Regulation of Asparagine-linked Oligosaccharide Processing

OLIGOSACCHARIDE PROCESSING IN Aedes albopictus MOSQUITO CELLS*

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We have examined the synthesis and processing of asparagine-linked oligosaccharides from Aedes albopictus C6/36 mosquito cells. These cells synthesized a glucose-containing lipid-linked oligosaccharide with properties identical to that of Glc3Man3GlcNAc2-PP-dolichol. Results of brief pulse label experiments with [3H]mannose were consistent with the transfer of Glc3Man3GlcNAc2 to protein followed by the rapid removal of glucose residues. Pulse-chase experiments established that further processing of oligosaccharides in C6/36 cells resulted in the removal of up to six α-linked mannosyl residues yielding Man3GlcNAc2 whose structure is identical to that of the trimannosyl "core" of N-linked oligosaccharides of vertebrate cells and yeast. Complex-type oligosaccharides were not observed in C6/36 cells.

When Sindbis virus was grown in mosquito cells, Man3GlcNAc2 glycans were preferentially located at the two glycosylation sites which were previously shown to have complex glycans in virus grown in vertebrate cells. These Man3GlcNAc2 structures are the most extensively processed oligosaccharides in A. albopictus, and as such, are analogous to the complex glycans of vertebrate cells. We suggest that determinants of oligosaccharide processing which reside in the polypeptide are universally recognized despite evolutionary divergence of the oligosaccharide-processing pathway between insects and vertebrates.

Asn-linked glycosylation begins with the en bloc transfer in the rough endoplasmic reticulum of a lipid-linked oligosaccharide, Glc3Man3GlcNAc2 from dolichol carrier to appropriate asparagine residues (reviewed in Ref. 1). Processing of oligosaccharides commences in the rough endoplasmic reticulum with the removal of glucose residues. In vertebrate cells, removal of up to four α-linked mannosyl residues in the Golgi apparatus yields high mannose-type oligosaccharides on the mature glycoprotein. Further modifications in the Golgi apparatus result in the formation of complex-type oligosaccharides.

Studies of viral and cellular glycoproteins demonstrate that individual glycosylation sites exhibit marked selectivity for either high mannose or complex-type oligosaccharides (reviewed in Ref. 2). These observations suggest that oligosaccharide processing is regulated in some fashion although relatively little is known about factors which control processing. Recently, we (3) and others (4–6) have provided support for the hypothesis that the extent of oligosaccharide processing is primarily determined by the accessibility of oligosaccharides to cellular processing enzymes. In these studies, a correlation between accessibility of oligosaccharides to endo H1 in native proteins and extents of oligosaccharide processing was established.

We have examined the synthesis and processing of Asn-linked oligosaccharides from Aedes albopictus C6/36 cells in order to ask two questions. First, what is the general pathway for oligosaccharide processing in insect cells? There is evidence to suggest that the processing pathway has diverged between insects and vertebrates since N-linked oligosaccharides from Aedes aegypti cells are deficient in sialic acid, galactose, and fucose (7–9). Second, if oligosaccharide structures are different in insect and vertebrate cells, do extents of oligosaccharide processing at individual glycosylation sites of C6/36-grown Sindbis virus parallel extents of processing at these same sites in virus grown in BHK, CHO, and CHF cells?

A study of oligosaccharide processing in C6/36 cells reveals that Man3GlcNAc2 structures, which arise from high mannose oligosaccharides, constitute the most extensively processed oligosaccharides in these cells. When Sindbis virus is grown in mosquito cells, Man3GlcNAc2 is preferentially located at two glycosylation sites which have complex-type oligosaccharides in Sindbis virus grown in vertebrate cells (10). These results suggest that extents of oligosaccharide processing are dictated by the glycoprotein itself and are independent of the cellular processing pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—A. albopictus C6/36 cells and Aedes pseudoscutellaris MOS-61 cells, obtained from Dr. S. Buckley (Yale Arbovirus Research Unit, New Haven, CT), were maintained as monolayers at 28 °C in Leibovitz-15 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum and 10% tryptose phosphate broth (Gibco). Drosophila Schneider line L-2 cells (11) were provided by and maintained in the laboratory of Dr. M. L. Pardue (MIT, Cambridge, MA). The cells were grown as suspension cultures at 25 °C in Dulbecco's modified MEM supplemented with 10% heat-inactivated fetal calf serum, 0.5% lactalbumin hydrolysate, MEM nonessential amino acids, 50 units/ml penicillin, and 50 µg/ml of streptomycin (all from Gibco).

Viral Infection and Carbohydrate Labeling—Wild type Sindbis

The abbreviations used are: endo H, endo-β-N-acetylglucosaminidase H; endo D, endo-β-N-acetylglucosaminidase D; BHK, baby hamster kidney; CEF, chicken embryo fibroblasts; CHO, Chinese hamster ovary; EDTA, (ethylenedinitritilo)tetraacetic acid; HPLC, high performance liquid chromatography; L-15, Leibovitz-15; MEM, minimal essential medium; MeOH, methanol; Dol-PP, dolichol pyrophosphate.

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Regulation of Oligosaccharide Processing

Purified Sindbis virions were isolated, and E1 and E2 glycoproteins were prepared as described previously (10).

Carbohydrate Labeling of Cells—C6/36 cells were pulse labeled as described previously (12) with minor modifications. Subconfluent cultures of C6/36 cells (3.0-3.5 x 10^6 cells/60-mm plate) were washed 3 times with 5 ml of prewarmed glucose-free MEM (Gibco) prior to labeling. Cultures to be labeled for 2.5 min or less were incubated with 0.7 ml of labeling medium (L-15 containing dialyzed heat-inactivated fetal calf serum, 5% tryptose phosphate broth, and 1.5 mCi/ml of [3H]mannose) on a sheet of plastic floated in a 28°C water bath. Cultures to be labeled for 5 min or longer were incubated in a 5% CO2 incubator at 28°C after addition of 1.5 ml of labeling medium containing 700 μCi/ml of [3H]mannose. At 10-min intervals, the plates were gently tilted to ensure uniform distribution of labeling medium. Cells were harvested in CHCl3/MeOH as described below. In some cases, cells were preincubated with 10 μg/ml of tunicamycin for 1 h prior to labeling with [3H]mannose or [35S]methionine in the same medium. Cells labeled with [3H]galactose were incubated in glucose-free MEM containing 700 μCi/ml of [3H]galactose. C6/36 and MO5-61 cells were labeled with [3H]mannose for 16-20 h at 28°C in 5 ml of L-15 containing 10% heat-inactivated fetal calf serum, 10% tryptose phosphate broth, and 500 μCi of [3H]mannose.

At the end of the labeling period, the cells were washed 3 times with 5 ml of phosphate-buffered saline at 4°C and lysed in 1 ml of lysis buffer (1% Nonidet P-40, 0.15 M NaCl, 5 mM EDTA, 0.1 M Tris-Cl, pH 7.4, 50 mM Na2CO3, and 5 mM phenylmethylsulfonyl fluoride). Schneider line L-2 cells (6.6 x 10^6 cells/60-mm plate) were gently tilted to ensure uniform distribution of labeling medium. Cultures to be labeled for 2.5 min or less were incubated with 0.7 ml of labeling medium (L-15 containing dialyzed heat-inactivated fetal calf serum, 5% tryptose phosphate broth, and 1 mM mannose) which predominated in the short pulse were chased to a large oligosaccharide migrating as Glc3Man9GlcNAc2 (data not shown). In contrast to vertebrate cells (12), intermediates in the biosynthesis of lipid-linked oligosaccharides containing 5 or 8 mannose residues did not accumulate in significant quantities in C6/36 cells. When cells prelabeled for 2.5 min with [3H]mannose were washed and incubated in nonradioactive medium, small lipid-linked oligosaccharides which predominated in the short pulse were chased to a large oligosaccharide migrating as Glc4Man9GlcNAc2 (Fig. 1B).

The kinetics of appearance and disappearance of this large lipid-linked oligosaccharide was studied by pulse labeling C6/36 cells for varying lengths of time with [3H]mannose or prelabeling cells for 5 min with [3H]mannose followed by chases in nonradioactive medium. The amount of label incorporated into Glc4Man9GlcNAc2 was quantitated after gel filtration chromatography (data not shown). Incorporation of [3H]mannose into the large lipid-linked oligosaccharide increased exponentially at early times and reached a maximum out as described by Hough and Jones (18) in ethyl acetate/pyridine/water (8:2:1).

Results

Incorporation of [3H]mannose into Lipid-linked Oligosaccharides—[3H]mannose was incorporated into the lipid-linked oligosaccharides of C6/36 cells after short pulse labels of A. albopictus cells. Lipid-linked oligosaccharides were extracted from labeled cells in CHCl3/MeOH/H2O (10:10:3) and analyzed by gel filtration chromatography after mild acid hydrolysis. When C6/36 cells were pulse-labeled for 2.5 min, the majority of the [3H]mannose-labeled oligosaccharides migrated on Bio-Gel P4 columns as structures containing fewer than 5 mannose residues (Fig. 1A). With increasing lengths of incubation, C6/36 cells accumulated a single large lipid-linked oligosaccharide which migrated as Glc3Man9GlcNAc2 (data not shown). In contrast to vertebrate cells (12), intermediates in the biosynthesis of lipid-linked oligosaccharides containing 5 or 8 mannose residues did not accumulate in significant quantities in C6/36 cells. When cells prelabeled for 2.5 min with [3H]mannose were washed and incubated in nonradioactive medium, small lipid-linked oligosaccharides which predominated in the short pulse were chased to a large oligosaccharide migrating as Glc4Man9GlcNAc2 (Fig. 1B).

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![Fig. 1. Gel filtration chromatography of mild acid-hydrolyzed lipid-linked oligosaccharides from C6/36 cells labeled with [3H]mannose. Subconfluent C6/36 cells were incubated with 1.5 mCi/ml of [3H]mannose (3 plates labeled sequentially and pooled) and harvested before or after a chase in nonradioactive medium. Cells were harvested in CHCl3/MeOH (2:1), washed, and extracted in CHCl3/MeOH/H2O (10:10:3) to solubilize lipid-linked oligosaccharides. The oligosaccharides were deacetylated by mild acid hydrolysis and analyzed by gel filtration on Bio-Gel P4. A, oligosaccharide lipid after 2.5-min pulse label; B, after a 2.5-min chase following a 2.5-min pulse label. Arrows indicate elution positions of standards. G, Glc; M, Man; N, GlcNAc.](http://www.jbc.org/)

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at 10 min of continuous labeling. The result is qualitatively similar to that observed in CSF, NIL-8, and CHO cells (12). If prelabeled cells were chased in nonradioactive medium, the large lipid-linked oligosaccharide disappeared in a manner similar to that seen in CHO cells (12). Initially, there was a small increase in incorporation of label presumably due to incorporation of prelabeled precursors such as GDP-Man or Dol-PP-Man into lipid-linked oligosaccharide. When the pool of prelabeled precursors was exhausted, [3H]mannose lipid-linked oligosaccharide levels decreased exponentially with a half-time of approximately 5–7 min.

Glc3ManGlcNAc2-Dol-PP is postulated to be the predominant lipid-linked oligosaccharide in C6/36 cells based on a number of criteria including: extraction of the intact lipid-linked oligosaccharide in CHCl3/MeOH/H2O (10:10:3) (19); gel filtration chromatography of the lipid-linked oligosaccharide before and after endo H digestion (20); partial resistance of the endo H-treated oligosaccharide to α-mannosidase digestion which was identical to that of authentic Glc3ManGlcNAc2 (20); demonstration of the presence of Glc residues in the lipid-linked oligosaccharide after in vivo labeling of C6/36 cells with [3H]galactose (15); adsorption of the intact lipid-linked oligosaccharide to DEAE-acetate characteristic of Dol-PP (14); and inhibition of lipid-linked oligosaccharide synthesis by tunicamycin, an inhibitor of GlcNAc-Dol-PP formation (data not shown).

Incorporation of [3H]mannose into C6/36 Glycoproteins—C6/36 cells were pulse-labeled for short periods of time with [3H]mannose. The cells were then extracted in CHCl3/MeOH/H2O (10:10:3) to remove lipid-linked oligosaccharides before or after a chase in nonradioactive medium. The glycoprotein fraction was digested with Pronase, endo H, and endo D and analyzed by gel filtration chromatography. An endo H-released oligosaccharide co-migrating with Glc3ManGlcNAc2 standard was observed when cells were labeled for 1.5 min (data not shown). When cells were pulse-labeled for 2.5 or 5 min, the levels of the oligosaccharide migrating as Glc3ManGlcNAc2 decreased while levels of smaller oligosaccharides migrating as Glc2Man2GlcNAc2, Glc2ManGlcNAc2, Man2GlcNAc2, and ManGlcNAc2 increased. These results are most consistent with the en bloc transfer of Glc3ManGlcNAc2 to an asparagine residue of the polypeptide acceptor followed by the rapid removal of the three terminal glucose residues yielding a Man3GlcNAc2 oligosaccharide. Since levels of lipid-linked oligosaccharides containing 1 or 2 glucose residues are very low in C6/36 cells, it is unlikely that transfer of lipid-linked Glc3ManGlcNAc2 is occurring.

Processing of C6/36 Protein-linked Oligosaccharides—Processing of C6/36 oligosaccharides was examined by gel filtration chromatography of protein-linked oligosaccharides from cells labeled in pulse-chase experiments. Actively growing cultures of cells were labeled with [3H]mannose for 5 min and then washed and chased in nonradioactive medium for varying lengths of time. [3H]Mannose-labeled oligosaccharides released by endo H and endo D treatment of Pronase glycopeptides are shown in Fig. 2.

Endo H-released oligosaccharides from cells labeled for 5 min migrated as Glc2Man2GlcNAc2, Glc2ManGlcNAc2, or Man2GlcNAc2 (Fig. 2A). When cells were labeled for 5 min followed by a 5-min chase, no new oligosaccharides were observed, but the relative amount of Man3GlcNAc2 increased. After a 10-min chase, an oligosaccharide migrating as Man4GlcNAc2 appeared, and the amount of Glc2Man2GlcNAc2 was much decreased. New oligosaccharide species containing 3 to 7 mannose residues were prominent after a 25-min chase (Fig. 2D). (Evidence will be presented below which establishes that the majority of protein-linked oligosaccharides in C6/36 cells are α-mannosidase sensitive and are either high mannose structures released by endo H containing 5 to 9 mannose residues or small endo D-released Man3GlcNAc2 oligosaccharides.) With increasing length of chase (Fig. 2, E and F), the relative amount of the smaller oligosaccharides containing 3 to 7 mannose residues increased. The length of time required for the appearance of extensively processed protein-linked oligosaccharides such as Man3GlcNAc2 contrasted sharply with initial processing events involving the rapid removal of glucose residues. A significant observation was the complete absence of complex-type oligosaccharides in C6/36 cells. Regardless of labeling conditions used, Glc2Man2GlcNAc2 was the largest oligosaccharide observed. It is possible that a small number of oligosaccharides present in mature glycoproteins contain at least 1 glucose residue. After a 2-h chase (Fig. 2F), a small amount of material migrated as Glc2Man2GlcNAc2.

The fact that Man3GlcNAc2 oligosaccharides appeared simultaneously with larger high mannose oligosaccharides containing 8 or 7 mannose residues (Fig. 2D) suggested that removal of mannose residues, once initiated, was a relatively efficient process. In spite of the efficiency of processing of some oligosaccharides, however, other oligosaccharide chains were relatively resistant to extensive processing as evidence by the large number of oligosaccharides containing 7 to 9 mannose residues (48% of total protein-linked oligosaccharides).

A new finding that C6/36 cells synthesize mostly high mannose-type oligosaccharides and Man3GlcNAc2 structures was...
Regulation of Oligosaccharide Processing

extended to other insect cell lines. *A. pseudoscutellaris* MOS-61 cells and Drosophila Schneider line L-2 cells were labeled for 16-20 h with [3H]mannose. The proteins from cell lysates were digested with Pronase and the glycopeptides and endo H and endo D-released oligosaccharides analyzed by gel filtration chromatography (Fig. 3). In all three cell lines, C6/36, MOS-61, and Schneider cells, no significant accumulation of complex-type oligosaccharides was observed.

Partial Characterization of Protein-linked Oligosaccharides—[3H]Mannose-labeled oligosaccharides released by endo H and endo D from CS/36 glycopeptides were resolved by gel filtration chromatography. Fractions corresponding to oligosaccharides migrating as Man₉GlcNAc₂ (Fraction I), Man₇GlcNAc₂ (Fraction II), and Man₅GlcNAc₂ (Fraction III) were pooled and exhaustively digested with α-mannosidase (data not shown). Fraction I oligosaccharides yielded the disaccharide Man,GlcNAc and free mannose in the ratio of 1:7.94 with 6% of the total [3H]-counts per min eluting as α-mannosidase-resistant material. Clearly, the vast majority (>90%) of the oligosaccharides in this fraction are authentic high mannose oligosaccharides containing 7 to 9 mannose residues. Similarly, fraction III oligosaccharides yielded exclusively the disaccharide and free mannose in the ratio of 1:7.94 confirming that the smallest oligosaccharide in CS/36 cells is Man₅GlcNAc₂, a substrate for cleavage by endo D, but not endo H (21, 22). Surprisingly, oligosaccharides from fraction II yielded a heterogeneous array of products when treated with α-mannosidase. Approximately 10% of the total [3H]mannose counts per min migrated as the disaccharide; however, 44% of the [3H]-counts per min migrated as α-mannosidase-resistant material. Clearly, however, the vast majority of oligosaccharides in CS/36 cellular glycoproteins are authentic high mannose oligosaccharides or Man₇GlcNAc₂ since fraction II glycans constitute only 14% of total [3H]mannose.

Processing of Oligosaccharides from Sindbis Virus Grown in C6/36 Cells—Regulation of oligosaccharide processing in *A. albopictus* C6/36 cells was studied by analyzing the oligosaccharides of Sindbis virus grown in C6/36 cells. [3H]Mannose-labeled Pronase glycopeptides and oligosaccharides from Sindbis virus grown in C6/36 cells were analyzed by gel filtration chromatography (Fig. 4). As expected, [3H]mannose-labeled Pronase glycopeptides when resolved on Bio-Gel
P4 gel filtration columns migrated as relatively small structures indicative of the absence of glycopeptides bearing complex-type oligosaccharides. Analysis of [3H]mannose-labeled oligosaccharides released by endo H and/or endo D digestion of glycopeptides (Fig. 4, B, C, and D) demonstrated that viral oligosaccharides were similar in composition to cellular oligosaccharides. Thus, all [3H]mannose-labeled oligosaccharides migrated on Bio-Gel P4 columns as either endo H-released high mannose-type structures containing 4 to 9 mannose residues or small Man9GlcNAc2 structures released by endo D. The inability to incorporate [3H]galactose or fucose in C6/36-grown virus is consistent with the absence of complex-type oligosaccharides in these cells (data not shown). Quantitation of the data shown in Fig. 4 allowed an approximation of the relative frequency of individual oligosaccharide species in Sindbis virus E1 or E2 glycoproteins. Normalization for the number of mannose residues in each oligosaccharide species (defined by elution position on Bio-Gel P4 gel filtration columns, susceptibility to endo H or endo D cleavage, and α-mannosidase digestion, presented below) revealed that extensively processed Man9GlcNAc2 oligosaccharides constituted approximately 59% of the total oligosaccharide chains in E1 and E2.

Confirmation of viral oligosaccharides as being high mannose structures or Man9GlcNAc2 was obtained by exhaustive α-mannosidase digestion of endo H- and endo D-released oligosaccharides. [3H]Mannose-labeled oligosaccharides migrating as Man9GlcNAc2, ManGlcNAc2, Man6GlcNAc2, Man5GlcNAc2, or Man4GlcNAc2 (Fractions I-V, respectively) were individually pooled from a preparative P4 column and exhaustively digested with jack bean α-mannosidase. The digestion products were analyzed by gel filtration chromatography (data not shown). Large Sindbis virus oligosaccharides from fraction I yielded exclusively the disaccharide, Man6GlcNAc2, and free mannose in a ratio of 1:8.96. Similarly, oligosaccharides from fraction II which migrated as Man5GlcNAc2, yielded exclusively the disaccharide and free mannose. As expected, α-mannosidase digestion of fraction V oligosaccharides confirmed that the smallest oligosaccharides present in E1 and E2 were endo D-sensitive Man9GlcNAc2 structures. The only observed digestion products, Man8GlcNAc2 and free mannose, were released in a ratio of 1:199. Interestingly, oligosaccharides migrating as Man9GlcNAc2 (fraction III) and Man7GlcNAc2 (fraction IV) yielded, in addition to the disaccharide and free mannose, α-mannosidase-resistant material. The majority of the oligosaccharides in fractions III and IV were Man6GlcNAc2 and Man5GlcNAc2, respectively. Nevertheless, the α-mannosidase-resistant material constituted 14% of the total counts per min in fraction III and 21-23% of the counts per min in fraction V.

Oligosaccharide Composition at Individual Glycosylation Sites of Sindbis Virus—The oligosaccharide composition at each of the four glycosylation sites of Sindbis virus was determined by reverse phase HPLC separation of E1 and E2 tryptic glycopeptides and gel filtration analysis of oligosaccharides. [3H]Mannose-labeled tryptic glycopeptides from Sindbis virus grown in C6/36 cells or separated E1 or E2 glycoproteins were resolved by reverse phase HPLC (Fig. 5). As described previously for Sindbis virus grown in CEF, BHK, and CHO cells (10), five characteristic tryptic peptides were resolved number I through V in order of elution. HPLC peaks III and V represent the two glycosylation sites of E1. HPLC peak II represents the first glycosylation site in E2; HPLC peaks I and IV represent the second E2 glycosylation site.

Glycopeptides from peaks 1, III, and IV eluted at identical acetonitrile concentrations in CEF- and C6/36-derived virus. However, elution of material from peaks II and V was retarded in C6/36-derived virus relative to virus grown in CEF cells (Fig. 5A). Since the same plaque-purified Sindbis virus stock used to infect CEF and C6/36 cells, differences in oligosaccharide composition (described below) account for the anomalous migration on reverse phase HPLC of tryptic glycopeptides from C6/36-grown virus. The doublet which constitutes HPLC peak V resulted from incomplete trypsin digestion. As expected, the ratios of the faster and slower eluting material from peak V increased with increasing trypsin digestion, oligosaccharide compositions of the leading and trailing peaks were identical, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of E1 glycoprotein preparations established the absence of contaminating glycoproteins.

The composition of oligosaccharides at individual glycosylation sites of Sindbis virus was determined by pooling [3H]mannose-labeled tryptic glycopeptides migrating as individual

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**Fig. 5.** Reverse phase HPLC of tryptic glycopeptides from E1 and E2 glycoproteins of C6/36-grown Sindbis virus. [3H]Mannose-labeled Sindbis virus or preparations of E1 or E2 glycoproteins were digested with trypsin and co-chromatographed on reverse phase HPLC with tryptic peptides from [14C]GlcN-labeled virus grown in CEF cells. Fractions of 0.3 ml were collected at a flow rate of 1.0 ml/min in an ascending gradient of acetonitrile in 0.1 M NaH2PO4, pH 2.2. A, tryptic peptides from C6/36-grown virus; B, tryptic peptides from E1 of C6/36-grown virus; C, tryptic peptides from E2 of C6/36-grown virus. Arrows indicate elution positions of [14C]GlcN-labeled tryptic peptides.
The majority of the oligosaccharides glycopeptides represent the same glycosylation site in E2 (10). On P4 gel filtration columns, was determined by quantitation mannose residues present. The relative frequency of each of the oligosaccharide species, defined by migration on P4 gel filtration columns, was determined by quantitation of the data in Fig. 6 and normalization for the number of mannose residues present.

Tryptic peptides from HPLC peaks I and IV have very similar oligosaccharide compositions confirming that these glycopeptides represent the same glycosylation site in E2 (10). The majority of the oligosaccharides (>80%) are large high mannose structures containing 7 to 9 mannose residues. A small number of extensively processed Man₆GlcNAc₂ oligosaccharides (approximately 12-18% of total chains) is also present. Oligosaccharides from peak II glycopeptides have almost exclusively Man₆GlcNAc₂ structures (>90% of total chains) with a small number of oligosaccharides migrating as Man₄GlcNAc₁ and Man₆GlcNAc₂ after digestion by endo H and endo D. Likewise, oligosaccharides from peak V peptides are also largely Man₆GlcNAc₂ structures, although 15–25% of the oligosaccharides appear to be larger heterogeneous structures. Oligosaccharides from the second glycosylation site in E1, represented by HPLC peak III, reflect the entire spectrum of oligosaccharide species with oligosaccharides migrating as Man₄GlcNAc₁ being the most predominant (approximately 30% of total chains).

The anomalous migration on reverse phase HPLC of peptides from peaks II and V in C6/36-grown virus is probably due to the marked differences in oligosaccharide composition of insect-derived virus and virus grown in vertebrate cells. The former have small uncharged Man₆GlcNAc₂ glycans at these particular glycosylation sites whereas the latter have large sialic acid-containing complex-type oligosaccharides.

Although the structures of α-mannosidase-resistant oligosaccharides are unknown at present, it is reasonable to suppose that they arise relatively late in the processing pathway and represent extensively processed structures. An approximation of the distribution of these α-mannosidase-resistant oligosaccharides among the four glycosylation sites of Sindbis virus was made. Endo H- and endo D-released [³H]mannose-labeled oligosaccharides from tryptic peptides isolated by reverse phase HPLC were exhaustively digested with α-mannosidase and analyzed by gel filtration chromatography (data not shown). The ratios of α-mannosidase-resistant material to Man₆GlcNAc₂ disaccharide were 0.05, 0.10, 0.26, and 0.65 for HPLC peaks I + IV, II, III, and V, respectively. Taken together, our results revealed that the two “complex” glycosylation sites (II and V) had almost exclusively Man₆GlcNAc₂ and α-mannosidase-resistant structures whereas the “high mannose” site (I + IV) was biased toward larger high mannose oligosaccharides. The “variable” site (III) had a mixture of high mannose, Man₆GlcNAc₂, and α-mannosidase-resistant oligosaccharides. Thus, extents of oligosaccharide processing in C6/36 cells paralleled those in vertebrate cells with respect to Sindbis virus glycoproteins.

**DISCUSSION**

Glc₃Man₆GlcNAc₂ was identified as the predominant lipid-linked oligosaccharide synthesized by A. albopictus C6/36 cells based on a number of criteria including extraction of the intact lipid-linked oligosaccharide in CHCl₃/MeOH/H₂O (10:10:3), adsorption to DEAE-cellulose, inhibition of synthesis by tunicamycin, gel filtration chromatography of the hydrolyzed lipid-linked oligosaccharide before and after endo H digestion, partial resistance of the lipid-linked oligosaccharide to α-mannosidase digestion, and demonstration of the presence of glucose residues in the oligosaccharide. These results are consistent with findings of Quesada Allue (23) who demonstrated the presence of glucose-containing lipid-linked oligosaccharide in fruit flies. Thus, Glc₃Man₆GlcNAc₂-Dol-PP appears to be the major lipid-linked oligosaccharide synthesized in insect cells as well in vertebrate cells (20, 24) and yeast (25, 37). In contrast to lipid-linked oligosaccharide synthesis in vertebrate cells in which Man₆GlcNAc₂ and Man₆GlcNAc₃ intermediates accumulate (12), lipid-linked intermediates in C6/36 cells are rapidly elongated.

When C6/36 cells were labeled with [³H]mannose for 1.5
Regulation of Oligosaccharide Processing

The synthesis of oligosaccharides involves multiple steps. In vertebrate cells, glycosylation of nascent polypeptides occurs in the rough endoplasmic reticulum, followed by oligosaccharide processing in the Golgi apparatus. The removal of glucose residues and the addition of mannose residues are characteristic of all vertebrate cells. The extent of these processes can be influenced by the accessibility of oligosaccharides to enzymes in the Golgi apparatus.

In C6/36 cells, the initial steps in oligosaccharide processing take place in the Golgi apparatus. The observation that removal of mannose residues in C6/36 cells takes place very slowly, i.e. not before 10 min post-glycosylation, suggests that later processing steps in C6/36 cells take place in the Golgi apparatus as well. A similar delay in the onset of processing of protein-linked Man\textsubscript{GlcNAC} oligosaccharides has been observed in secondary CEF cells (13).

Regulation of oligosaccharide processing in A. albopictus was examined by analyzing the oligosaccharides at individual glycosylation sites of Sindbis virus grown in C6/36 cells. The Man-linked oligosaccharides of Sindbis virus reflected the repertoire of cellular oligosaccharides as judged by gel filtration chromatography of \textsuperscript{3}H)mannose-labeled viral oligosaccharides before and after endo H digestion (Fig. 4). α-mannosidase treatment, and incorporation of various radiolabeled sugars (data not shown). While most of the oligosaccharides (approximately 90%) are either high mannose oligosaccharides or Man\textsubscript{GlcNAC}, a small number of oligosaccharides migrating as Man\textsubscript{GlcNAC} after endo H digestion are resistant to α-mannosidase.

The composition of oligosaccharides at individual glycosylation sites of Sindbis virus E1 and E2 glycoproteins was determined by reverse phase HPLC separation of tryptic glycopeptides from C6/36-grown virus labeled with \textsuperscript{3}H)mannose (Figs. 5 and 6). As described previously (10), peptides from HPLC peaks I and IV represent a single glycosylation site at Asn 318 of E2; peak II peptides represent the other glycosylation site at Asn 196. Peak III peptides and peak V peptides represent the two glycosylation sites of E1 at Asn residues 256 and 169, respectively.

Examination of endo H- and endo D-released oligosaccharides at each of the four sites (Fig. 6) and estimates of the frequency of α-mannosidase-resistant oligosaccharides at each of these sites yield a striking result. Sites which have extensively processed complex-type oligosaccharides in virus grown in CEF, BHK, or CHO cells, represented by HPLC peaks II and V peptides, have exclusively Man\textsubscript{GlcNAC}\textsubscript{2} and α-mannosidase-resistant oligosaccharides in insect-grown virus. Similarly, the glycosylation site which has high mannose oligosaccharides in virus grown in vertebrate cells (peak I + IV) has a high mannose oligosaccharide and very few Man\textsubscript{GlcNAC}\textsubscript{2} glycans in C6/36-grown virus. Finally, at the site which has high mannose oligosaccharides in CEF-grown virus and complex oligosaccharides in BHK-grown virus (peak III), an intermediate level of processing occurs in insect-grown virus with Man\textsubscript{GlcNAC} oligosaccharides predominating.

Previously, we demonstrated a correlation between extents of processing of Sindbis virus oligosaccharides and accessibility of these oligosaccharides to endo H in intact virions (3). Similar correlations between oligosaccharide accessibility and processing were observed by others in human β-glucuronidase (5, 6) and carbonyl reagents and invertase of yeast (4). These results support the hypothesis that the extent of oligosaccharide processing is primarily determined by the accessibility of oligosaccharides to cellular processing enzymes. Here, we demonstrate that when Sindbis virus is grown in insect cells, Man\textsubscript{GlcNAC}\textsubscript{2} glycans are preferentially located at the two glycosylation sites which have predominantly complex oligosaccharides in virus grown in vertebrate cells. These Man\textsubscript{GlcNAC}\textsubscript{2} oligosaccharides represent the most extensively processed oligosaccharides in A. albopictus, and as such, are analogous to the complex glycan of vertebrate cells. We suggest, therefore, that determinants of processing which reside in the polypeptide are universally recognized despite evolutionary divergence of the oligosaccharide-processing pathway between insects and vertebrates.

Although sterically accessibility of oligosaccharides is important in processing regulation, other factors may also be involved. Recognition of specific polypeptide conformations by a processing enzyme may direct the synthesis of unusual glycans such as the phosphorylated oligosaccharides of lysosomal enzymes (29), polygalactosamine structures (30–32), or sulfated oligosaccharides in embryonic tissue (33, 34). The location of a glycosylation site with respect to the NH\textsubscript{2} or COOH terminus of the polypeptide may also have a role in processing since complex-type sites are preferentially located toward the NH\textsubscript{2} terminus, while high mannose sites are located toward the COOH terminus (2). Differences in rates of intracellular transport of glycoproteins (35) may also affect oligosaccharide processing. Finally, host-dependent variation in oligosaccharide composition of Sindbis virus (10, 36) suggests that differences among cell types in specific activity of enzymes, substrate specificities, and enzyme levels would influence the extent of oligosaccharide processing.

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P Hsieh and P W Robbins


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