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A Structural Polypeptide of the Baculovirus *Autographa californica* Nuclear Polyhedrosis Virus Contains O-Linked *N*-Acetylglucosamine

MARC WHITFORD AND PETER FAULKNER*

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario K7L 3N6, Canada

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A structural glycopeptide, gp41, derived from the occluded virus of the baculovirus *Autographa californica* nuclear polyhedrosis virus was characterized. The peptide specifically bound wheat germ agglutinin but was not recognized by a panel of seven other lectins. Reactivity with wheat germ agglutinin was eliminated by treatment of gp41 with beta-*N*-acetylglucosaminidase, indicating that *N*-acetylglucosamine (GlcNAc) was present as terminal residues. gp41 was efficiently galactosylated by galactosyltransferase only in the presence of Nonidet P-40, suggesting that GlcNAc residues are not exposed on the surface of the virion. Metabolic labelling of gp41 with [^{3}H]GlcNAc occurred in the presence of tunicamycin. The carbohydrate was released by alkaline borohydride treatment and comigrated with *N*-acetylglucosaminitol in descending paper chromatography. The data indicate that gp41 contains single residues of GlcNAc O glycosidically linked to the polypeptide chain. Evidence suggesting that gp41 is located in the region between the envelope membrane and the capsid (defined here as the tegument) of the occluded virus is also presented.

Two distinct types of virions are produced during replication in insects and in cell cultures of the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV). Although genetically identical, they differ in relative infectivities in cells in vitro and in insect larvae (22, 36). Extracellular viruses (ECV) are virions which are formed by the budding of single nucleocapsids through the plasma membrane; they are considered to be the vehicle for the systemic spread of infection within the insect. A second type of baculovirus virions, the occluded virions (OV), obtain their envelope in the nuclei of infected cells. One or more nucleocapsids may share a common envelope; in morphogenesis, these bundles become embedded within occlusion bodies (OB). Larvae of susceptible insect species become infected when ingested OB disassemble in the lumen of the midgut and OV bundles are released. OV structural polypeptides are involved in at least two binding functions: (i) they recognize and bind to gut epithelial cells to initiate cell infections (21), and (ii) the surfaces of the envelopes appear to have a close affinity for the developing polyhedrin lattice in OV morphogenesis. The two virus phenotypes differ in their glycoprotein compositions. An abundant glycoprotein, gp67, having a role in virus-cell interactions (35) is present in ECV but not in OV. Similarly, a major glycoprotein not found in ECV has been identified in OV (32): Stiles and Wood described a 42-kDa OV glycoprotein (41K OV glycoprotein) that was metabolically labelled with *N*-[^{3}H]acetylglucosamine ([^{3}H]GlcNAc) but not [^{3}H]mannose. Labelling was not inhibited by tunicamycin, suggesting the presence of O-linked oligosaccharide side chains. To date, the OV glycoprotein has not been further characterized with respect to its structure or function in the life cycle of AcNPV.

Peptide glycosylation is generally thought to occur via the secretory pathway in eukaryotic cells, resulting in glycoproteins that are largely restricted to the surface and lumenal compartments of cells (16, 24, 28). However, there has been a growing body of evidence to suggest that glycoproteins do exist in the cytoplasm and nucleoplasm (14, 15). Many of these glycoproteins are of a novel type, in which GlcNAc is O glycosidically linked to a serine or threonine residue (14, 15). This study was done to characterize a major OV glycoprotein of AcNPV. We show that this glycoprotein contains O-linked GlcNAc and that it is most likely localized in the tegument of OV.

MATERIALS AND METHODS

Virus and cell cultures. A plaque-purified isolate of AcNPV, HR3 (5), was propagated in *Spodoptera frugiperda* IPLB-SF-21 (SF) cells (34) by use of TC100 (8) supplemented with 10% fetal calf serum. OB were recovered from HR3-infected SF cells (18). OV were released from OB by treatment with alkali (1) and concentrated by centrifugation through a 20% sucrose cushion. For recovery of nucleocapsids, OV were resuspended in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–300 mM NaCl–1% (vol/vol) Nonidet P-40 (NP-40) for 24 h at 37°C, with occasional mixing (33). The suspension was clarified for 1 min in a Microfuge, layered onto a 1-ml cushion of 20% sucrose, and centrifuged for 1 h at 32,000 × *g* with an SW60 rotor. Hybridoma cell lines were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 0.05 mM 2-mercaptoethanol.

MAb production. Monoclonal antibodies (MAb) 3.10 and 5.15 were generated as described previously (38) with whole OV as an immunogen. MAb 3.10 specifically immunoprecipitated a 41K OV polypeptide which could be metabolically labelled by [^{3}H]GlcNAc. MAb 5.15 specifically reacted with this 41K polypeptide in immunoblots. MAb 3.10 and 5.15 also recognized a 111K OV polypeptide with properties identical to those of gp41. MAb AcV12 (17) specifically reacted with major capsid protein p39. Hybridoma supernatants were used throughout the study.

Metabolic labelling of OV. Nearly confluent SF cells were infected at a multiplicity of 5 to 10. At 17 h postinfection,

* Corresponding author.

cells were starved for 1 h in TC100 lacking glucose or methionine, as required. Cells were labelled for 4 h with [^{3}H]GlcNAc (50 μCi/ml), [^{3}H]mannose (50 μCi/ml), or [^{35}S]methionine (25 μCi/ml), after which complete TC100 was added. OB were harvested at 3 days postinfection. In some cases, 10 μg of tunicamycin per ml was added at infection.

Immunoprecipitation. HR3-infected SF cells were labelled with [^{3}H]GlcNAc (100 μCi/ml) or [^{35}S]methionine (50 μCi/ml) in TC100 lacking glucose or methionine at 18 h postinfection for 4 h. Harvested cells (3×10^7 to 5×10^7) were washed with phosphate-buffered saline (PBS), solubilized in 1 ml of 20 mM Tris-HCl (pH 9.0)–137 mM NaCl–0.92 mM $MgCl_2$–10% (vol/vol) glycerol–1% (vol/vol) NP-40–2 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min, and clarified by centrifugation for 30 min in a Microfuge. NP-40 extracts were preabsorbed with protein A–Sepharose CL-4B (Pharmacia, Nepean, Ontario, Canada) (5 mg/ml) which had been coated with rabbit anti-mouse immunoglobulin sera (Sigma, St. Louis, Mo.). Aliquots (200 μl) of the preabsorbed extracts were added to 5 mg of protein A-Sepharose CL-4B coated with MAb 3.10. Immunoprecipitates were washed three times with 10 mM Tris-HCl (pH 7.6)–1% (vol/vol) NP-40–1% (wt/vol) sodium deoxycholate–0.1% (wt/vol) sodium dodecyl sulfate (SDS)–1 mM EDTA–2 mM PMSF and once with PBS. The precipitates were resuspended in electrophoresis sample buffer (0.0623 M Tris-HCl [pH 6.8], 1% SDS, 10% glycerol, 2% 2-mercaptoethanol, 0.01% bromophenol blue), and the suspensions were heated in a boiling water bath for 5 min. gp41 was resolved on 12% SDS-polyacrylamide gel electrophoresis (PAGE) gels as described by Laemmli (23).

Immunoblotting. Immunoblotting was performed as previously described (38).

Lectin blotting. Polypeptides were resolved on SDS-polyacrylamide slab gels (23) and transferred by electrophoresis to polyvinylidene difluoride (PVDF) membranes (Millipore Canada Ltd., Mississauga, Ontario, Canada). Membranes were blocked for 30 min with PBS–0.05% Tween 20 (PBST) and then incubated with biotinylated wheat germ agglutinin (WGA), lentil lectin, peanut agglutinin, soybean agglutinin, *Helix pomatia* agglutinin, winged pea agglutinin, or *Ricinus communis* agglutinin (all at 10 μg/ml) or concanavalin A (2 μg/ml) (biotinylated lectins were purchased from Sigma). After being washed in PBST, the blots were incubated with streptavidin-conjugated alkaline phosphatase in PBST for 20 min and developed. For beta-*N*-acetylglucosaminidase digestion, blocked membranes were incubated in PBST containing 2.5 U of beta-*N*-acetylglucosaminidase (Sigma) per ml at 37°C for 24 h, washed, and reacted with biotinylated WGA. For fluorography, membranes were sprayed with En^{3}Hance (Du Pont Canada Ltd., Mississauga, Ontario, Canada) and exposed to Kodak XAR film.

Mild alkaline borohydride treatment. gp41 was electroluted from SDS-polyacrylamide gel slices. The polypeptide was concentrated by use of a Centricon 10 apparatus (Amicon Division, W. R. Grace & Co., Danvers, Mass.) and treated with 2 mCi of NaB^3H_4 (Du Pont) in 0.05 M NaOH for 18 h at 37°C (31). The sample was placed on ice, diluted 10-fold with distilled H_2O, and brought to pH 5.0 by the dropwise addition of 4 N acetic acid. Methylborate was removed by several evaporations in methanol–1 N acetic acid, and the sample was desalted through small columns containing AG 50W-X8 (H^+ form) and AG 2-X8 (Cl^- form) (both from Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada) in distilled H_2O. Sample and wash fractions were lyophilized and resuspended in a small volume of distilled H_2O.

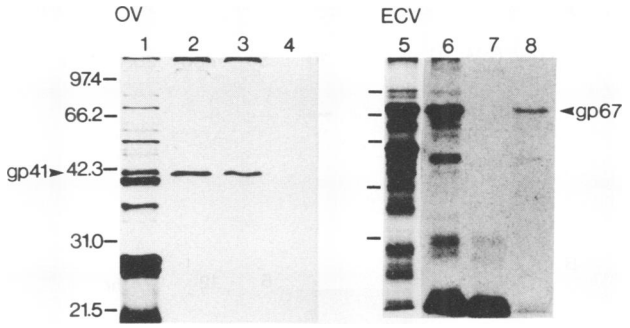

FIG. 1. Metabolic labelling of AcNPV OV and ECV proteins. OV (lanes 1 to 4) and ECV (lanes 5 to 8) were labelled with [^{35}S]methionine (lanes 1 and 5), [^{3}H]GlcNAc (lanes 2 and 6), [^{3}H]GlcNAc in the presence of 10 μg of tunicamycin per ml (lanes 3 and 7), and [^{3}H]mannose (lanes 4 and 8). Proteins were separated by 12% SDS-PAGE and examined by fluorography. Molecular masses in kilodaltons are indicated to the left of lanes 1 and 5.

Descending paper chromatography. Descending paper chromatography was done on Whatman no. 1 paper, and the chromatogram was developed with ethanolamine-pyridine-water (8:2:1) (11). The chromatogram was sprayed with En^{3}Hance and exposed to Kodak XAR film.

GalTase assay. OV were dialyzed against 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.3) and labelled as described previously (2). In brief, 10 μl of 20 mM galactose–30 mM NaCl–10 mM $MnCl_2$–10 mM HEPES (pH 7.3)–2 mM PMSF with or without 1% NP-40 was added to 10 μl of virus. The labelling reaction was started by adding 10 mU of galactosyltransferase (GalTase) (Sigma) and then 1 μCi of UDP-[^{3}H]Gal in 2 μl of 25 mM 5'-AMP, and the mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition of electrophoresis sample buffer.

TX-114 extraction. Phase separation of proteins in Triton X-114 (TX-114) was based on the methods described by Bordier (3). [^{3}H]GlcNAc- and [^{35}S]methionine-labelled OV in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA were brought to 0.15 M NaCl. One volume of 2% TX-114–TNE (pH 7.5) (TNE is 10 mM Tris-HCl [pH 7.5], 0.15 M NaCl, and 1 mM EDTA) was added, and the samples were kept on ice for 20 min before centrifugation for 20 min at 4°C in a Microfuge. The supernatants were transferred to fresh tubes, and phase separation was induced by incubating the tubes at 37°C for 3 min and then centrifuging the samples for 3 min at $300 \times g$ and 37°C. The aqueous phase was removed and kept on ice. One volume of ice-cold TNE (pH 7.5) was added to the detergent phase, and the phase separation was repeated. The second aqueous phase was combined with the first one. A 1/10 volume of 10% TX-114–TNE (pH 7.5) was added to this fraction, and phase separation was induced. Phase separation was performed once more on both phases. The final aqueous and detergent fractions were examined by SDS-PAGE and fluorography.

RESULTS

Metabolic labelling. AcNPV OV polypeptides metabolically labelled with [^{3}H]GlcNAc were examined by fluorography. Radioactivity was incorporated into 41K and 111K polypeptides (Fig. 1, lane 2). Labelling was not inhibited in

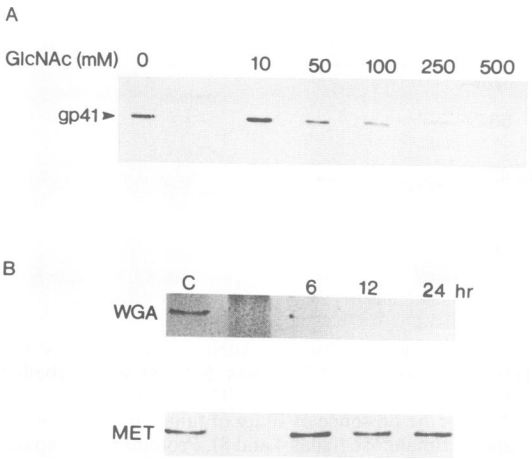

FIG. 2. Properties of gp41 binding to WGA. (A) Samples of OV proteins were separated by 12% SDS-PAGE, transferred to PVDF membranes, and probed with 10 μg of biotinylated WGA per ml. Incubations were performed in the presence of GlcNAc (0 to 500 mM, indicated above each lane), a competitive inhibitor of WGA. (B) PVDF membranes bearing immunoprecipitated [^{35}S]methionine-labelled gp41 (upper panel, WGA) were either not treated (C) or treated for 6, 12, or 24 h with *N*-acetylglucosaminidase and probed with biotinylated WGA. The lower panel (MET) is a fluorogram of the upper panel.

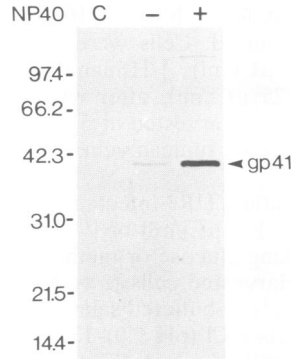

FIG. 3. Enzymatic labelling of glycoproteins in vitro with [^{3}H]galactose. OV proteins were labelled with [^{3}H]galactose from UDP-[^{3}H]galactose by use of GalTase in the absence (−) or presence (+) of NP-40, as indicated. Lane C is a negative control in which GalTase was omitted from the reaction mixture. Samples were subjected to 12% SDS-PAGE, and proteins were detected by fluorography. Molecular masses of protein standards are indicated on the left in kilodaltons.

the presence of 10 μg of tunicamycin per ml (Fig. 1, lane 3). In contrast, labelling of gp67 in ECV with [^{3}H]GlcNAc was completely inhibited in the presence of tunicamycin (Fig. 1, lane 7), as would be expected for a polypeptide containing N-linked oligosaccharide side chains (29). No OV polypeptides were labelled with [^{3}H]mannose (Fig. 1, lane 4), again in contrast to gp67 (Fig. 1, lane 8). These results indicate that the carbohydrate moieties on the 41K and 111K polypeptides are not covalently bound by an N-glycosidic linkage. The 41K protein (designated gp41) likely corresponds to a 42K OV polypeptide described by Stiles and Wood (32). This polypeptide was labelled by [^{3}H]GalNAc but not [^{3}H]mannose, and labelling was insensitive to tunicamycin.

The pattern of incorporation of labelled carbohydrate into the 111K polypeptide was identical to that of gp41. MAb 3.10 coprecipitated this polypeptide with gp41 (data not shown). However, gp111 was not investigated further.

Lectin blotting of OV polypeptides. As an approach to identifying the carbohydrate attached to gp41, PDVF membranes bearing OV polypeptides were treated with a panel of eight lectins, including WGA, concanavalin A, lentil lectin, peanut agglutinin, soybean agglutinin, *H. pomatia* agglutinin, winged pea agglutinin, and *R. communis* agglutinin. Bound lectins were detected following treatment of the filters with streptavidin-conjugated alkaline phosphatase. Only WGA, which recognizes GlcNAc, its oligomers, and sialic acid (9, 10), bound to gp41. No other OV proteins were recognized by any of the lectins. To ensure that the WGA-gp41 reaction was specific, we included GlcNAc, a competitive inhibitor of WGA, during treatment of a blot with biotinylated WGA. As the concentration of the competitor increased, there was a corresponding decrease in WGA binding to gp41 on the blot (Fig. 2A).

To further define the gp41-WGA interaction, we incubated PDVF membranes bearing immunoprecipitated [^{35}S]methionine-labelled gp41 with *N*-acetylglucosaminidase (hexosaminidase), which removes terminal GlcNAc residues from carbohydrate side chains on glycoproteins (25). Treatment of gp41 with this enzyme resulted in the loss of WGA binding to gp41 (Fig. 2B). The loss of reactivity was not due to the removal of protein from the membranes, as indicated by a fluorogram of the lectin blot (Fig. 2B). These results demonstrate that the carbohydrate side chain of gp41 has terminal GlcNAc residues.

In vitro labelling of gp41 by GalTase. To confirm that gp41 contains terminal GlcNAc residues, we performed in vitro labelling with GalTase. GalTase, in the absence of alpha-lactalbumin, transfers the [^{3}H]galactose residue of UDP-[^{3}H]galactose to glycoproteins containing terminal GlcNAc residues (4). When OV were incubated with GalTase in the presence of UDP-[^{3}H]galactose, gp41 was efficiently galactosylated when NP-40 was included in the reaction mixture (Fig. 3). However, labelling was greatly reduced when NP-40 was omitted. These results confirm the presence of terminal GlcNAc residues on gp41 and further indicate that the carbohydrate residues on gp41 are located internally in OV. Similar results have been reported for a human cytomegalovirus polypeptide containing O-linked GlcNAc (2).

Determination of the carbohydrate side chain linkage in gp41. Immunoprecipitated gp41 was treated with mild alkali in the presence of $NaB^{3}H_4$, which specifically releases O glycosidically linked side chains from glycoproteins as the corresponding alditols (beta-elimination) (30, 31). The released material was recovered, resuspended in distilled H_2O, and fractionated on a descending paper chromatogram. The sample comigrated exactly with the authentic *N*-acetylglucosaminitol standard (Fig. 4). On the basis of these results it was concluded that gp41 contains O glycosidically linked GlcNAc.

Location of gp41 in OV. Although the GalTase labelling experiment indicated that the O-linked GlcNAc residues of gp41 are not exposed on the surface of the virion, the location of gp41 in the virion is unknown. The glycoprotein could be a component of the capsid or an integral membrane protein or may be present in the region between the nucleocapsid and the virus envelope (12, 13), referred to here as the tegument (2). To locate gp41, we performed the following experiments. OV were treated with 1% NP-40–300 mM NaCl and subjected to ultracentrifugation through a sucrose cush-

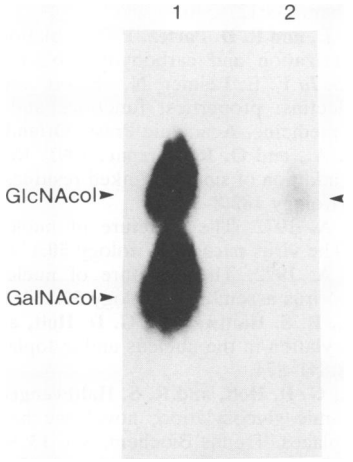

FIG. 4. Descending paper chromatography of beta-elimination products from gp41. O-linked carbohydrate was released from gp41 by treatment of purified gp41 with 0.05 M NaOH. The carbohydrate was reduced and labelled by use of NaB^3H_4. Products (lane 2) were analyzed by desending paper chromatography, and the chromatogram was developed with ethanolamine-pyridine-water (8:2:1). Labelled standards (*N*-acetylglucosaminitol [GlcNAcol] and *N*-acetylgalactosaminitol [GalNAcol]; lane 1) were prepared from the appropriate reducing sugar by treatment with 0.05 M NaOH in the presence of NaB^3H_4.

ion. This technique has been used to recover intact nucleocapsids (33). Samples of OV and nucleocapsids were run on an SDS-polyacrylamide gel, transferred to Immobilon, and probed with MAb. MAb AcV_{12} (17), which specifically reacts with the major capsid protein (p39), bound to a 39K polypeptide in the OV and nucleocapsid samples. In contrast, MAb 5.15 recognized a 41K polypeptide in the OV sample but not in the nucleocapsid sample (Fig. 5), indicating that gp41 is not an integral capsid protein.

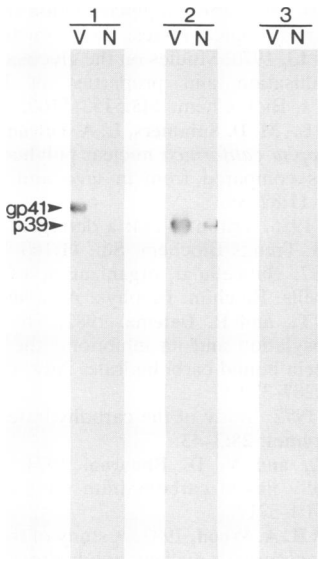

FIG. 5. Immunoblot analysis of virion and nucleocapsid proteins. OV (V) or nucleocapsids (N) were run in parallel on 12% SDS-polyacrylamide gels, transferred to Immobilon, and probed with MAb 5.15 (lane 1), AcV_{12} (lane 2), or no primary antibody (lane 3).

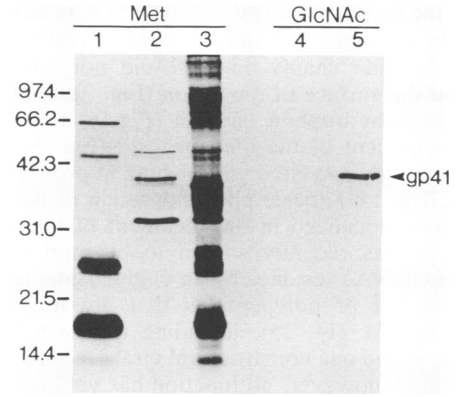

FIG. 6. TX-114 phase separation of OV polypeptides. OV polypeptides labelled with [^{35}S]methionine (lanes 1 and 2) or [^{3}H]GlcNAc (lanes 4 and 5) were separated by use of TX-114 into detergent (lanes 1 and 4) and aqueous (lanes 2 and 5) phases and analyzed by SDS-PAGE and fluorography. Lane 3 shows total [^{35}S]methionine-labelled OV proteins. Molecular masses of standards are indicated on the left in kilodaltons.

TX-114 phase separation was performed to determine whether gp41 is an integral membrane protein. [^{35}S]methionine- and [^{3}H]GlcNAc-labelled OV were solubilized in TX-114, and the mixture was separated into detergent and aqueous phases. Integral membrane proteins should be recovered in the detergent phase (although several exceptions have been reported), while hydrophilic proteins should remain in the aqueous phase (27). The results show that gp41 was partitioned into the aqueous phase (Fig. 6, lane 5) and could not be detected in the detergent phase (Fig. 6, lane 4), suggesting that gp41 is not an integral membrane protein. In support of this conclusion, no distinct hydrophobic clusters characteristic of integral membrane protein signal and anchor peptides were located on the deduced amino acid sequence of gp41 (37). Taken together, these results suggest that gp41 is a hydrophilic protein located in the tegument of OV.

DISCUSSION

We demonstrated that a 41K OV structural protein (gp41) contains one or more O glycosidically linked GlcNAc residues and probably corresponds to a 42K OV glycoprotein identified by Stiles and Wood (32). This protein, like gp41, was labelled in the presence of [^{3}H]GalNAc but not [^{3}H]mannose, and labelling was not inhibited by tunicamycin, suggesting the presence of O glycosidically linked carbohydrate side chains. Two other studies (7, 26) reported that the major OV glycoprotein had a molecular weight of approximately 42,000 and was not labelled by [^{3}H]mannose. However, these studies also reported a number of minor glycoproteins not found by Stiles and Wood (32). These authors suggested that the labelled sugar may have been metabolized before being incorporated into virus proteins, as a result of the long labelling times used.

gp41 does not appear to be a component of ECV. MAb 3.10 and 5.15 did not cross-react with ECV in an enzyme-linked immunosorbent assay (data not shown), and no ECV-specific glycoprotein corresponding to gp41 was labelled with [^{3}H]GlcNAc (Fig. 1), detected by WGA (Fig. 2), or galactosylated by GalTase (Fig. 3).

The findings presented here also suggest that gp41 is

located in the tegument of OV, referred to here as the region between the nucleocapsid and the virion envelope (12, 13). The GlcNAc side chains on gp41 did not appear to be exposed on the surface of the virion (Fig. 3), and gp41 was identified as a hydrophilic protein (Fig. 6) that is not an integral component of the nucleocapsid (Fig. 5). To date, only one other glycosylated viral tegument protein has been described. The 149K basic phosphoprotein of human cytomegalovirus is localized in the tegument of the virion (2). This protein was recently shown to be modified by the addition of GlcNAc residues by an O-glycosidic linkage (2).

A diverse set of polypeptides that are modified with O-linked GlcNAc (14, 15), including two structural viral proteins (2, 6) and one nonstructural viral proteins (11), have been identified; however, no function has yet been assigned to this modification. It has been suggested that this modification may have a regulatory function (14, 15), and a recent study describes results consistent with this hypothesis (20). The authors found that the apparent levels of O-linked GlcNAc in many nuclear proteins increased rapidly after the activation of T lymphocytes, while the apparent levels of O-linked GlcNAc in a set of cytoplasmic proteins decreased rapidly after the activation of these cells. Levels of O-linked GlcNAc-modified proteins returned to control levels in the nucleus and cytoplasm after several hours. These changes may, for example, play a role in the regulation of transcription by the addition or removal of O-linked GlcNAc from transcription factors (19). Examination of the role of gp41 in the life cycle of AcNPV may help define the function of the modification.

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