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Structure of O-Glycosidically Linked Oligosaccharides Synthesized by the Insect Cell Line Sf9

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The O-glycosidically linked oligosaccharides on the pseudorabies virus (PRV) glycoprotein gp50 synthesized by three different cell lines were studied. The intact membrane protein (gp50) was expressed in Vero cells and in the insect cell line Sf9. In addition, a truncated, secreted form lacking the transmembrane and cytoplasmic domains (gp50T), was expressed in CHO and Sf9 cells. The protein, both in intact and truncated form, synthesized by the two mammalian cells contained only the disaccharide Galβ1-3GalNAc, either unsubstituted or substituted with one or two sialic acid residues. By contrast, the major O-linked structure on gp50 and gp50T synthesized by Sf9 cells was the monosaccharide GalNAc. The Sf9 cells also linked lower amounts of Galβ1-3GalNAc to gp50 (12%) and gp50T (26%). None of the structures synthesized by Sf9 cells contained sialic acid. Measurements of the two relevant glycosyltransferases revealed that while all three cell lines contain comparable levels of UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase activity, there is a greater variation in the levels of UDP-Gal:N-acetylgalactosamine, β1-3 galactosyltransferase, with the Sf9 cells containing the lowest level.

Key words: insect cells, O-glycosidic glycosylation, glycosyltransferases, O-linked oligosaccharides, O-glycosylation

Studies of the oligosaccharide structures on glycoproteins synthesized by insect cells have revealed that these cells do not synthesize complex type N-linked oligosaccharides [1–5]. Comparison of the oligosaccharide structures on Sindbis Virus glycoproteins expressed in mammalian and insect cells have shown that insect cells frequently substitute truncated high-mannose structures at sites where mammalian cells would add complex type structures [3]. Thus insect cells appear to synthesize only high-mannose type N-linked oligosaccharides and truncated derivatives thereof. Activity measure-

Abbreviations used: GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; Gal, galactose; Man, mannose; Fuc, Fucose; NeuNAc, N-aetylneuraminic acid; MEM, minimal essential medium; DMEM, Dulbeccos modified Eagle's medium; HEPES, N-2-hydroethylpiperazine-N-2-ethanesulfonic acid; FCS, fetal calf serum; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Sf9, *Spodoptera frugiperda* cell line 9.

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ments of several glycosyltransferases involved in the addition of terminal sugars to N-linked oligosaccharides have demonstrated that insect cells contain negligible levels of N-acetylglucosaminyl-, galactosyl-, and sialyltransferases [2]. Consequently, they appear to lack completely the enzyme machinery needed for the assembly of complex type oligosaccharides.

Little is known about O-glycosidic glycosylation in insect cells. It has been reported that the cells can incorporate radioactive oligosaccharide precursors into glycoproteins also in the presence of tunicamycin [2,6,7]. Analysis of the monosaccharide composition of glycoproteins synthesized by insect cells have in some cases demonstrated the presence of N-acetylgalactosamine [1,2,8]. Other investigators have shown that some glycoproteins isolated from insect cells can be probed with N-acetylgalactosamine specific lectins [1,2,9,10,11]. Finally, Kress demonstrated that the oligosaccharide structures on the larval glue protein LGP 1 in *Drosophila* are linked to threonine residues on the protein [6].

We compared the structures of the O-linked oligosaccharides on the pseudorabies virus (PRV) envelope protein gp50 expressed in the insect cell line Sf9 with the oligosaccharides on the same protein expressed in the two mammalian cell lines CHO and Vero. PRV gp50 is a particularly appropriate protein with which to study O-linked glycosylation, since it is a glycoprotein that contains no N-linked glycosylation sites. We have previously sequenced the gp50 gene, and expressed it in CHO cells [12] and insect cells [13]. Two forms of the protein were studied, the intact gp50 and a truncated, secreted derivative lacking the transmembrane and cytoplasmic domains. The truncated protein, gp50T, was studied in CHO cells since these cells are not susceptible to PRV infections and the cloned intact protein is expressed at very low levels. Similarly, the intact protein is expressed at lower levels in Sf9 cells; however in this system preliminary experiments indicated quantitative differences in the oligosaccharide structures between the two gp50 forms. Thus, both proteins were investigated. In Vero cells the intact gp50 produced by PRV infection was analyzed.

Our results show that the two mammalian cell lines both synthesize approximately the same distribution of three O-linked oligosaccharides, the major two of which contain terminal sialic acid. The Sf9 cells synthesize smaller nonsialylated structures, the majority of which is the monosaccharide GalNAc. The relevance of these findings in relation to the activity levels of glycosyltransferases involved is discussed.

MATERIALS AND METHODS

Materials

UDP-(1-^{3}H)N-acetylgalactosamine (8.7 Ci/mmol), UDP-[1-^{3}H]-galactose (11.4 Ci/mmol), D-[1,6-^{3}H]-glucosamine hydrochloride (44.8 Ci/mmol), D-[4,5-^{3}H]-galactose (45.1 Ci/mmol), D-[^{14}C,U]-glucose (265 mCi/mmol), sodium boro [^{3}H]hydride (407 mCl/mmol), and EN^{3}HANCE were purchased from New England Nuclear. ATP, UDP-N-acetylgalactosamine, UDP-galactose, bovine submaxillary mucin, 2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-D-galactopyranose (Galβ1-3GalNAc), chymostatin, leupeptin, antipain, pepstatin, aprotinin, Nonidet P-40, Triton X-100, taurodeoxycholate, Amberlite MB-3, and jack bean β-galactosidase were from Sigma. Bovine testis β-galactosidase and endo-α-N-acetylgalactosaminidase were obtained from Boehringer. Pronase was from Calbiochem. Ham's F12 medium, Dulbecco's Modified Eagle's Medium (DMEM), Grace's insect medium, heat-inactivated fetal

calf serum, nonessential MEM amino acids, penicillin, and streptomycin were obtained from Gibco. Preparation of the monoclonal anti-gp50 antibody 3A-4 has been described [12]. All other reagents were from standard sources.

Cells

The clones CHOgp50 and CHOgp50T were cultured in DMEM, 10% FCS, 0.1 mM MEM nonessential amino acids, 20 mU penicillin/ml, 20 μg streptomycin/ml. CHO cells were cultured in Hamm's F12 medium, 10% FCS, 10 mM HEPES, 20 mU penicillin/ml, 20 μg streptomycin/ml. Vero cells were cultured in DMEM, 10% FCS, 20 mU penicillin/ml, 20 μg streptomycin/ml. Sf9 cells were cultured in Grace's insect medium supplemented with 10% FCS, 20 mU penicillin/ml, 20 μg streptomycin/ml, and 2.5 μg fungizone/ml [14].

Baculovirus Constructions

Preparation of the two baculovirus constructions AcNPV-gp50 and AcNPV-gp50T was carried out as described previously [13]. Briefly, the gp50 gene [12] was cloned into the BamHI restriction site of the transfer vector pAc373 by adding BamHI linkers to the NarI site (at the amino terminal end of the gene) and the MaeIII site (at the carboxy terminal end of the gene) before insertion. This plasmid, designated pAc50, when inserted into AcNPV results in a recombinant virus capable of producing the intact gp50 molecule. To produce the truncated version of gp50, (gp50T), a phase termination codon was inserted into the gene at the Sau3A site 47 amino acids from the carboxy terminal end. The construct was then cloned into pAc373 to produce the plasmid pAc50T, which upon insertion into AcNPV resulted in a virus producing a secreted form of gp50 lacking the transmembrane and cytoplasmic domains. Insertion of pAc-gp50 and pAc-gp50T into AcNPV-E2 was done using standard baculovirus expression vector technology [14].

CHO Cell Constructions

CHO cells producing intact gp50 have been described [12]. The CHO cell line producing truncated gp50 was produced by the identical expression vector, with insertion of the truncated gp50 gene described above.

Preparation of [^{3}H]Galactose, [^{3}H]Glucosamine, and [^{14}C]Glucose-Labeled gp50, and gp50T From Cultured Cells

CHO cells. The CHOgp50T clone was grown in a 25 cm^2 tissue culture flask to approximately 80% confluency (~2.2 × 10^6 cells); the medium was removed and 5 ml of glucose-poor (1 mM) DMEM containing 10% FCS, 10 mM HEPES, 0.1 mM MEM nonessential amino acids, and 100 μCi/ml [^{3}H]glucosamine or [^{3}H]galactose was added. The cells were cultured in the labeled medium for 16–18 hours. At the end of the labeling period, the culture medium was collected and any cells present were removed by low-speed centrifugation. The cell-free culture medium was subsequently substituted with 0.3 TIU/ml aprotinin and used directly for immunoprecipitation.

Vero cells. Approximately 1 × 10^7 cells cultured in a 75 cm^2 tissue culture flask were infected with pseudorabies virus, Rice strain, at a moi of 5 PFU/cell in DMEM containing 2% FCS. One hour post infection the medium was removed and replaced with DMEM containing 0.55 mM (one tenth the normal concentration) glucose, 2% FCS,

and 100 μCi/ml [^{3}H]glucosamine or [^{3}H]galactose. The infected cells were harvested 16 h post infection by scraping the cells from the monolayer into the medium and sedimenting them by low-speed centrifugation. The pelleted cells were washed once with ice-cold PBS and lysed in 1 ml PBS containing 1% Triton X-100, 1% taurodeoxycholate, 0.1% SDS, 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin, and 0.1 TIU/ml aprotinin (buffer A). After incubation for 1 h on ice, the lysate was centrifuged at 105,000g for 45 min. The supernatant was diluted with 1 ml PBS containing 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin, and 0.1 TIU/ml aprotinin and used for immunoprecipitaiton. The pellet was discarded.

Sf9 cells. Approximately 6×10^6 cells grown in a 75 cm^2 tissue culture flask were infected with AcNPV-gp50 or AcNPV-gp50T at a moi of 10 PFU/cell. Infected cells were labeled 24–48 h post infection in Grace's insect medium containing 0.4 mM (one tenth the normal concentration) glucose, 2% FCS, and 100 μCi/ml [^{3}H]glucosamine or 50 μCi/ml [^{14}C]glucose. For isolation of gp50, the cells were harvested by scraping them into the medium followed by sedimentation by low-speed centrifugation. Following washing with PBS the sedimented cells were solubilized in buffer A and the lysate was prepared for immunoprecipitation as outlined for Vero cells. When isolating gp50T, the medium from the infected, labeled cells was collected and prepared for immunoprecipitation as described for CHO cells (see above).

Immunoprecipitation and Preparation of Radiolabeled Glycopeptides and Oligosaccharides

When immunoprecipitating from lysed cells, the diluted cell lysate (see above) was pre-cleared by incubation with 200 μl (100 μl/ml lysate) protein A-Sepharose for 90 min. After sedimenting and discarding the protein A-Sepharose, 8 μl anti-gp50 antibodies was added/ml lysate and the mixture was incubated overnight. Immunoprecipitation from conditioned cell culture medium was carried out as follows: 800 μl of medium was supplemented with Triton X-100, taurodeoxycholate, and SDS to 0.5%, 0.5%, and 0.05% concentration, respectively. The sample was pre-cleared by incubation with 80 μl protein A-Sepharose for 90 min. Following sedimentation of the gel the supernatant was supplemented with 8 μl anti-gp50 antibodies and incubated overnight. The antigen-antibody complex was isolated by adding 40 μl of protein A-Sepharose and incubating for 2–3 h, after which the gel was sedimented and thoroughly washed using a procedure outlined by Dunphy et al. [15]. All incubations were carried out at 4°C on a tube rotator. The precipitated gp50 was released from the protein A-Sepharose-antibody complex by boiling for 5–10 min in an equal volume (to the gel volume) 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenolblue, 0.3M β-mercaptoethanol. After boiling the gel was sedimented and the supernatant was loaded directly on SDS-PAGE. SDS-PAGE and fluorography of the dried gels was carried out as described [16]. The acrylamide concentration was 10%. The position of the immunoprecipitated gp50 on the dried gel was determined by fluorography (see above). The protein band was cut out of the gel and radiolabeled oligosaccharides were released either as glycopeptides by pronase treatment, as described by Cummings et al. [17], or by subjecting the gel slices directly to alkaline hydrolysis in the presence of sodium borohydride, essentially as described by Carlson [18]. Briefly, the gel slice was cut into small fragments and suspended in 2 ml H_2O, 2 ml 100 mM NaOH containing 1M $NaBH_4$ was added, and the sample was incubated at 45°C for 15–24 hours. Following hydrolysis the sample was

passed over a column of Dowex 50W-X8 (10 ml bed volume) to remove sodium. Borates were removed by repeated evaporations with methanol-4 M acetic acid (9:1). This latter method was used for most experiments in this study since it was found that pronase digestion of gp50 and gp50T results in a large portion of the released radiolabeled glycopeptides being resistant to alkaline sodium borohydride treatment. Using direct treatment with alkaline sodium borohydride, the recovery of the radioactivity loaded on the gel was approximately 80%.

Analysis of the O-linked Oligosaccharides on Gp50 and Gp50T

In vivo labeled oligosaccharides released with alkaline sodium borohydride treatment from immunoprecipitated gp50 and gp50T were separated on QAE-Sephadex chromatography as described [19]. After removal of terminal sialic acid by mild acid treatment [20], the neutral oligosaccharides were separated on descending paper chromatography in ethyl acetate:pyridine:glacial acetic acid:H_2O (5:5:1:3) for 20 hours. Strong acid hydrolysis was performed in 2 N HCl at 100° for 4 hours. Analysis of aminosugar composition on borate impregnated paper was performed as described in [21]. The solvent system used was 1-butanol:pyridine:H_2O (6:4:3). Neutral sugars were separated on descending paper chromatography in ethyl acetate:pyridine:H_2O (8:2:1). The radioactive standard Galβ1-3GalNAc-ol was prepared by reduction of Galβ1-3GalNac with NaB[$^3H_4$] as described in [22]. Nonradioactive standards were detected using a silver nitrate dip assay [23]. Chromatography on Biogel P-2 was done on a 1.5 × 100 cm column equilibrated in 0.1 M NH_4HCO_3. One hundred twenty 1.3 ml fractions were collected. V_0 and V_e were determined using bovine serum albumin and [^{3}H]mannose, respectively. Conditions for digestion with bovine testis and jack bean β-galactosidase have been described [24]. Digestion with endo-α-N-acetylgalactosaminidase was carried out as follows. After washing the protein A-Sepharose-antibody-antigen complex as outlined above, the gel was washed twice in 400 μl 20 mM Tris-Maleate, pH 6.0. Following washing, the gel was suspended in 150 μl 20 mM Tris-Maleate, pH 6.0, and supplemented with 6 mU (40 mU/ml) endo-α-N-acetylgalactosaminidase. After incubation at 37°C overnight, the gel was sedimented by centrifugation and the supernatant containing the released oligosaccharides was collected. The gel was subsequently washed twice with 400 μl H_2O and the combined washes were pooled with the initial supernatant, evaporated and loaded on the Biogel P-2 column. Control experiments showed that the release of the disaccharide on gp50 and gp50T under these conditions was almost quantitative.

Enzyme Assays

Assays for UDP-GalNAc:polypeptide, N-acetylgalactosaminyltransferase was carried out essentially as described in [25]. The amount of radioactivity was 40,000 cpm/assay and the UDP-GalNAc concentration 15 μM. Assays for UDP-Gal:N-acetylgalactosamine, β1-3 galactosyltransferase was performed as described in [26] with the following modifications: bovine serum albumin was omitted, the amount of radioactivity was 90,000 cpm/assay and the UDP-Gal concentration was adjusted to 40 μM, 2 × Km [27].

Two sources of enzyme were used. A total cell lysate prepared by solubilizing cells (approx. 6×10^7/ml) in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50% glycerol, 2% Triton X-100, 10 μg/ml each of leupeptin, antipain, chymostatin, and pepstatin and 0.1

TIU/ml aprotinin. The crude membrane fractions were prepared as described in [28]. The isolated, washed membranes were solubilized in the same buffer as the cell lysate at approximately 8 mg protein/ml.

The Sf9 cells used for enzyme assays had been cultured under serum-free conditions [29] for greater than 6 months.

Protein Determination

Protein concentration was measured as described by Lowry [30].

RESULTS

Structural Analysis of the Oligosaccharide Structures on gp50 and gp50T

QAE-Sephadex chromatography of oligosaccharides released by alkaline sodium borohydride treatment from in vivo [^{3}H]galactose labeled gp50T and gp50 isolated from CHO and Vero cells respectively, resulted in three separate fractions (Fig. 1A,B). Approximately 11% and 16% of the structures synthesized by CHO and Vero cells, respectively, did not interact with the column, 53% and 48% eluted with 20 mM NaCl, and thus contained one charge, and the remaining 35% and 36% contained two charges [19]. Mild acid or neuraminidase treatment of the charged material followed by reapplication to the QAE-Sephadex column resulted for both runs in a quantitative shift of the radioactivity to the run-through fraction (data not shown).

In a similar experiment using gp50 oligosaccharides isolated from in vivo [^{3}H]glucosamine labeled CHO and Vero cells, the charged molecules released by mild acid treatment were isolated on the QAE-Sephadex column and identified on paper chromatography; more than 80% of the radioactivity co-migrated with the N-acetylneuraminic acid standard (Table I). Thus, the majority of the oligosaccharides synthesized by both CHO and Vero cells contains one or two terminal sialic acid residues. Oligosaccharides released from gp50T isolated from [^{3}H]glucosamine labeled Sf9 cells on the other hand, did not interact with the QAE-Sephadex column at all (Fig. 1C), and no sialic acid could be detected on paper chromatography after mild acid treatment. Analysis of gp50 oligosaccharides synthesized by Sf9 cells yielded an identical profile on the QAE-Sephadex column (data not shown).

Separation on paper chromatography of oligosaccharides released by alkaline sodium borohydride treatment of gp50T and gp50 isolated from CHO and Vero cells and subjected to mild acid hydrolysis resulted in both cases in one symmetric peak co-migrating with the Galβ1-3GalNAc-ol standard (Fig. 2A,B). A similar separation of oligosaccharides isolated from Sf9 gp50 and gp50T produced in both cases two peaks (Fig. 2C,D). The majority of the radioactivity in the insect produced oligosaccharides co-migrated with the GalNAc-ol standard; smaller amounts, 12–16% for gp50 and 25–26% for gp50T, were however also detected in the position of Galβ1- 3GalNAc-ol. The quantitative differences in the two oligosaccharide structures between gp50 and gp50T appears to be significant. In three separate experiments gp50T consistently contained at least 50% more of the structure co-migrating with Galβ1-3GalNAc-ol (data not shown).

Strong acid hydrolysis of the [^{3}H]glucosamine labeled oligosaccharides from all three cell types followed by separation of the released monosaccharides on paper chromatography failed to detect any other aminosugar than GalNAc (Table I).

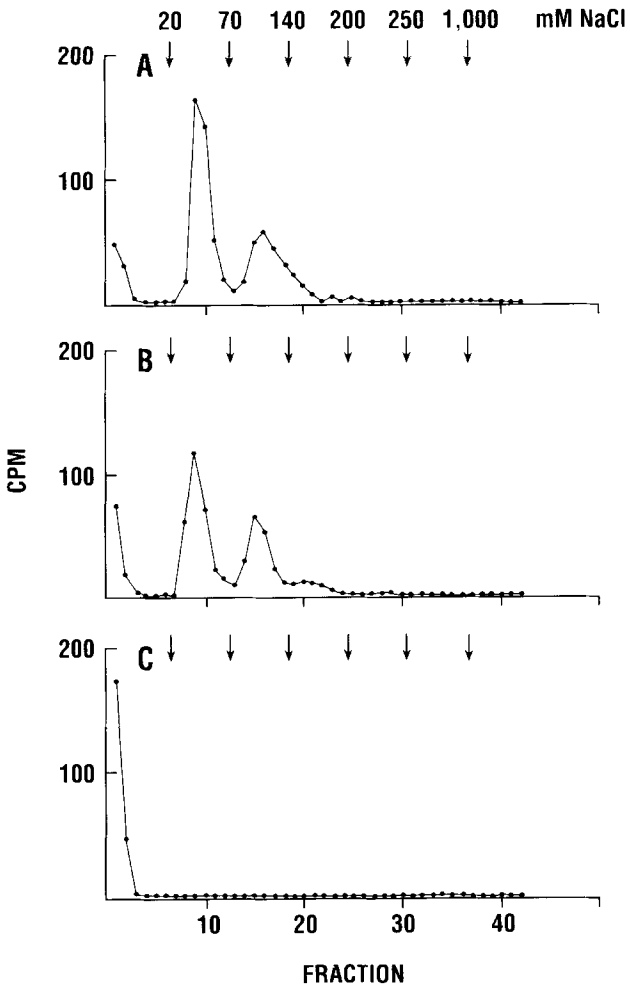

Fig. 1. QAE-Sephadex chromatography of [^{3}H]galactose and ^{3}H-glucosamine-labeled gp50 and gp50T oligosaccharides. Cells were labeled in vivo with [^{3}H]galactose (CHO and Vero) and [^{3}H]glucosamine (Sf9). Gp50 and gp50T were isolated by immunoprecipitation and O-linked oligosaccharides were released by alkaline borohydride treatment of the antigen band after separation on SDS-PAGE. The released oligosaccharides were applied to QAE-Sephadex columns and the bound oligosaccharides were eluted with increasing concentrations of NaCl as described in Materials and Methods. Aliquots of each fraction were counted for radioactivity. **A and C:** Oligosaccharides isolated from gp50T synthesized by CHO and Sf9 cells respectively. **B:** Oligosaccharides isolated from gp50 synthesized by Vero cells.

A similar experiment carried out on gp50T and gp50 isolated from CHO and Vero cells labeled in vivo with [^{3}H]galactose demonstrated that the only neutral sugar present in the putative disaccharide structure(s) synthesized by these cells is galactose (Table I).

The two structures on gp50T synthesized by Sf9 cells labeled in vivo with [^{14}C]glucose were separated on a Biogel P-2 column (Fig. 3A) and the two major peaks (a and b) were identified on paper chromatography (Fig. 3A, inset); the small, earlier eluting peak(s) contains unhydrolysed or incompletely hydrolysed glycopeptides and the material in the small radioactivity peak close to V_e co-migrates with galactose on paper

TABLE I. Identification of Component Monosaccharides in PRV gp50 and gp 50T Oligosaccharides Synthesized by CHO, Vero, and Sf9 Cells*

	% of total radioactivity[a]		
	CHO	Vero	Sf9
Neutral sugars			
Gal	90	77	86
Man	0	0	0
Fuc	0	0	0
Amino sugars			
GalNAc	75	81	85
GlcNAc	6[b]	4[b]	0
Sialic acids			
NeuNAc	84	81	ND[c]

*Neutral sugars were determined by *in vivo* labeling of CHO and Vero cells with [^{3}H]galactose, Sf9 cells with [^{14}C]glucose. Gp50 and gp50T oligosaccharides were isolated as described in the legend to Figure 1 and in the case of structures synthesized by CHO an Vero cells, subjected directly to strong acid hydrolysis. For Sf9 gp50T, the disaccharide was isolated on a Biogel P-2 column (see legend to Fig. 3) prior to hydrolysis. The hydrolysates were separated on paper chromatography in ethyl acetate:pyridine:H_2O (8:2:1). For analysis of aminosugar content gp50 and gp50T were labeled in vivo with [^{3}H]glucosamine, immunoprecipitated and separated on SDS-PAGE. Labeled glycopeptides were released by pronase digestion of the antigen bands and subjected to strong acid hydrolysis following the procedure outlined in Materials and Methods. The resulting monosaccharides were separated on descending paper chromatography in 1-butanol:pyridine:H_2O (6:4:3) using borate impregnated papers. The sialic acid content in the charged oligosaccharides isolated from CHO gp50T and Vero gp50 (see legend to Fig. 1) was determined by hydrolysis under mild acid conditions followed by separation on QAE-Sephadex chromatography as described in Materials and Methods. Charged monosaccharides were identified on descending paper chromatography in isoamylacetate:glacial acetic acid:H_2O (3:3:1).

[a]Numbers represent percent of total radioactivity on chromatogram recovered from the migration position of the monosaccharide.

[b]Some background radioactivity was present in the position of GlcNAc; no GlcNAc peak was detected.

[c]ND, not determined.

chromatography. The neutral sugar composition of the fractions corresponding to the putative disaccharide was determined on paper chromatography following strong acid hydrolysis. Table I shows that the only neutral sugar present in this fraction is galactose. Thus the composition of the gp50 disaccharide synthesized by all three cell lines appears to be Gal-GalNAc.

Further experiments demonstrated that the disaccharide on gp50 and gp50T synthesized by Sf9 cells could be released from the peptide by digestion with endo-α-N-acetylgalactosaminidase (Fig. 3B), an enzyme reported to specifically cleave the linkage between GalNAc on the structure Galβ1-3GalNAc and Ser/Thr on the peptide [31,32]. In addition digestion of the released disaccharide with exoglucosidases showed that the released disaccharide was readily cleaved by bovine testis β-galactosidase while jack bean β-galactosidase only cleaved the structure slowly and incompletely (Fig. 4C,B). These results are consistent with a β1-3linkage between the two monosaccharides. Bovine testis β-galactosidase readily cleaves β1-3 and β1-4 linkages but β1-6 linkages only slowly (33) while jack bean β-galactosidase cleaves β1-4 and β1-6 linkages but β1-3 linkages only slowly under the conditions used [34]. The disaccharide on gp50T and gp50 synthesized by CHO and Vero cells showed the same sensitivity to endo-α-N-acetylgalactosaminidase and bovine testis and jack bean β-galactosidases as the structure synthesized by Sf9 cells (data not shown).

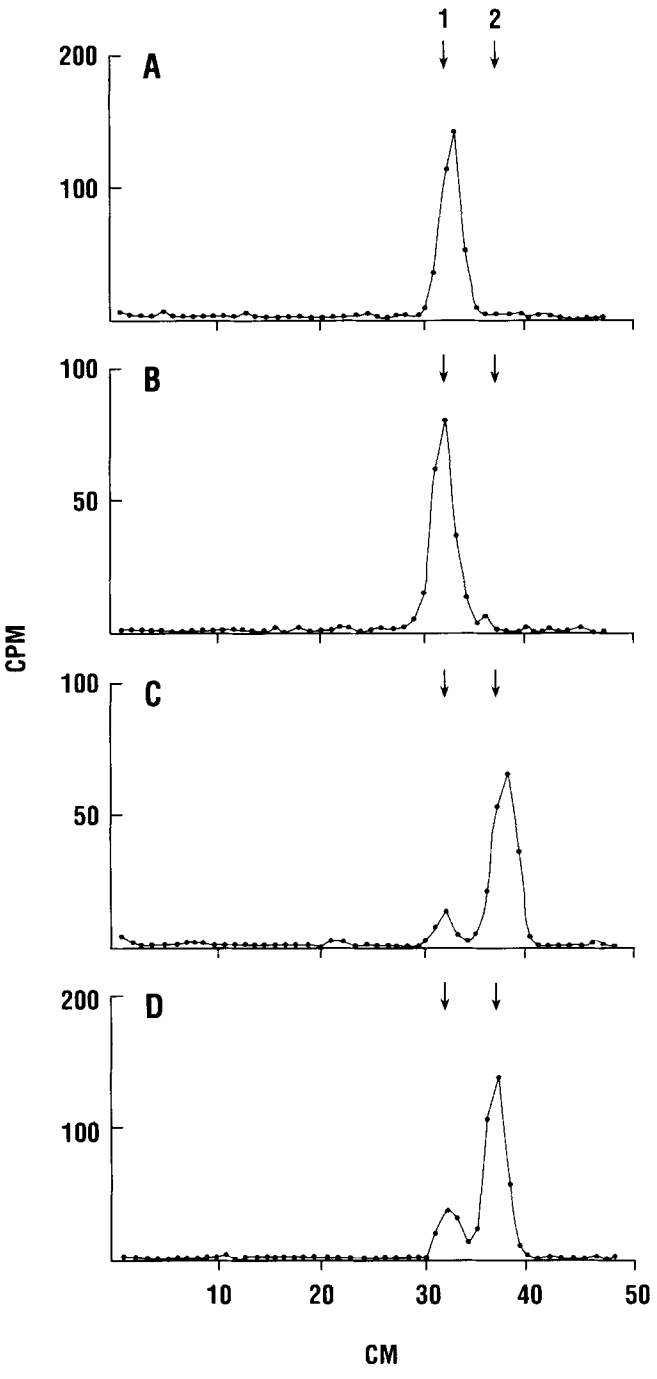

Fig. 2. Paper chromatography of ^{3}H-glucosamine-labeled gp50 and gp50T oligosaccharides. Cells were labeled in vivo with [^{3}H]glucosamine and the O-linked oligosaccharides on gp50 and gp50T were isolated as outlined in the legend to Figure 1. After removal of terminal sialic acid by mild acid treatment, the oligosaccharides were separated on descending paper chromatography in pyridine:ethyl acetate:glacial acetic acid:H_2O (5:5:1:3) as outlined in Materials and Methods. **A and D:** Oligosaccharides isolated from gp50T synthesized by CHO and SF9 cells, respectively. **B and C:** Oligosaccharides isolated from gp50 synthesized by Vero and Sf9 cells, respectively. The migration of standards is indicated 1, Galβ1-3GalNAc-ol; 2, GalNAc-ol.

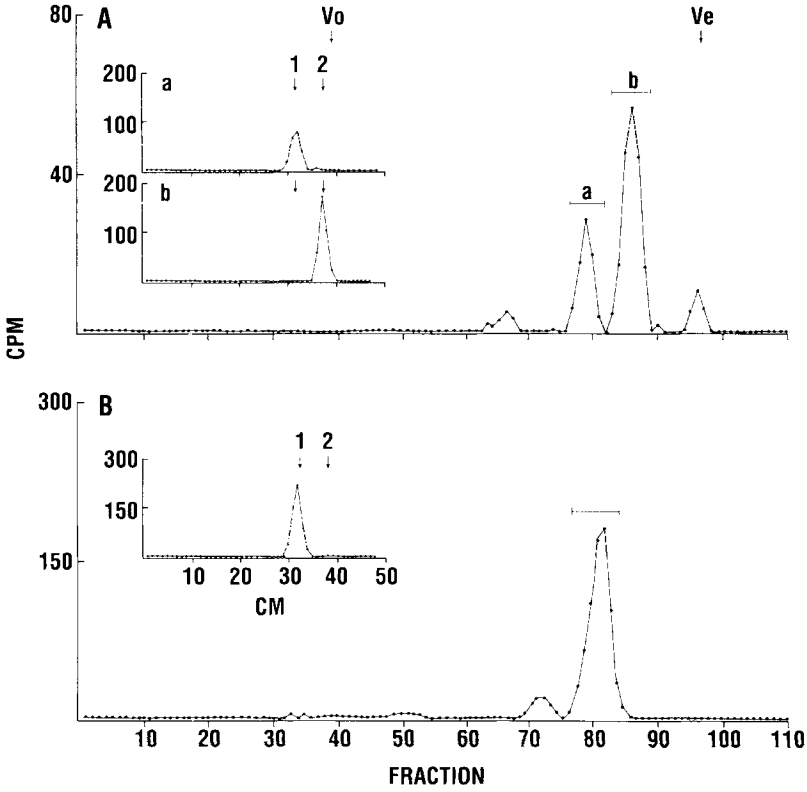

Fig. 3. Biogel P-2 chromatography of gp50T oligosaccharides synthesized by Sf9 cells. **A:** Oligosaccharides on gp50T synthesized by in vivo ^{14}C-glucose-labeled Sf9 cells were isolated as outlined in the legend to Figure 1 and analyzed by Biogel P-2 chromatography as described in Materials and Methods. Aliquots from each fraction were counted for radioactivity. The two major peaks (a and b) were pooled as indicated and identified on paper chromatography (insets a and b). **B:** The gp50T was immunoprecipitated from the culture medium of Sf9 cells labeled in vivo with [^{3}H]glucosamine as outlined in the legend to Figure 1. The washed immunoprecipitate was digested with endo-α-N-acetylgalactosaminidase and the released oligosaccharides were separated on the Biogel P-2 column (see above). The major peak was pooled and identified on paper chromatography (inset). The standards for paper chromatography were 1, Galβ1-3GalNAc-ol; 2, GalNAc-ol.

Assays for Glycosyltransferases Involved in O-Linked Oligosaccharide Biosynthesis

In order to confirm the presence of a galactosyltransferase involved in O-linked oligosaccharide biosynthesis in Sf9 cells and to try to understand why the insect cell line only substituted between 12% and 25% of the available terminal GalNAc residues with galactose, we decided to determine the activity levels of the two enzymes involved in the synthesis of Galβ1-3GalNAc in all three cell lines. Control experiments showed that both assays used were linear with incubation time and the amount of added enzyme (both when assaying total cell lysates and isolated membrane fractions), for all three cell lines. Very little transfer to endogenous acceptors was observed (data not shown). Analysis of the reaction products showed that >94% (in Sf9 cells 100%) of the radioactivity transferred in the galactosyltransferase assay by enzyme from all three cell

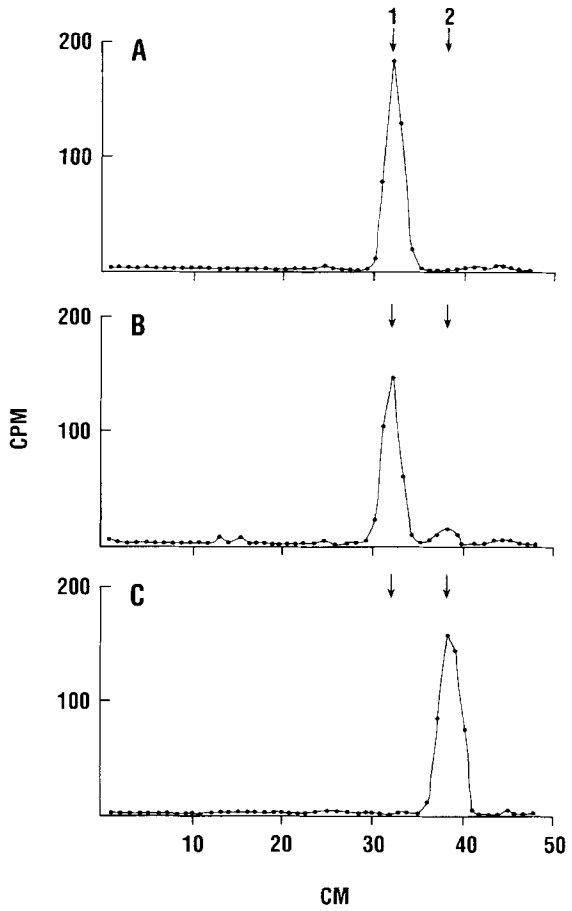

Fig. 4. Digestion of Sf9 gp50T oligosaccharides with β-galactosidases. The Sf9 gp50T oligosaccharide recovered from the pool of the major peak on Biogel P-2 chromatography (see legend to Fig. 3B) was digested with β-galactosidases as outlined in Materials and Methods. The resulting digests were analyzed on descending paper chromatography. **A:** The undigested disaccharide. **B and C:** Products from digestion with jack bean and bovine testis β-galactosidase, respectively. Standards are indicated by the arrows: 1, Galβ1-3GalNAc; 2, GalNAc.

lines was incorporated in Galβ1-3GalNAc. For the GalNac-transferase assay, essentially all the radioactivity transferred to the acceptor could be recovered as GalNAc-ol after alkaline sodium borohydride treatment (data not shown).

When comparing the activity levels of the N-acetylgalactosaminyl and galactosyltransferase in CHO, Vero, and Sf9 cells it is apparent that while all three cell lines contain varying but comparable levels of GalNAc-transferase, there is a greater variation in the levels of Gal-transferase with the lowest levels found in the Sf9 cells (Table II).

DISCUSSION

The pseudorabies virus protein gp50 contains no consensus sequence for N-linked glycosylation [12]. However, previous work has shown that CHO and Vero cells can

TABLE II. UDP-GalNAc:Polypeptide N-Acetylgalactosaminyltransferase and UDP-Gal:N-Acetylgalactosamine β1-3 Galactosyltransferase Activities in Different Cell Lines*

	Activity (mU/mg)			
	GalNAc-transferase		Gal-transferase	
Cell line	Total cell protein	Membrane protein	Total cell protein	Membrane protein
CHO	101 ± 15[a]	392 ± 44[a]	69 ± 8[b]	257 ± 43[b]
Vero	232 ± 33	481 ± 57	309 ± 34	620 ± 61
Sf9	159 ± 23	253 ± 45	19 ± 4	32 ± 6

*Enzyme preparations and assays were carried out as described in Materials and Methods. The values are given as means ± S.E. (n = 4).

[a]1 unit equals 1 nmole N-acetylgalactosamine transferred to apomucin per min under assay conditions.

[b]1 unit equals 1 nmole galactose transferred to asialomucin per min under assay conditions.

incorporate [^{14}C]glucosamine into this protein in vivo [12,35]. In addition it has been demonstrated that the molecular size of gp50 is not affected by incubation of the producing cell with tunicamycin but that incubation with monensin results in a 15 kDa reduction [12].

The current data shows that gp50 both in intact form and as a truncated derivative does contain O-linked oligosaccharides. When synthesized by a mammalian cell the O-linked oligosaccharides on the protein are essentially restricted to one primary structure, Galβ1-3GalNAc, which may be substituted with one or two terminal sialic acid residues. When synthesized by the insect cell line Sf9, the protein still contains O-linked oligosaccharides but, in analogy with N-linked structures synthesized by insect cells [2,3,36], these structures do not contain any terminal sialic acid. In addition Sf9 cells appear to substitute only a portion of the GalNAc residues linked to the protein with galactose. This finding is consistent with observations made by Butters and Hughes when studying oligosaccharides synthesized by *Aedes aegypti* cells. Alkaline sodium borohydride treatment of glycopeptides isolated from these cells labeled in vivo with [^{14}C] glucosamine in the presence of tunicamycin resulted in the release of structures with a mobility coinciding with V_t on Biogel P-6 chromatography, thus indicating the presence of only small O-linked structures [2].

In contrast to what has been reported for the galactosyltransferase(s) involved in the synthesis of N-linked oligosaccharides in insect cells [2], Sf9 cells do contain measurable levels of a β1-3 galactosyltransferase active on O-linked structures (Table II). The relatively low levels of this activity in the Sf9 cells may be related to the observed low percentage of O-linked structures substituted with galactose. The reason for the higher degree of substitution on gp50T versus gp50 is not known. Assuming that the low levels of galactosyltransferase constitutes a limiting factor, a possible explanation is that the longer intracellular transit time of gp50T [13], allows for a longer exposure to the enzyme.

The existence of terminal GalNAc residues on insect proteins from several different species has been demonstrated previously using N-acetylgalactosamine specific lectins [1,2,9,11]. Thus, the inability to add terminal sialic acid to O-linked structures may be a general characteristic among insect cells, as has been reported for N-linked structures. This finding is in agreement with observations made by Warren who failed to detect any sialic acid at all in three different insect species [37]. Whether or not the low

levels of β1-3 galactosyltransferase and the incomplete substitution of O-linked GalNAC with galactose also is a general characteristic of insect cells awaits further experimentation.

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