, their effect varies depending on the particular protein. The N-linked oligosaccharides of BHV-1 glycoproteins have been shown to be important for the transport of these proteins to the cell surface and the production of infectious virions in virus-infected cells grown in the presence of tunicamycin (24). By using site-directed mutagenesis to analyze the role of N-linked glycans present on the gIV glycoprotein of BHV-1, it was possible to determine the effect each potential site of N-linked oligosaccharide addition had on the antigenicity, processing, and transport of the BHV-1 gIV glycoprotein in eukaryotic cells.

An analysis of protein sequences has shown that all potential N-linked glycan acceptor sites are not usually glycosylated (10). The amino acid sequence of the BHV-1 gIV glycoprotein contains three potential sites for the addition of N-linked oligosaccharides (10, 23). Two lines of evidence suggest that N-linked oligosaccharides are not attached to the third predicted site at amino acid 411. First, wild-type gIV and gN3 proteins migrate to similar positions in the gel. Second, the gN1N2 protein and gIV protein synthesized in the presence of tunicamycin, with or without PNGase F treatment, showed no size difference. A plausible explanation can be that because of its location in the cytoplasmic domain of the protein (23), the acceptor site at amino acid 411 of gIV may not be properly oriented and accessible to the glycosylation machinery (10).

The wild-type gIV and gN3 proteins appeared as single bands, while the gN1 and gN2 proteins were detected as two distinct bands. The two bands could result from gIV molecules with different levels of glycosylation, as is evident from the O-glycosylation studies. A similar observation was made earlier for herpes simplex virus type 1 gD (22).

A number of studies have postulated that N-linked glycans influence proper folding, disulfide bond formation, and oligomerization of proteins (17, 22, 30), thus affecting the formation of discontinuous epitopes. A previous report has shown that carbohydrates attached to gIV do not form part of any antigenic epitopes of the protein (26). In addition, studies have shown that gIV synthesized in *Escherichia coli* lacked most of the discontinuous epitopes and induced a serum neutralizing antibody response approximately 100-fold lower than the wild-type gIV produced by BHV-1 (28). This has led to speculation that N-linked glycans may be important for proper folding of gIV protein, as glycosylation of protein does not occur in *E. coli*. Our results provide evidence that N-linked glycans are important for proper folding of the gIV protein.

The absence of glycans at amino acid 41 did not show any significant effect on the conformation of the protein, as MAbs against discontinuous epitopes recognized gN1 protein essentially in a manner similar to that of the wild-type gIV. In contrast, the absence of glycans at amino acid 102 (gN2) affected the conformation of the protein. In particular, the reactivity of three MAbs, 2C8, 4C1, and 10C2, all of which inhibit attachment of the virus to cells (9), was significantly reduced when their reactivity to gN2 was compared with that to wild-type gIV. A previous report regarding the reactivity of these MAbs, with enzymatically deglycosylated mature gIV (26), and the present data suggest that the *N*-glycans at amino acid 102 may be required for the formation rather than the maintenance of these epitopes. Alternatively, it is also possible that the observed phenotype of gN2 may result from the change in the amino acid at the glycosylation site rather than the absence of the *N*-glycans (21, 22); however, a similar reactivity pattern of these MAbs with gIV synthesized in the presence of tunicamycin argues against this.

Essentially all MAbs displayed less reactivity with gN1N2 than with gIV synthesized in the presence of tunicamycin. Also, the level of the precursor form of the proteins tended to be greater in gN1N2 than in gIV synthesized in the presence of tunicamycin. This further alteration of confor-

mation in gN1N2 may in part be due to the amino acid change.

The potency of the serum neutralizing antibody response to gIV depends on conformation of the protein, as most of the neutralizing MAbs against gIV recognize discontinuous epitopes (9). The serum neutralizing antibody response induced by the mutant proteins, in particular, gN2 and gN1N2, was significantly lower than that by the wild-type gIV. This confirms the earlier conclusion that the N-linked glycans, particularly at amino acid 102, help in proper folding of gIV, as none of the domains which elicit virus-neutralizing antibodies are composed of carbohydrates (26).

Wild-type gIV and the glycosylation mutants were processed from the precursor to product form by the addition of O-linked oligosaccharides and sialic acid. However, the rate of processing of wild-type gIV was faster than that for the mutant proteins (gN1, gN2, and gN1N2). Moreover, the processing of mutant proteins was not complete even after a 90-min chase. This may be due to improper folding of the protein, which may promote the retention of the mutant protein in the endoplasmic reticulum much longer than the properly folded wild-type gIV. The results indicate that the absence of glycans alters but does not prevent the processing of the protein from the precursor to the product form.

The wild-type gIV and mutant proteins (gN1, gN2, gN3, and gN1N2) were detected on the surface of the infected cells. However, gIV protein synthesized in the presence of tunicamycin was never detected on the surface of the cell, although it appeared to accumulate in the Golgi, as did the gN1N2 protein. Similarly, gIV synthesized in BHV-1-infected cells, grown in the presence of tunicamycin, was not detected on the cell surface (24). One possible explanation is that gIV synthesized in the presence of tunicamycin is modified differently than gIV synthesized in the absence of the drug. Support for such a possibility is forthcoming from studies in which immunoglobulin G synthesized in the presence of tunicamycin from hybridoma cells was shown to possess sulfated tyrosine residues, a modification not present on the glycoprotein synthesized in the absence of the drug (3). In addition, tunicamycin is a mixture of components, some of which exhibit biological activities other than mere inhibition of N-linked glycosylation (16).

The data presented above indicate that the absence of *N*-glycans in gN2 and gN1N2 affects the conformation of the protein, probably by interfering with proper folding. This change affects the formation of the discontinuous epitopes and reduces the ability to induce a potent neutralizing antibody response but does not prevent the processing and transport of these mutant proteins to the cell surface. Whether this conformational change affects the ability of the N-linked glycosylation mutants to function in BHV-1 virions remains to be seen.

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