Unusual Neutral Oligosaccharides in Mature Sindbis Virus Glycoproteins are Synthesized from Truncated Precursor Oligosaccharides in Chinese Hamster Ovary Cells

By SANDRA K. DAVIDSON* AND LAWRENCE A. HUNT

Department of Microbiology & Immunology, University of Louisville School of Medicine, Louisville, Kentucky 40292, U.S.A.

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SUMMARY

We have previously demonstrated the presence of unusual small asparaginyl-oligosaccharides [(Man)_3GlcNAc_2-ASN] in the mature glycoproteins of Sindbis virus released from both wild-type and lectin-resistant Chinese hamster ovary cells, but the mechanism of synthesis of these structures was not determined. Gel filtration and endo-\(\beta\)-N-acetylglucosaminidase analyses of Pronase-digested glycopeptides from \(^3H\)mannose-labelled Sindbis virus released at different times after infection of a phytohaemagglutinin-resistant line of Chinese hamster ovary cells demonstrated that these small asparaginyl-oligosaccharides were present in similar relative amounts in virus released throughout the virus infection, rather than arising primarily at late times when cytopathic effects were maximal. Similar analyses of pulse-labelled, cell-associated viral glycopeptides suggested that these small oligosaccharides on mature virus glycoprotein resulted from the normal \(\alpha\)1,2-mannosidase processing of truncated precursor oligosaccharides (containing five rather than nine mannoses), rather than from aberrant processing or degradation of the full-size precursor oligosaccharides or normal intermediates.

INTRODUCTION

Sindbis virus is a lipid-enveloped, RNA-containing virus belonging to the togavirus group, and the mature virion contains two major envelope glycoprotein species, E1 and E2, with approximate molecular weights of 50000 to 55000 (Strauss & Strauss, 1977). E2 is derived by proteolytic cleavage of cell-associated precursor polypeptide PE2 (Schlesinger & Schlesinger, 1972), which also results in a small protein species, E3, that is released free into the medium (Welch & Sefton, 1979).

The nucleic acid sequence of the 26S viral mRNA of Sindbis virus indicates there are two potential glycosylation sites (ASN-X-SER/THR) for both E1 and E2 (Rice & Strauss, 1981). E1 and E2 have asparagine-linked oligosaccharides of both the diantennary complex acidic-type \([\text{NeuNAc} + \text{Gal-GlcNAc}]_2\text{(Man)}_3\text{GlcNAc}_2\text{(-fucose)-ASN}\] and mannose-rich, neutral-type \([\text{Man}])_5\text{GlcNAc}_2\text{-ASN}\] (Burke & Keegstra, 1979; Hakimi & Atkinson, 1980; Hunt, 1981). Both types are derived from a common precursor oligosaccharide (Robbins et al., 1977; Sefton, 1977) and are apparently synthesized using host cell machinery. The initial step occurs in the rough endoplasmic reticulum with the en bloc transfer of a large glucose-containing oligomannosyl structure \([\text{Glc}_3\text{(Man)}_9\text{GlcNAc}_2]\) to a lipid intermediate to the nascent polypeptide, followed by the rapid removal of glucose (Robbins et al., 1977; Hunt et al., 1978; Li et al., 1978; Tabas et al., 1978). In the synthesis of complex acidic-type structures, the \(\alpha\)-1,2-linked mannoses are removed in the Golgi membranes by \(\alpha\)-mannosidase I to give a \(\text{(Man)}_5\text{GlcNAc}_2\) structure (Fig. 1b) (Tabas & Kornfeld, 1979; Tulsiani et al., 1982). The trimming of two additional mannoses by \(\alpha\)-mannosidase II (Tulsiani et al., 1982) follows the addition of the first branch \(N\)-acetylglucosamine residue (Fig. 1c) by \(N\)-acytlyglucosaminyltransferase I (GlcNAc transferase I) (Tabas & Kornfeld, 1978; Harpaz & Schachter, 1980).
Fig. 1. Asparaginyl-oligosaccharide processing. (a) Structure of the precursor oligosaccharide of CHO cells (Li et al., 1978). The truncated structure (lacking the four mannose units within the dotted box) has been reported as a lipid-linked precursor oligosaccharide in glucose-starved CHO cells (Rearick et al., 1981) and class E Thy-1-negative mouse lymphoma cells (Kornfeld et al., 1979). (b) This intermediate in the synthesis of complex acidic-type oligosaccharides is the most highly processed oligosaccharide in mature viral or cellular glycoproteins in CHO-PhaR and CHO-15B cells lacking GlcNAc transferase I (Li & Kornfeld, 1978; Robertson et al., 1978; Hunt, 1980a, b, 1981). The truncated precursor is similarly processed by α-mannosidase I (Tabas & Kornfeld, 1979) to give the three-mannose structure (lacking the two mannose units within the dotted box). (c) This intermediate results from GlcNAc transferase I and subsequent processing by α-mannosidase II (Tabas & Kornfeld, 1978; Tulsiani et al., 1982).

Complex acidic-type structures are then formed in the Golgi membranes by the addition of N-acetylglucosamine, galactose, sialic acid, and fucose residues (Hunt & Summers, 1976b). Recent evidence indicates that proteolytic cleavage of PE2 to E2 and E3 occurs in or near Golgi membranes at a time when final processing of oligosaccharides occurs, prior to association of mature glycoprotein with the plasma membrane (Bonatti & Cancedda, 1982; Hakimi & Atkinson, 1982).

Two lectin-resistant Chinese hamster ovary (CHO) cell lines (CHO-PhaR selected for resistance to phytohaemagglutinin, and CHO-15B selected for resistance to ricin) are deficient in GlcNAc transferase I activity (Gottlieb et al., 1975; Narasimhan et al., 1977), and both mature viral and cellular glycoproteins accumulate (Man)3GlcNAc2-size oligosaccharides (Fig. 1b) in place of complex acidic-type oligosaccharides (Li & Kornfeld, 1978; Robertson et al., 1978; Hunt, 1980a, b, 1981). Recently we have demonstrated the presence of unusual small oligosaccharides [(Man)3GlcNAc2-ASN; Fig. 1b] in the mature glycoproteins of Sindbis virus harvested from both wild-type CHO cells and CHO-PhaR cells, but the mechanism of synthesis of these structures was not determined (Hunt, 1981). The virus from the lectin-resistant cells contained more of these oligosaccharides than virus from the wild-type cells, presumably because this small oligosaccharide (Fig. 1b, c) can be processed by an alternative pathway (independent of α-mannosidase II) to complex acidic-type oligosaccharides (Kornfeld et al., 1979). These (Man)3GlcNAc2 oligosaccharides have not been reported for other cellular or viral glycoproteins.
The first objective of the present studies was to determine when these structures appear in the glycoproteins of released Sindbis virus. The second objective was to determine whether these unusual oligosaccharides resulted from the aberrant processing of full-size precursor oligosaccharides or \((\text{Man})_9\text{GlcNAc}_2\) structures that accumulate on viral and cellular proteins in GlcNAc transferase I-deficient cells (Fig. 1b) or, alternatively, from the normal \(x\)-mannosidase I processing of a truncated precursor oligosaccharide containing five instead of the normal nine mannoses. These studies have utilized Sindbis virus-infected \(\text{CHO-Pha}\) cells rather than wild-type \(\text{CHO}\) cells because (i) the lectin-resistant cells are apparently identical to the wild-type cells in the initial stages of lipid- and protein-linked oligosaccharide synthesis and processing (Hunt, 1980a, b) and (ii) the analysis of oligosaccharide processing would not be complicated by the further maturation of \((\text{Man})_9\text{GlcNAc}_2\) and \((\text{Man})_9\text{GlcNAc}_2\) structures (Fig. 1b, c) into complex acidic-type oligosaccharides containing a three-mannose core.

**METHODS**

**Growth of cells and viruses.** The phytohaemagglutinin-resistant \(\text{CHO}\) cell line and the parental \(\text{CHO}\) cell line from which it was selected were obtained from Dr P. Stanley, Albert Einstein College of Medicine. This cell line, \(\text{Gat}^{-}\text{Pha}^+\), was a clone selected from \(\text{Gat}^{-}\) (auxotroph requiring glycine, adenosine, and thymidine) for resistance to phytohaemagglutinin (Pha) and is referred to as \(\text{CHO-Pha}\) (Robertson et al., 1978; Hunt, 1980a, b). The nomenclature and genetic characterization have been described previously in more detail (Stanley et al., 1975; Stanley & Siminovitch, 1977). The cells were grown in monolayer culture at 37 °C in Eagle’s minimum essential medium (MEM; Gibco) containing 10% foetal bovine serum (Gibco) and supplemented with 10 mg/l each of glycine, adenosine, and thymidine.

Unlabelled Sindbis virus and the Indiana serotype of vesicular stomatitis virus (VSV) were purified and the titre determined by plaque assay as described previously (Robertson et al., 1978; Hunt, 1981).

**Radiolabelling of virus and cells.** A confluent \(\text{CHO-Pha}\) monolayer culture was infected with 20 p.f.u./cell of purified Sindbis virus, and radiolabelling was started 1 h post-infection with 100 \(\mu\)Ci/ml \([2^3\text{H}]\)mannose (15-8 Ci/mmol; New England Nuclear) in MEM containing 2% foetal bovine serum and one-fifth the normal glucose concentration (0-2 mg/ml instead of 1-0 mg/ml). To obtain radiolabelled Sindbis virus released at different times after infection, medium was harvested at the end of 3-h labelling periods beginning at 4 h after infection (1 to 4 h, 4 to 7 h, 7 to 10 h) and also at 24 h after infection (10 to 24 h). After each harvesting of virus-containing medium, MEM of the same composition as above except containing 50 \(\mu\)Ci/ml \([2^3\text{H}]\)mannose was added to the infected cells. Released virus was purified by clarification of medium at 1000 \(g\) for 2 min, followed by centrifugation at 10000 rev/min for 10 min in a Beckman JA20 rotor, and then virus was pelleted through a cushion of 20% (w/w) sucrose in NET buffer (0-1 M-NaCl, 1 mM-EDTA, and 10 mM-Tris-HCl pH 7-4) by centrifugation at 45000 rev/min for 10 min at 4 °C in a Beckman 75Ti rotor. The virus pellet was resuspended in NET buffer. At the end of the 24 h incubation, virus-infected cells were harvested by scraping with a rubber policeman into unlabelled MEM, and these cells were combined with loose cells pelleted at 1000 \(g\) from the medium harvested at 24 h after infection. Homogenates containing the cell-associated glycoproteins were prepared from these combined cells as previously described for VSV-infected HeLa cells (Hunt & Summers, 1976a).

Radiolabelled precursor viral glycoproteins and lipid-linked oligosaccharides were obtained by pulse-labelling virus-infected \(\text{CHO-Pha}\) monolayers (20 p.f.u./cell Sindbis or VSV) from 4-5 to 5 h after infection at 37 °C in MEM containing 100 \(\mu\)Ci/ml \([2^3\text{H}]\)mannose, 2% foetal bovine serum and one-fifth the normal glucose concentration. At the end of the labelling period, the cells were harvested with a rubber policeman into unlabelled MEM at 0 °C and pelleted by centrifugation at 1000 \(g\) for 2 min. Homogenates containing the cell-associated glycoproteins were prepared from half of the virus-infected cells, and the other half was used to prepare lipid-linked oligosaccharides.

**Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) of radiolabelled glycoproteins.** Aliquots (0-05 ml) of purified virus or cellular fractions were subjected to electrophoresis in polyacrylamide slab gels using the discontinuous buffer system of Laemmli (1970). After electrophoresis, the slab gels were simultaneously fixed and stained in a solution consisting of 0-04% (w/v) Coomassie Brilliant Blue R (Sigma), 50% (v/v) methanol, and 7% (v/v) glacial acetic acid. Destaining was carried out in a solution of 5% (v/v) methanol and 7% (v/v) glacial acetic acid. After treatment for fluorography in En'Hance (New England Nuclear), the gels were dried and exposed at -70 °C using Kodak X-Omat R or X-Omat L film.

Purified viral E1 and E2 were isolated by preparative electrophoresis in 8% (w/v) polyacrylamide cylindrical gels (11-5 × 0-5 cm) using the same buffer system. After electrophoresis, gels were frozen, cut into 1-mm fractions with a BioRad gel slicer and incubated overnight in 1 ml distilled water at 25 °C. A 0-1 ml portion of each fraction was removed for liquid scintillation counting to determine the position of the \(^3\text{H}\)-labelled viral glycoproteins, and peak fractions were pooled and lyophilized.
Preparation of radiolabelled viral glycopeptides and lipid-derived oligosaccharides. Radiolabelled glycoprotein was precipitated with 2 vol. n-butanol from Nonidet P40 (NP40; Shell Chemical Co.)-treated virus or homogenate fractions (Hunt & Summers, 1976b). The precipitated glycoproteins were washed with ethanol, air-dried, and digested with 10 mg/ml Pronase (Calbiochem) as described previously (Etchison et al., 1977). Lipid-linked oligosaccharides were prepared by selective organic extraction of cell pellets, and oligosaccharides were released from lipid by mild acid hydrolysis (Hunt, 1980b).

Glycosidase digestion of glycopeptides. Pronase-digested glycopeptides were desalted on either a 120 × 1.5 cm or a 120 × 1.0 cm column of Sephadex G15/50 (Pharmacia) before glycosidase digestion (Hunt & Summers, 1976b). Digestion of glycopeptides and lipid-derived oligosaccharides with endo-β-N-acetylglucosaminidase D and H (ENDO-D and ENDO-H) or α-mannosidase were performed as described previously (Etchison et al., 1977; Hunt et al., 1978). Purified ENDO-D from Diplococcus pneumoniae and ENDO-H from Streptomyces griseus were purchased from Miles Laboratories, and Canavalia ensiformis (jack bean) α-mannosidase was purchased from Sigma. With the digestion conditions used in the present studies, the jack bean α-mannosidase preparation from Sigma lacked detectable β-mannosidase activity or other exoglycosidase activities (neuraminidase, galactosidase-glucosaminidase). Preparations of jack bean α-mannosidase purchased from Boehringer-Mannheim, however, contained β-mannosidase activity.

Analysis of virion- and cell-associated glycopeptides. Glycopeptides were analysed by gel filtration through columns (120 × 1.5 cm) of BioGel P-4 (minus 400 mesh; BioRad) along with unlabelled markers (blue dextran, stachyose, and mannose) and 14C-labelled gel filtration markers (Hunt & Summers, 1976b; Hunt et al., 1978). The glycopeptides were eluted with 0.05 M-ammonium acetate, pH 6. The internal standards were not utilized as mol. wt. standards, but were useful in comparing different samples run separately on the same column or on different columns. Prior to running the unknown 3H-labelled samples, each column was calibrated with various 3H-labelled oligosaccharides as described previously (Hunt, 1981).

Following ENDO-H digestions, neutral oligosaccharide products were separated from endoglycosidase-resistant radiolabelled glycopeptides and unlabelled N-acetylglucosamine-peptide products by chromatography on a Dowex 1 × 2 (formate) anion-exchange column (5 × 0.6 cm) (Robertson et al., 1978). The neutral oligosaccharides were collected by washing the column with water. The column was then washed with 2 M-formic acid to elute the bound glycopeptides. Aliquots (0-1 ml) of each 1 ml fraction were assayed for the presence of radiolabel by liquid scintillation counting. Fractions containing the neutral oligosaccharides or the glycopeptides were separately pooled.

RESULTS

Analysis of [3H]mannose-labelled glycoproteins and glycopeptides from Sindbis virus released during different time periods after infection of CHO-PhaR cells

To determine when the unusual small asparaginyl-oligosaccharides were produced and released in mature virus, virus was harvested from the medium at the end of 3 h intervals (1 to 4, 4 to 7, 7 to 10 h after infection) and also at 24 h post-infection. Sindbis virus E1 and E2 glycoproteins were the only radiolabelled proteins observed with SDS–polyacrylamide gel analysis of purified [3H]mannose-labelled virus harvested at these intervals. A representative autoradiograph of released virus is shown in Fig. 2(c). Viral PE2, E1 and E2 glycoproteins were the only major radiolabelled species present in the cell-associated material at the end of the 1 to 24 h post-infection labelling period (Fig. 2b). The majority of the radiolabelled virus was released during the 7 to 10 h interval, with only minimal amounts of virus apparently released before 4 h after infection (Fig. 3). This material released early (1 to 4 h) after infection contained insufficient radiolabel for additional analysis.

Pronase-digested glycopeptides from the different virion samples were analysed by gel filtration before and after digestion with ENDO-D in order to determine the relative amount of the small asparaginyl-oligosaccharides [(Man)3GlcNAc2-ASN] compared to the predominant oligosaccharide species [(Man)5GlcNAc2-ASN] that accumulates in mature virion and cellular glycoproteins synthesized in the GlcNAc transferase I-deficient CHO cells (Li & Kornfeld, 1978; Robertson et al., 1978; Hunt 1980b, 1981). ENDO-D cleaves oligosaccharides between the two N-acetylglucosamine residues proximal to the asparaginyl-peptide moiety and is specific for small oligomannosyl core structures (three to five mannoses, Fig. 1b) (Tai et al., 1975).

Another endo-β-N-acetylglucosaminidase, ENDO-H, can cleave the five-mannose core structure (Fig. 1b) and also oligosaccharides with larger oligomannosyl cores of six to nine mannoses (Fig. 1a) (Tarentino et al., 1974). As shown in Fig. 4(a) for the 7 to 10 h virus sample, the majority of radiolabelled virion glycopeptides were sensitive to ENDO-D, and the
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Fig. 2. SDS-PAGE of [3H]mannose-labelled polypeptides from Sindbis virus-infected CHO-Pha cells and released virus. Radiolabelled proteins were subjected to electrophoresis in either a 10% polyacrylamide (a to c) or 8% polyacrylamide slab gel (d, e). (a, d) 14C-methylated protein markers: (1) phosphorylase B (92.5K), (2) bovine serum albumin (68K), (3) immunoglobulin G heavy chain (50K), (4) ovalbumin (43K) and (5) carbonic anhydrase (29K). (b) Cell-associated fraction from continuously labelled (1 to 24 h post-infection) virus-infected cells. (c) Purified virus released between 7 and 10 h post-infection. (e) Cell-associated fraction from 30 min pulse-labelled (4.5 to 5 h post-infection) virus-infected cells.

Fig. 3. Radiolabel in Sindbis virus released at different times after infection. The total amount of [3H]mannose label in proteins from virus harvested at 4, 7, 10 and 24 h post-infection was estimated by determining the amounts of acid-insoluble radioactivity in 0.05 ml portions of purified virus. The numbers in parentheses refer to the ratio of radiolabel in (Man)_5GlcNAc_1-size to (Man)_3GlcNAc_1-size oligosaccharides released by ENDO-D digestion of glycopeptides from virus [ct/min in (Man)_5GlcNAc_1 divided by ct/min in (Man)_3GlcNAc_1].

Fig. 4a. Enzymatic digestion products of the Man_6GlcNAc oligosaccharide eluted in two major peaks: one peak co-eluted with the 14C-labelled (Man)_5GlcNAc_1 marker, and the second peak eluted several fractions before the stachyose marker, in the position expected for (Man)_3GlcNAc_1. Similar profiles were obtained for the ENDO-D-digested glycopeptides from the 4 to 7 and 10 to 24 h samples (data not shown). The ratios of radiolabel in these ENDO-D-released oligosaccharides [(Man)_3GlcNAc_1 : (Man)_5GlcNAc_1] from the different virus samples are shown in Fig. 3. The ratio was slightly higher for virus released earlier in infection (4 to 7 or 7 to 10 h post-infection) compared to virus released late in infection (10 to 24 h). The ratios of radiolabel in the ENDO-D-released oligosaccharides (fractions 86 to 105, Fig. 4a) to the ENDO-D-resistant glycopeptides (fractions 63 to 85, Fig. 4a) were 3:1:1:0, 2:8:1:0, and 2:3:1:0 for the virus released 4 to 7, 7 to 10 and 10 to 24 h after infection respectively. These results suggested that these unusual small asparaginyl-oligosaccharides were produced throughout Sindbis virus infection and did not result from non-specific breakdown of the virion protein-linked oligosaccharides in the culture medium or from abnormal intracellular processing that developed late in infection when cytopathic effects were maximal.

To confirm that the radiolabelled products of ENDO-D digestion were (Man)_5GlcNAc_1 and (Man)_3GlcNAc_1 structures, these individual species were obtained by preparative gel filtration of the ENDO-D digested glycopeptides from the 7 to 10 h virus sample and were re-analysed by gel filtration after digestion with x-mannosidase (data not shown). The ratios of radiolabel in the free mannose to the Man_6GlcNAc disaccharide were 3:8:1:0 for the (Man)_5GlcNAc_1-size oligosaccharide and 2:0:1:0 for the (Man)_3GlcNAc_1-size oligosaccharide, consistent with (Man)_5GlcNAc and (Man)_3GlcNAc_1 structures, respectively. The ENDO-D-resistant glycopeptides from the same preparative gel filtration of the 7 to 10 h virus glycopeptides were digested with ENDO-H; approximately three-quarters of these radiolabelled glycopeptides was sensitive to ENDO-H, and the neutral products eluted in the positions of (Man)_6GlcNAc_1-size oligosaccharides (data not shown).
Fig. 4. BioGel P-4 gel filtration of [3H]mannose-labelled glycopeptides and oligosaccharides from Sindbis virus released 7 to 10 h after infection. (a) Virion glycopeptides were analysed separately on the same column before (©) and after (●) digestion with ENDO-D, and the profiles were superimposed by alignment of the peak elution positions of the three unlabelled internal standards (solid vertical arrows, from left to right: blue dextran, stachyose and mannose). (b) ENDO-D-treated glycopeptides from purified E1 glycoprotein (●) and purified E2 glycoprotein (©) were analysed separately on the same column, and the profiles superimposed as described for (a). The italic numbers (5, 3) refer to the peaks that elute in the positions of neutral oligomannosyl core structures [(Man)₃GlcNAc] with five and three mannoses, respectively.

The unusual small neutral oligosaccharides were present in large amounts in both E1 and E2 virion glycoproteins (Fig. 4b). The ratios of radiolabel in the (Man)₃GlcNAc₁ and (Man)₅GlcNAc₁ oligosaccharides released by ENDO-D were 1.1:1.0 for E1 and 1.5:1.0 for E2. The relative amount of these ENDO-D-released oligosaccharides compared to the ENDO-D-resistant glycopeptides was greater for E1 than for E2: the ratios of radiolabel in the (Man)₃GlcNAc₁ and (Man)₅GlcNAc₁ oligosaccharides (fractions 90 to 103, Fig. 4b) versus the ENDO-D-resistant glycopeptides (fractions 63 to 89, Fig. 4b) were 3.8:1.0 for E1 and 0.7:1.0 for E2. To ensure that the low ratio of ENDO-D-sensitive to ENDO-D-resistant glycopeptides for E2 was not due to incomplete digestion, the digestion was repeated with a twofold higher enzyme to substrate ratio, and identical results were obtained.

Analysis of cell-associated glycopeptides from continuously labelled Sindbis virus-infected CHO-Phaβ cells.

It was of interest to determine the relative amounts of these three-mannose- and five-mannose-containing oligosaccharides in the glycoproteins that were cell-associated at the end of the infection (Fig. 2b) and, in addition to determine whether any other unusual [3H]mannose-labelled oligosaccharides were present. The ENDO-D-released (Man)₃GlcNAc₁- and (Man)₅GlcNAc₁-size oligosaccharides from the cell-associated glycopeptides were present in a ratio of 0.9:1.0 (Fig. 5a), similar to the ratios for virus released 4 to 24 h after infection (Fig. 3).
Fig. 5. BioGel P-4 gel filtration of [3H]mannose-labelled glycopeptides and oligosaccharides from Sindbis virus-infected cells. The conditions of gel filtration in this figure and in Fig. 6 and 7 were identical to those in Fig. 4, except that an additional internal standard was included: [14C] glucosamine-labelled (Man)$_5$ GlcNAc$_1$ oligosaccharide from CHO-Pha$^a$ cell glycopeptides (indicated by the dotted vertical arrow). (a) Glycopeptides from cell-associated protein (labelled 1 to 24 h post-infection) were analysed separately on the same column before (O) and after (●) digestion with ENDO-D, and the profiles were superimposed. An equivalent ENDO-D digested sample was subjected to preparative gel filtration, and the fractions containing the ENDO-D resistant glycopeptides (fractions 64 to 88), (Man)$_5$ GlcNAc$_1$-size oligosaccharides (fractions 91 to 94), and (Man)$_3$ GlcNAc$_2$-size oligosaccharides (fractions 98 to 102) were pooled, as indicated by the solid horizontal bars, for further analyses. The ENDO-D resistant glycopeptides were then digested with ENDO-H and separated into ENDO-D and H resistant glycopeptides and ENDO-H released neutral oligosaccharides prior to additional gel filtration. (b) Superimposed profiles of ENDO-D and H resistant glycopeptides before (O) and after (●) additional α-mannosidase digestion. (c) Superimposed profiles of ENDO-H released neutral oligosaccharides before (O) and after (●) additional α-mannosidase digestion.

Fig. 6. BioGel P-4 gel filtration of [3H]mannose-labelled precursor glycopeptides and oligosaccharides from pulse-labelled virus-infected cells. The cell-associated glycopeptides from Sindbis virus-infected cells (labelled 4.5 to 5 h post-infection) were digested with ENDO-H and then separated into ENDO-H resistant glycopeptides and ENDO-H released neutral oligosaccharides prior to additional gel filtration. (a) Superimposed profiles of ENDO-H resistant glycopeptides from Sindbis virus-infected cells before (O) and after (●) additional α-mannosidase digestion. (b) Superimposed profiles of ENDO-H released neutral oligosaccharides from Sindbis virus-infected cells before (O) and after (●) additional α-mannosidase digestion. (c) Cell-associated glycopeptides from VSV-infected cells (labelled 4.5 to 5 h post-infection) were similarly digested with ENDO-H and separated into ENDO-H released neutral oligosaccharides (O) and ENDO-H resistant glycopeptides (●) prior to separate gel filtration analysis on the same column.
However, the ratio of radiolabel in ENDO-D-released oligosaccharides (fractions 89 to 105, Fig. 5a) to ENDO-D-resistant glycopeptides (fractions 64 to 88, Fig. 5a) was much lower than the ratios for mature virus (0.5:1.0 versus 2.3 to 3.1:1.0). This result was expected because the [3H]mannose-labelled, cell-associated proteins were a mixture of both precursor (PE2 and E1) and mature forms (E1 and E2) of the viral glycoproteins (Fig. 2b), and the precursor glycoproteins should have contained unprocessed oligomannosyl core structures (Fig. 1a) that were resistant to ENDO-D. The ENDO-D-resistant glycopeptides from cell-associated protein (equivalent to fractions 64 to 88 in Fig. 5a) were separated into an ENDO-H-resistant fraction representing approximately 45% of the radioactivity (Fig. 5b) and an ENDO-H-released oligosaccharide fraction representing approximately 55% of the radioactivity (Fig. 5c). A comparison of the profiles of the endoglycosidase-resistant glycopeptides in Fig. 5(a) and (b) indicated that the lower molecular weight glycopeptides in Fig. 5(a) were also resistant to ENDO-H, whereas the higher molecular weight glycopeptides in Fig. 5(a) were sensitive to ENDO-H and were the source of the (Man)6-9GlcNAc1-size neutral oligosaccharide products in Fig. 5(c).

Additional information concerning the composition and structure of the various ENDO-D- and ENDO-H-released oligosaccharides and the ENDO-D- and ENDO-H-resistant glycopeptides from the cell-associated viral glycoproteins was obtained by digestions with α-mannosidase and gel filtration of the radiolabelled digestion products. The ratios of radiolabelled free mannose to radiolabelled Man 4 GlcNAc disaccharide for the two major ENDO-D-released oligosaccharide species were similar to the results with the 7 to 10 h virion glycopeptides (data not shown): 3.7:1.0 for (Man)3GlcNAc1 (fractions 90 to 95, Fig. 5a) and 1.9:1.0 for (Man)4GlcNAc1 (fractions 97 to 103, Fig. 5a). The neutral oligosaccharides released by ENDO-H also exhibited a (Man)6-α-Man 4 GlcNAc1 structure, with a ratio of 6:4:1.0 for radiolabelled mannose: Man 4 GlcNAc1 disaccharide (Fig. 5c). A minor fraction of these ENDO-H-released oligosaccharides may have been terminally glucosylated (Fig. 1a), since a small amount of label was observed in the position (fractions 84 to 90, Fig. 5c, broken line) expected for the α-mannosidase-resistant core of Glc1(Man)6GlcNAc1 oligosaccharides (Hunt, 1980b).

Almost all of the radiolabel in the ENDO-D- and ENDO-H-resistant glycopeptides (Fig. 5b) was converted by α-mannosidase to a major peak of free mannose and several minor peaks of lower molecular weight glycopeptides (fractions 80 to 95, Fig. 5b, broken line) with a ratio of 2.8:1.0 for free mannose: small glycopeptides. Because essentially all of the terminal α-linked mannoses were removed from the neutral oligosaccharides (Fig. 5c) under the same digestion conditions, we presumed that the small glycopeptides (fractions 80 to 95, Fig. 5b) represented Man 4 GlcNAc2-asparaginyl peptides, with the size heterogeneity reflecting differences in the peptide moiety. The size, ENDO-D- and ENDO-H-resistance, and α-mannosidase sensitivity of the glycopeptides displayed in Fig. 5(b) were consistent with glycopeptides containing truncated (Man)4GlcNAc2 oligosaccharides and/or (Man)6GlcNAc2 oligosaccharides derived by partial processing of the five-mannose structure (Fig. 1a). Such truncated oligosaccharides satisfy the structural requirements for efficient binding to concanavalin A (ConA) columns (two mannose residues attached to the same residue and unsubstituted at the 3, 4 or 6 positions (Harpaz & Schachter, 1980), a portion of these glycopeptides was also chromatographed on ConA-agarose according to the procedure of Hunt (1982). All of the radiolabelled glycopeptides were bound and subsequently eluted with a low concentration (10 mM) of α-methyl mannoside (data not shown), characteristic of small to medium size oligomannosyl cores versus larger oligomannosyl cores containing seven to nine mannoses (Hunt, 1982). These combined results suggested that the (Man)3GlcNAc2-asparaginyl-oligosaccharides may have been derived by normal α-mannosidase processing of truncated precursor oligosaccharides (Fig. 1a) that were equivalent to the major precursor oligosaccharide species observed in glucose-starved CHO cells (Gershman & Robbins, 1981; Rearick et al., 1981) or mutant mouse lymphoma cells (Chapman et al., 1980).

Analysis of pulse-labelled glycopeptides from Sindbis virus- and VSV-infected CHO-Phaκ cells

To determine whether truncated precursor oligosaccharides (Fig. 1a) were being transferred from lipid to newly synthesized protein, Sindbis virus-infected CHO-Phaκ cells were pulse-
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labelled for 30 min with [3H]mannose. SDS-PAGE of the cell-associated protein indicated that the precursor glycoprotein PE2 and glycoprotein E1 were the only major radiolabelled species (Fig. 2e). Following ENDO-H digestion of the glycopeptides from these pulse-labelled cells, approximately 60% of the radiolabel was present in neutral oligosaccharide products (Fig. 6b, solid line) that eluted in a major peak of (Man)_9GlcNAc_1-size oligosaccharides and a shoulder of higher molecular weight oligosaccharides in the position expected for Glc(Man)_9GlcNAc_1 (Hunt, 1980b). A minor peak co-eluted with the 14C-labelled (Man)_5GlcNAc_1 marker, indicating that only a small amount of the asparagine-linked oligosaccharides had been completely processed following transfer from lipid to protein during the 30 min labelling period. Approximately 40% of the radiolabel in the pulse-labelled glycopeptides was resistant to ENDO-H and eluted in a single broad peak (Fig. 6a, solid line) that was similar in elution position to the ENDO-D- and ENDO-H-resistant glycopeptides (Fig. 5b) from the continuously labelled cells (1 to 24 h post-infection). A very small fraction of these glycopeptides (approximately 7% of the radiolabel) was sensitive to ENDO-D, with the neutral oligosaccharide product eluting as the fully processed, (Man)_3GlcNAc_1-size oligosaccharides (data not shown).

Following α-mannosidase digestion, radiolabel from the ENDO-H-released neutral oligosaccharides eluted in three peaks (Fig. 6b, broken line): a major peak of free mannose, a peak of Man_4GlcNAc disaccharides, and a peak (fractions 78 to 91) in the position of residual oligosaccharides from the α-mannosidase digestion of glucosylated precursor oligosaccharides (Hunt, 1980b). The radiolabel from the ENDO-H-resistant glycopeptides also eluted in three peaks following α-mannosidase digestion (Fig. 6a, broken line): a major peak of free mannose, smaller molecular weight glycopeptides (fractions 82 to 98) that presumably represented Man_4GlcNAc_2-asparaginyl peptides, and a peak of α-mannosidase-resistant glycopeptides (fractions 65 to 82) that eluted with the higher molecular weight half of the original glycopeptides (Fig. 6a, solid line) and presumably contained terminal glucose. The ratio of free mannose: presumed Man_4GlcNAc_2-asparaginyl peptides was 3:1, which was higher than the value for the ENDO-D- and ENDO-H-resistant glycopeptides (2.8:1.0) from the continuously labelled, cell-associated protein (Fig. 5c) and suggestive of a non-glucosylated and truncated oligosaccharide structure [(Man)_4_5Man_4GlcNAc_2-ASN, Fig. 1a] for the majority of these pulse-labelled, ENDO-H-resistant glycopeptides. The complete resistance to α-mannosidase of the largest (and presumably glucosylated forms) of these ENDO-H-resistant glycopeptides (fractions 65 to 82, Fig. 6a, broken line) was not unexpected, since the single mannose that is both terminal and α-linked in the glucosylated form of the truncated precursor oligosaccharide (Fig. 1a) is not removed by jack bean α-mannosidase under conditions that are sufficient to remove all α-linked mannoses from non-glucosylated oligosaccharides (Trowbridge & Hyman, 1979).

In contrast to the large amounts of ENDO-H-resistant glycopeptides for the Sindbis virus-infected cells, approximately 95% of the radiolabel in precursor glycopeptides from VSV-infected cells was ENDO-H-sensitive (Fig. 6c). The gel filtration profile of these ENDO-H-released oligosaccharides (Fig. 5c, solid line) was almost identical to the profile for the pulse-labelled Sindbis virus-infected cell sample in Fig. 6(b) and the profiles observed in earlier studies of ENDO-H-digested glycopeptides from 30 min labelled VSV-infected (Hunt, 1980a) and uninfected CHO-PhaR cells (Hunt, 1980b): a major peak of (Man)_9GlcNAc_1-size oligosaccharides, a partially resolved peak of Glc(Man)_9GlcNAc_1-size oligosaccharides, and a minor peak of (Man)_5GlcNAc_1-size oligosaccharides. The small amount of ENDO-H-resistant glycopeptides (fractions 60 to 80, Fig. 6c, broken line) eluted in approximately the same position as the ENDO-H-resistant glycopeptides from the Sindbis virus-infected cells (Fig. 6a).

Analysis of lipid-derived precursor oligosaccharides from Sindbis virus- and VSV-infected CHO-PhaR cells

Since truncated precursor oligosaccharides (Fig. 1a) were apparently present in significant amounts in the pulse-labelled glycoproteins of Sindbis virus-infected cells, but not in the pulse-labelled VSV-infected cells, the oligosaccharides from the lipid-linked fraction of these two cell cultures were also compared by gel filtration (Fig. 7). Whereas the major peak of lipid-derived oligosaccharides from the VSV-infected cells (Fig. 7, peak 1) eluted in the position expected for...
Fig. 7. BioGel P-4 gel filtration of lipid-derived oligosaccharides from pulse-labelled virus-infected cells. [3H]Mannose-labelled oligosaccharides (labelled 4-5 to 5 h post-infection) from Sindbis virus-infected cells (O) or VSV-infected cells (●) were separately analysed, and the profiles were superimposed. Only 0.1 ml of each 1.0 ml fraction was assayed for radioactivity in the analysis of the lipid-derived oligosaccharides of the Sindbis virus-infected cells, and fractions from major and minor peaks of radiolabel (indicated by roman numerals I to IV and solid horizontal bars) were pooled for further analyses.

The full size precursor oligosaccharides [Glc₃(Man)₉GlcNAc₂, Fig. 1a], the major peak of lipid-derived oligosaccharides from the Sindbis virus-infected cells (Fig. 7, peak III) eluted in the position expected for the smaller ENDO-D- and ENDO-H-resistant (Man)₅GlcNAc₂ oligosaccharides (Fig. 1a minus glucose). These oligosaccharides are considered to be normal intermediates in the assembly of the full size precursor oligosaccharides in CHO cells (Chapman et al., 1979) and are the major lipid-linked oligosaccharides in mutant mouse lymphoma cells (Trowbridge & Hyman, 1979) and glucose-starved CHO cells (Rearick et al., 1981). The profile of the Sindbis virus-infected cell oligosaccharides also included several minor peaks: (i) peak I oligosaccharides eluting in the same position as the major peak for the VSV-infected cell oligosaccharides (fractions 59 to 64), (ii) peak II oligosaccharides eluting midway between peak I and III (fractions 66 to 72), (iii) very low molecular weight oligosaccharides (peak IV and smaller, fractions 84 to 91), and (iv) free mannose (fractions 116 to 120). The VSV-infected cell oligosaccharides also contained lesser amounts of peak III [Man₅GlcNAc₂-size], peak IV, and oligosaccharides eluting between peaks I and III.

Individual oligosaccharide peaks from the Sindbis virus-infected cell sample were further analysed by ENDO-H and/or α-mannosidase digestion. The (Man)₅GlcNAc₂-size oligosaccharides (peak III) were resistant to ENDO-H digestion, but were completely digested by α-mannosidase to give radiolabelled free mannose and a (Man)₁GlcNAc₂-size radiolabelled oligosaccharide in a ratio of 3:9:1.0. The Glc₃(Man)₉GlcNAc₂-size oligosaccharides (peak I) were sensitive to ENDO-H digestion, and the released oligosaccharides eluted in the position of Glc₃(Man)₉GlcNAc₁. Approximately 80% of the radiolabel in peak II oligosaccharides was ENDO-H-sensitive and gave a (Man)₈GlcNAc₁-size product. Peak IV oligosaccharides were completely digested by α-mannosidase to give radiolabelled free mannose and a (Man)₁Glc-
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The unfractionated mixture of lipid-derived oligosaccharides from the Sindbis virus-infected cells was further analysed by ConA-affinity chromatography (data not shown). The ConA-unbound fraction contained the peak IV and smaller oligosaccharides, as expected for \((\text{Man})_{1-2}\text{GlcNAc}_2\) structures (Harpaz \& Schachter, 1980), whereas the ConA-bound and 10 mM-\(\alpha\)-methyl mannoside-eluted fraction contained essentially all of the major peak III oligosaccharides and part of the peak II oligosaccharides. The ConA-bound fractions that were eluted with 50 mM- and 100 mM-\(\alpha\)-methyl mannoside contained both peak I and peak II oligosaccharides, consistent with the tighter binding of larger oligomannosyl core structures \([\text{Glc}_3(\text{Man})_9\text{GlcNAc}_2\text{ and } (\text{Man})_9\text{GlcNAc}_2]\) to ConA-agarose (Hunt, 1982). The portion of peak II oligosaccharides that were similar to the major \((\text{Man})_5\text{GlcNAc}_2\) species (peak III) with respect to ENDO-H resistance and ConA affinity may have represented glucosylated forms of the truncated precursor oligosaccharide \([\text{Glc}_3(\text{Man})_5\text{GlcNAc}_2\text{, Fig. 1a}]\) that co-eluted on BioGel P-4 with \((\text{Man})_9\text{GlcNAc}_2\) oligosaccharides.

DISCUSSION

We have analysed the \([3H]\)mannose-labelled glycoproteins from Sindbis virus-infected CHO-Pha\(^R\) cells and from released virions to determine possible mechanisms of synthesis of the unusual neutral-type asparaginyl-oligosaccharides \([\text{(Man)}_3\text{GlcNAc}_2-\text{ASN}\] that were previously demonstrated in Sindbis virus glycoproteins (Hunt, 1981). The combined gel filtration and glycosidase digestion (ENDO-D, ENDO-H, \(\alpha\)-mannosidase) of Pronase-digested glycopeptides demonstrated that these small mature oligosaccharides were present in significant amounts in both E1 and E2 glycoproteins of Sindbis virus and in similar proportions in virus released at early and late times after infection. This indicated that these small oligosaccharides were not the products of abnormal intracellular processing that developed late in infection when cytopathic effects were maximal, or of extracellular degradation that occurred when released virus remained in the medium for extended periods of time.

The presence of both truncated (five mannose) and full size (nine mannose) precursor oligosaccharides (Fig. 1a) in the protein-linked and lipid-linked fractions of pulse-labelled Sindbis virus-infected cells indicated that the \((\text{Man})_3\text{GlcNAc}_2\) mature oligosaccharides (Fig. 1b) on Sindbis virus glycoproteins resulted from the normal \(\alpha1,2\)-specific mannosidase processing (Tabas \& Kornfeld, 1979; Tulsiani et al., 1982) of the truncated precursor oligosaccharides, analogous to the \(\alpha1,2\)-specific mannosidase processing of the full size precursor oligosaccharides that resulted in the ENDO-D- and ENDO-H-sensitive \((\text{Man})_5\text{GlcNAc}_2\) mature oligosaccharides (Fig. 1b). The size, ENDO-D- and ENDO-H-resistance, \(\alpha\)-mannosidase sensitivity, and ConA affinity of the truncated glycopeptides (Fig. 6a) were consistent with \(\text{Glc } + (\text{Man})_5\text{GlcNAc}_2\)-asparaginyl peptides. With the assumption of uniform labelling of all of the mannose residues, normalization of the radiolabel (division of ct/min by number of mannoses in oligosaccharide) suggested that approximately equal amounts of the truncated and full size precursor oligosaccharides \([\text{Glc } + (\text{Man})_5\text{GlcNAc}_2\] were present in the newly synthesized viral glycoproteins of the Sindbis virus-infected CHO-Pha\(^R\) cells. The profile of radiolabelled oligosaccharides from the lipid-linked fraction of the same pulse-labelled Sindbis virus-infected cells (Fig. 7) was similar to the profiles of lipid-derived oligosaccharides reported for uninfected CHO cells that were starved for glucose (Rearick et al., 1981; Gershman \& Robbins, 1981), with the major peak of full size precursor oligosaccharides \((\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\] replaced by a major peak of the truncated and non-glucosylated \((\text{Man})_5\text{GlcNAc}_2\) oligosaccharides (Fig. 1a). This five mannose-containing, lipid-linked oligosaccharide is considered to be a normal biosynthetic intermediate in the synthesis of the full size, lipid-linked oligosaccharide in CHO cells, but may also be directly glucosylated in minor amounts in uninfected CHO cells to give a \(\text{Glc}_3(\text{Man})_5\text{GlcNAc}_2\) oligosaccharide that can be transferred to protein (Chapman et al., 1979). The presence of only a small amount of lipid-derived oligosaccharides from the Sindbis virus-infected cells (Fig. 7) with the size, ENDO-H resistance, and ConA affinity characteristics of this \(\text{Glc}_3(\text{Man})_5\text{GlcNAc}_2\) species was not
surprising, since the non-glycosylated \((\text{Man})_5\text{GlcNAc}_2\) oligosaccharides accumulate in the lipid-linked fraction of cells even when the truncated oligosaccharides are essentially the only species transferred to protein (Trowbridge & Hyman, 1979; Rearick et al., 1981). One possible explanation for these observations is that the glucosylation of truncated oligomannosyl cores (five or seven mannose) occurs at a relatively slow rate compared to the glucosylation of the full size oligomannosyl core, so that this glucosylation step is rate-limiting compared to the transfer of the fully glucosylated truncated oligosaccharide to newly synthesized protein (Hunt, 1980b; Rearick et al., 1981).

Although uninfected CHO cells are apparently very sensitive to glucose starvation and undergo an abrupt shift from the full size precursor to the truncated \((\text{Man})_5\text{GlcNAc}_2\) species (Fig. 1a) in the lipid-linked oligosaccharide fraction (Rearick et al., 1981; Gershman & Robbins, 1981), glucose starvation did not seem to be a major reason for the synthesis of both truncated and full size precursor oligosaccharides in the present studies with Sindbis virus-infected CHO-Pha\(^8\) cells. The amount of glucose and serum in the labelling medium and the cell density used in the present experiments were significantly greater than the maximal values that led to any glucose starvation effects in the studies with the uninfected CHO cells (Rearick et al., 1981; Gershman & Robbins, 1981). In addition, the biosynthesis and transfer to protein of the truncated precursor oligosaccharides did not occur to any significant extent in an equivalent culture of CHO cells that was infected with VSV instead of Sindbis virus (Fig. 6c, 7). The molecular mechanism(s) responsible for the shift in oligomannosyl core size of the precursor oligosaccharides is unknown for both the glucose-starved CHO cells (Rearick et al., 1981; Gershman & Robbins, 1981) and the Sindbis virus-infected CHO cells in the present studies, but it may be related to the deficiency in synthesis of dolichol-phosphate-mannose that has been described for the class E Thy-1 negative lymphoma cell line (Chapman et al., 1980).

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