Selective Cleavage by Endo-β-N-acetylglucosaminidase H at Individual Glycosylation Sites of Sindbis Virion Envelope Glycoproteins*

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Endo-β-N-acetylglucosaminidase H (endo H) was used to probe the relative accessibility of the four asparagine-linked oligosaccharides of E1 and E2 glycoproteins in intact Sindbis virions. A mutant clone of Chinese hamster ovary cells, clone 15B, which has been shown to use a hamster N-acetylglucosaminyltransferase I, was used as the viral host. In this cell line, high mannose type oligosaccharides are synthesized normally, but formation of complex type glycans is blocked; thus, all N-linked glycans retain endo H-sensitive structures. Using high performance liquid chromatography to separate tryptic glycopeptides from E1 or E2, we examined endo H cleavage of oligosaccharides at individual glycosylation sites of intact Sindbis virions. Under nondenaturing conditions, exhaustive endo H digestion resulted in preferential cleavage at glycosylation sites which have complex type glycans in virus grown in wild type Chinese hamster ovary cells (see accompanying paper, Hsieh, P., Rosner, M. R., and Robbins, P. W. (1983) J. Biol. Chem. 258, 2548–2554). Thus, 74–80% of the oligosaccharides at a complex site in E2 and 60–63% of the glycans at a complex site in E1 were cleaved by endo H, but only 15–16% of the oligosaccharide chains at the high mannose site of E2 were released. At a site in E1 which has both high mannose and complex type oligosaccharides in virus from wild type Chinese hamster ovary cells, 23–25% of the oligosaccharides were cleaved by endo H. The selectivity of endo H cleavage was lost when virions were digested with pronase or incubated in detergent. Our findings support the hypothesis that the extent of oligosaccharide processing at glycosylation sites in Sindbis virus glycoproteins is determined primarily by the physical accessibility of oligosaccharides.

The asparagine-linked oligosaccharides of mammalian glycoproteins have heterogenous structures that generally fall into two categories: “high mannose” and “complex” (reviewed in Refs. 1 and 2). Both classes are synthesized from a common lipid-linked precursor, Glc3Man9GlcNAc2, which is transferred to polypeptides cotranslationally (3, 4). The common precursor is extensively modified to yield both complex and high mannose type oligosaccharides. The three terminal glucose residues (5) and possibly a single mannose residue (6) are removed in the endoplasmic reticulum, but subsequent oligosaccharide processing takes place in the Golgi apparatus. Removal of up to four α-1,2-linked mannose residues by Golgi mannosidases IA and IB (7, 8) generates mature high mannose glycans.

Synthesis of complex type oligosaccharides begins with modification of a high mannose precursor, Man6GlcNAc2; the first step is the addition of an N-acetylglucosamine residue to this protein-linked oligosaccharide (9, 10). Mature complex type glycans are produced from GlcNAcMan6GlcNAc2 by the removal of 2 more mannose residues and the addition of the peripheral sugars N-acetylgalactosamine, galactose, fucose, and sialic acid (reviewed in Ref. 11).

Regulation of oligosaccharide processing at individual glycosylation sites is poorly understood at present. Strain-dependent variations in oligosaccharide composition among closely related strains of influenza virus (12, 13) and murine leukemia virus (14) suggest that the primary amino acid sequence of a glycoprotein can affect the processing of its oligosaccharides. Earlier studies have identified two prerequisites for glycosylation at a specific asparagine residue: location within the sequence -Asn-X-Ser(Thr)- (15) and steric accessibility to the oligosaccharide transferase enzyme (16). By analogy, it is possible that a specific amino acid sequence determines whether a particular asparagine residue will carry high mannose or complex oligosaccharides, though no such sequence has ever been identified. An alternative hypothesis is that, while cell-specific enzymes may not recognize detailed protein structure they will preferentially process oligosaccharide chains which are stericly more accessible. The present study was designed to test this latter possibility.

The enzyme endo H was used to probe the relative exposure of oligosaccharides of the Sindbis virus envelope glycoproteins E1 and E2 in intact virions. Endo H is specific for high mannose oligosaccharides and cleaves between the two proximal GlcNAc residues (17). A mutant clone of CHO cells, clone 15B (18, 19), was used as the viral host. These cells lack Golgi N-acetylgalacosaminyltransferase I, so processing of oligosaccharides that are normally complex type is halted at the Man9GlcNAc2 stage, and all N-linked glycans remain sensitive to endo H. Using reverse phase HPLC to separate tryptic glycopeptides containing individual glycosylation sites from E1 and E2, we observed that oligosaccharides at sites which have complex oligosaccharides in virus grown in wild type cells were cleaved more readily by endo H than oligosaccharides at a site which has high mannose oligosaccharides (see accompanying paper (20)). This finding is consistent with the

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The abbreviations used are: endo H, endo-β-N-acetylglucosaminidase H; CHO, Chinese hamster ovary cells; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.
hypothesis that steric accessibility of oligosaccharides is an important factor in the control of oligosaccharide processing.

**EXPERIMENTAL PROCEDURES**

**Cell Preparation and Viral Infection—**CHO-K1 cells and clone 15B cells were cultured as monolayers in Alpha Minus minimal essential medium (DM-325, KC Biological) containing 5% fetal calf serum ( Gibco). CHO-K1 cells were obtained from Dr. R. Baker, Roswell Memorial Park, Buffalo, NY; clone 15B was obtained from Dr. S. Kornfeld, Washington University, St. Louis. Wild type Sindbis virus was obtained from Dr. B. Selton, Salk Institute, San Diego. The virus was passaged on wild type CHO cells and plaque-purified. The CHO-derived stock had a titer of 2 x 10⁶ plaque-forming units/ml assayed on secondary chicken embryo fibroblast cells.

For infection, confluent monolayers of cells grown in 490-cm² plastic roller bottles were infected with 0.1 plaque-forming unit/cell in 10 ml of medium per roller bottle. Virus was allowed to adsorb to cells for 1 h at 37 °C. Then 9 ml of medium containing 1 ml/ml [4-H]-mannose (20–30 Ci/mmol, New England Nuclear) was added, and the cells were incubated for 18–24 h at 37 °C.

Isolation of mature virions was carried out as described in the accompanying paper (20).

**Pronase and Endo H Digestion—**Pronase glycopeptides were prepared as described previously (21). Endo H was prepared according to the method of Tarentino and Maley (17). Glycopeptides and intact virions were incubated with endo H (3 μg/ml) for 4 h at 37 °C in 0.1 M Tris-C1, pH 6.8, unless otherwise noted. In control incubations, endo H was incubated without virions in the same buffer. Then endo H activity was assayed by the method of Tarentino and Maley (17), except that 0.1 M Tris-C1, pH 6.8, was used as the buffer.

**Separation of Free Oligosaccharides from Glycoproteins—**Chloroform/methanol/water extraction was carried out as described previously (21). In an Eppendorf tube, 750 μl of chloroform/methanol (3:2) and 50 μl of calf serum carrier were added to 100 μl of all oligosaccharides which had been incubated at 37 °C with or without endo H to achieve a final ratio of 3:2:1 chloroform/methanol/water. The mixture was agitated vigorously on a Vortex mixer and centrifuged for 5 min. The organic phase was washed once by the addition of 0.5 ml of water followed by vigorous agitation and centrifugation. The denatured protein was washed twice by sonification in 0.5 ml of water followed by centrifugation. The three aqueous washes were combined with the initial aqueous phase, dried under air, and resuspended in water prior to gel filtration analysis.

**Gel Filtration Chromatography—**Oligosaccharides and glycopeptides were resolved as described previously on columns (1 x 115 cm) of Bio-Gel P-4 (400 mesh, Bio-Rad) in 0.1 M pyridine acetate, pH 6.0 (22). Fractions of 9.5 ml were collected. Bovine serum albumin (150,000 daltons) was added to each tube as markers for the exclusion and inclusion volumes, respectively.

**Trypsin Digestion and Reverse Phase HPLC—**[3H]-Mannose-labeled virions were denatured and then digested with 30-μg aliquots of trypsin (Worthington, type TRTPCK) for 4 h at 37 °C and analyzed by reverse phase HPLC as described in the accompanying paper (20).

**RESULTS**

**Oligosaccharide Composition of Sindbis Virus Grown in Wild Type and Clone 15B CHO Cells—**Sindbis virus grown in [3H]-mannose-labeled wild type or clone 15B CHO cells was treated with pronase and endo H and the oligosaccharides and glycopeptides analyzed by gel filtration chromatography to achieve a final ratio of 3:2:1 chloroform/methanol/water. The mixture was agitated vigorously on a Vortex mixer and centrifuged for 5 min. The organic phase was washed once by the addition of 0.5 ml of water followed by vigorous agitation and centrifugation. The denatured protein was washed twice by sonification in 0.5 ml of water followed by centrifugation. The three aqueous washes were combined with the initial aqueous phase, dried under air, and resuspended in water prior to gel filtration analysis.

**Gel Filtration Chromatography—**Oligosaccharides and glycopeptides were resolved as described previously on columns (1 x 115 cm) of Bio-Gel P-4 (400 mesh, Bio-Rad) in 0.1 M pyridine acetate, pH 6.0 (22). Fractions of 9.5 ml were collected. Bovine serum albumin (150,000 daltons) was added to each tube as markers for the exclusion and inclusion volumes, respectively.

**Trypsin Digestion and Reverse Phase HPLC—**[3H]-Mannose-labeled virions were denatured and then digested with 30-μg aliquots of trypsin (Worthington, type TRTPCK) for 4 h at 37 °C and analyzed by reverse phase HPLC as described in the accompanying paper (20).

| Oligosaccharide species | CHO host | 15B host |
|-------------------------|---------|---------|
| Glc,MannGlcNAc<sup>b</sup> | 1790 | 6600 |
| +Man,GlcNAc<sup>b</sup> | 1870 | 8 |
| Man,GlcNAc<sup>b</sup> | 1900 | 8 |
| Man,GlcNAc<sup>b</sup> | 940 | 5 |
| Man,GlcNAc<sup>b</sup> | 920 | 6 |
| --- | --- | --- |
| 6% of the total oligosaccharide chains released under these nondenaturing conditions. Incubation of intact [3H]-mannose-labeled virions with endo H at an enzyme concentration of 3 μg/ml was carried out at 37 °C for 2, 4, or 16.5 h.

In order to separate the free oligosaccharides released by endo H from the residual protein-linked glycans, each reaction mixture was extracted with chloroform/methanol/water (3:2:1). The aqueous phase, which contained the free oligosaccharides, was analyzed directly by gel filtration chromatography (Fig. 1, A, C, and E). Denatured protein, containing residual, covalently linked oligosaccharides, was digested with pronase and endo H, and the resulting oligosaccharides were analyzed by gel filtration chromatography (Fig. 1, B, D, and F).

Clearly, the susceptibility of the 15B-grown virion oligosaccharides to endo H was not random; virtually all of the oligosaccharide chains released under these non-denaturing conditions were either Man,GlcNAc (90–95%) or Man,GlcNAc (4–6%) (Fig. 1, A, C, and D). Gel filtration analysis of endo H-released oligosaccharides and residual oligosaccharides indicated that approximately 85% of the Man,GlcNAc<sub>2</sub> chains present in intact virions were released by endo H. Since Man,GlcNAc<sub>2</sub> constitutes 59% of the total oligosaccharides of 15B-grown virions (Table I), the Man,GlcNAc<sub>2</sub> which was released by endo H constituted 40% of the entire population.
of oligosaccharides present in intact virions. By comparison, when virus is grown in wild type CHO cells, only 6% of the total oligosaccharide in the virions are Man,GlcNAc2 (Table I). The abundance of Man,GlcNAc2 oligosaccharides released by endo H from 15B-grown virions and the virtual absence of larger high mannose species released by endo H from these intact virions suggested that endo H preferentially cleaved potential complex type oligosaccharides whose processing was terminated at the Man,GlcNAc2 state in 15B host cells.

The preferential cleavage of Man,GlcNAc2 chains by endo H in this experiment was not due to an inherently higher activity of the enzyme toward this substrate than toward the larger high mannose chains, since increasing the length of exposure of virions to endo H had no significant effect on the extent of endo H cleavage. Furthermore, the selective cleavage of oligosaccharides was not the results of inactivation of endo H; the enzyme retained 90% of its initial activity after incubation for 16.5 h at 37 °C.

Three lines of evidence show that the chloroform/methanol/water extraction procedure yielded reproducible and accurate recovery of oligosaccharides. First, addition of the gel filtration profiles for endo H-released oligosaccharides and residual glycans for each set of incubations shown in Fig. 1 resulted in an oligosaccharide population identical with that obtained when whole virions were digested with pronase and then treated with endo H. Second, a small quantity of [14C]mannose (1800–2000 cpm) was added to each sample prior to chloroform/methanol/water extraction as an internal monitor for the recovery of endo H-released oligosaccharides. Greater than 90% of the [14C]mannose was routinely recovered in the inclusion volume after gel filtration chromatography of free oligosaccharides. Third, the same overall oligosaccharide composition was obtained when [3H]mannose-labeled virus grown in 15B cells was treated with pronase and endo H and analyzed directly by gel filtration chromatography as when oligosaccharides were prepared by chloroform/methanol/water extraction prior to gel filtration analysis (data not shown).

Oligosaccharide Composition at Individual Glycosylation Sites of Virus Grown in Clone 15B Cells—In the accompanying paper (20), we show that [3H]mannose-labeled tryptic peptides from Sindbis virus grown in chicken embryo fibroblasts, baby hamster kidney cells, or wild type CHO cells, when resolved by reverse phase HPLC, yield five characteristic peaks numbered I through V in order of elution. HPLC peaks I and IV were shown to contain the same glycosylation site from E2; thus these five peaks represent a total of four glycosylation sites in Sindbis virus, two in E1 (peaks III and V) and two in E2 (peaks I–IV and II). When tryptic glyco-

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**Fig. 1** Gel filtration of endo H-released and -resistant oligosaccharides of Sindbis virus grown in 15B cells. [3H]Mannose-labeled Sindbis virus from 15B cells was incubated with 3 μg/ml of endo H under nondenaturing conditions in 0.1 M Tris-Cl, pH 6.8, for 2, 4, or 16.5 h at 37 °C. Following extraction of virions in chloroform/methanol/water (3:2:1, v/v), the free oligosaccharides were analyzed directly by gel filtration chromatography. The denatured protein containing residual oligosaccharides was treated with pronase and endo H prior to gel filtration. Bovine serum albumin eluted at fraction 63; [14C]mannose eluted at fraction 173. Man,GlcNAc, Man-GlcNAc, and Man,GlcNAc, standards eluted at fractions 130, 117, and 112, respectively, on the Bio-Gel P-4 column used above. A, endo H-released oligosaccharides, 2-h incubation (65% of total Man,GlcNAc, chains); B, endo H-resistant oligosaccharides, 2-h incubation; C, endo H-released oligosaccharides, 4-h incubation (65% of total Man,GlcNAc, chains); D, endo H-resistant oligosaccharides, 4-h incubation; E, endo H-released oligosaccharides, 16.5-h incubation (65% of total Man,GlcNAc,); F, endo H-resistant oligosaccharides, 16.5-h incubation.

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**Fig. 2** HPLC of glycopeptides from Sindbis virus grown in wild type CHO or 15B cells. [3H]Mannose-labeled Sindbis virus grown in CHO or 15B cells was digested with a total of 60 μg of trypsin for 4 h at 37 °C as described under "Experimental Procedures." The tryptic peptides were resolved by reverse phase HPLC in a 0–50% gradient of acetonitrile in solvent A, 0.1 M sodium phosphate, pH 2.2. HPLC peaks are identified as I–V in order of elution. A, tryptic glycopeptides from virus grown in wild type CHO cells; B, tryptic glycopeptides from virus grown in 15B cells.
peptides from [\(^{3}H\)]mannose-labeled Sindbis virus grown in clone 15B cells were resolved by HPLC, the same four glycosylation sites were observed (Fig. 2).

To determine the oligosaccharide composition at each glycosylation site in Sindbis virus grown in 15B cells, HPLC fractions corresponding to a single glycosylation site were pooled. In repeated trials, peaks I and II were well resolved and separated by a fraction which contained no greater than 12% of the total counts/min in either peak alone. Peaks IV and V were separated by a single fraction which contained glycopeptides from both sites. Material from this fraction was analyzed separately from the other peak fractions which were free of contaminating glycopeptides. The [\(^{3}H\)]mannose-labeled glycopeptides were treated with pronase and endo H and analyzed by gel filtration chromatography. The results of such an analysis, shown in Table II, are consistent with the replacement of the complex type oligosaccharides found in wild type CHO cells by Man\(_5\)GlcNAc\(_2\) in clone 15B cells. In virus grown in wild type CHO cells, E\(_1\) has complex type oligosaccharides at one site (HPLC peak V) and high mannose type oligosaccharides at a second site (peak II). When the same virus is grown in 15B cells, both glycosylation sites of E\(_1\) (HPLC peaks III and V) have predominantly Man\(_5\)GlcNAc\(_2\) oligosaccharides; in E\(_2\), the high mannose site (peaks I+IV) has larger high mannose structures in addition to Man\(_5\)GlcNAc\(_2\), while at the complex type site (peak II), Man\(_5\)GlcNAc\(_2\) oligosaccharides predominate. A small amount of material at three of the four sites (peaks I+IV, II, and V) has been tentatively identified on the basis of migration of gel filtration chromatography, as large, incompletely processed high mannose oligosaccharides containing terminal glucose residues. As was noted for virus grown in wild type CHO cells, three of the four glycosylation sites (HPLC peaks I+IV, II, and V) are glycosylated at roughly the same frequency in 15B-grown virus; the remaining site (peak III) is glycosylated about 80% as often as the other three sites.

**HPLC Analysis of Selective Endo H Cleavage—Having**

**TABLE II**

| Oligosaccharide compositions at individual glycosylation sites of Sindbis virus grown in 15B cells |
|---|
| \[^{3}H\]Mannose-labeled tryptic peptides from virions grown in 15B cells were separated by reverse phase HPLC as described in Fig. 2. Fractions containing radiolabeled glycopeptides from a single HPLC peak were pooled, desalted on Sephadex G-10, and lyophilized. The resulting material was treated with pronase and endo H and analyzed by gel filtration chromatography as described in Fig. 1. The per cent of total oligosaccharides was determined for each glycosylation site by normalizing for the number of mannose residues in each chain. |

| Oligosaccharide species | Radioactivity for HPLC peak number | Per cent of total oligosaccharides for HPLC peak number |
|---|---|---|
| \[^{3}H\]Mannose-labeled tryptic peptides from virions grown in 15B cells were separated by reverse phase HPLC as described in Fig. 2. Fractions containing radiolabeled glycopeptides from a single HPLC peak were pooled, desalted on Sephadex G-10, and lyophilized. The resulting material was treated with pronase and endo H and analyzed by gel filtration chromatography as described in Fig. 1. The per cent of total oligosaccharides was determined for each glycosylation site by normalizing for the number of mannose residues in each chain. |

| Oligosaccharide species | Radioactivity for HPLC peak number | Per cent of total oligosaccharides for HPLC peak number |
|---|---|---|
| Glc,Man\(_5\)GlcNAc | 10,840 | 8,530 | 160 | 2,900 | 18 | 21 | 1 | 12 |
| +Man\(_5\)GlcNAc | 10,040 | 190 | 19 | 1 | 1 |
| Man\(_5\)GlcNAc | 7,600 | 680 | 16 | 2 |
| Man\(_5\)GlcNAc | 2,230 | 1,560 | 6 | 6 |
| Man\(_5\)GlcNAc | 12,840 | 16,280 | 18,010 | 11,670 | 39 | 75 | 85 |
| +Man\(_5\)GlcNAc | 340 | 580 | 300 | 1 |

\(^{a}\) Glucose-containing high mannose oligosaccharides have been tentatively identified on the basis of migration of gel filtration chromatography. Their compositions have not been determined.

\(^{a}\) Not detected.

**TABLE III**

**Composition of oligosaccharides released by endo H**

In two independent experiments, \[^{3}H\]mannose-labeled Sindbis virus grown in clone 15B cells were incubated under nondenaturing conditions in 0.1 M Tris-Cl, pH 6.8, for 4 h at 37 °C in the presence of 3 µg/ml of endo H. After extraction with chloroform/methanol/water (3:2:1), free oligosaccharides released by endo H were analyzed by gel filtration chromatography as described in Fig. 1. Denatured protein containing residual, covalently linked glycans was digested with trypsin and analyzed by HPLC (Table IV). Oligosaccharides resolved by gel filtration were identified by comparison of their relative elution constants (K\(_{\text{e}}\) values) with those of known standards. The per cent of total released oligosaccharides was determined by normalizing for the number of mannose residues in each chain.

| Oligosaccharide species | Experiment | \[^{3}H\] cpm | Per cent of total released oligosaccharides |
|---|---|---|---|
| Man\(_5\)GlcNAc | +Endo H | 70 | <1 |
| Man\(_5\)GlcNAc | +Endo H | 80 | <1 |
| Man\(_5\)GlcNAc | +Endo H | 120 | <1 |
| +Man\(_5\)GlcNAc | +Endo H | 60 | <1 |
| Man\(_5\)GlcNAc | +Endo H | 140 | 2 |
| Man\(_5\)GlcNAc | +Endo H | 100 | 1 |
| +Man\(_5\)GlcNAc | +Endo H | 110 | 1 |
| Man\(_5\)GlcNAc | +Endo H | 5,960 | 90 |
| Man\(_5\)GlcNAc | +Endo H | 8,000 | 93 |
| +Man\(_5\)GlcNAc | +Endo H | 306 | 6 |
| +Man\(_5\)GlcNAc | +Endo H | 2 | 4 |
| Total | +Endo H | 6,680 | 2 |
| Total | +Endo H | 8,875 | 2 |

**TABLE IV**

**HPLC analysis of glycopeptides containing residual oligosaccharides after endo H treatment of intact virions**

\[^{3}H\]Mannose-labeled virions grown in 15B cells were incubated with or without endo H under nondenaturing conditions followed by extraction as described in Fig. 1. Gel filtration analysis of the released oligosaccharides from two independent trials are shown in Table III. Denatured protein containing covalently linked residual glycans (+endo H) or total virion oligosaccharides (−endo H) was digested with trypsin and the tryptic peptides resolved by HPLC as described in Fig. 2. Recoveries of \[^{3}H\] counts/min after HPLC were 70–74%.

| HPLC peak | Experiment | \[^{3}H\] cpm | Per cent of total oligosaccharides released \(^{a}\) |
|---|---|---|---|
| I+IV | −Endo H | 2,410 | 669 |
| | +Endo H | 5,170 | 719 |
| | +Endo H | 740 | 16 |
| | +Endo H | 7,620 | 15 |
| II | −Endo H | 2,120 | 570 |
| | +Endo H | 2,070 | 74 |
| | +Endo H | 910 | 15 |
| III | −Endo H | 1,050 | 3,310 |
| | +Endo H | 3,310 | 80 |
| | +Endo H | 3,040 | 59 |
| V | −Endo H | 2,800 | 803 |
| | +Endo H | 800 | 23 |
| | +Endo H | 2,030 | 60 |
| | +Endo H | 2,780 | 63 |
| Total | −Endo H | 10,990 | 110 |
| | +Endo H | 20,490 | 20,490 |

\(^{a}\) The per cent of total oligosaccharides released was calculated for each glycosylation site as follows. The fraction of endo H-released Man\(_5\)GlcNAc was calculated for each site. This fraction was compared to the percentage of total chains attributable to Man\(_5\)GlcNAc, for each site (Table II) to arrive at the percentage of total oligosaccharides released. For purposes of this table, it was assumed that all endo H-released \[^{3}H\] counts/min were attributable to Man\(_5\)GlcNAc.

For example, for HPLC peak V, trial I, the initial total radioactivity in peak V is 4,570 cpm (−endo H). From Table II: 78% of radioactivity in peak V is associated with Man\(_5\)GlcNAc. 0.78 \times 4,570 cpm = 3,590 cpm of Man\(_5\)GlcNAc initially. From above: 2,540 cpm of Man\(_5\)GlcNAc were released by endo H. 2,540 cpm released/3,590 cpm initial = 71% of Man\(_5\)GlcNAc, chains released by endo H at peak V. From Table II: 85% of total chains at peak V are Man\(_5\)GlcNAc. Therefore, 0.71 \times 85% = 60% of total chains at peak V were released by endo H.
characterized the oligosaccharide composition at individual glycosylation sites of Sindbis virus grown in 15B cells, we examined the availability of these oligosaccharides to endo H under nondenaturing conditions. In two independent experiments, [3H]mannose-labeled virions from 15B cells were incubated under nondenaturing conditions with or without endo H for 4 h at 37 °C. After chloroform/methanol/water extraction, the aqeous phase containing free oligosaccharides from the endo H-treated sample was analyzed directly by gel filtration chromatography (Table III). The denatured proteins from the endo H-treated and control (=endo H) samples were treated with trypsin and analyzed separately by reverse phase HPLC (Table IV).

The results of this analysis confirmed that oligosaccharides at complex type glycosylation sites were preferentially released by endo H (Table IV). Thus, 74-80% of the oligosaccharides at the complex site in E2 (peak II) and 60-63% of the oligosaccharides at a complex site in E1 (peak V) were cleaved by endo H, but only 15-16% of the oligosaccharide chains at the high mannose site of E2 (HPLC peaks I+IV) were released. At a site in E1 which has been high mannose and complex type oligosaccharides in virus grown in wild type CHO cells (peak III), 23-26% of the oligosaccharides were cleaved.

**Analysis of Oligosaccharides Liberated by Endo H in the Presence of Detergent**—To explore the possible role of tertiary polypeptide conformation or glycoprotein-lipid interactions in determining the accessibility of oligosaccharides, we incubated virions with endo H in the presence of ionic or nonionic detergents. [3H]Mannose-labeled virions grown in 15B cells were heated at 100 °C for 2 min in 0.2% SDS and 1% β-mercaptoethanol or suspended in 0.2% NP-40 at room temperature prior to addition of endo H. Detergent was present during the incubation with endo H, which was performed as described in the legend to Fig. 1. A control sample was incubated for 4 h at 37 °C without detergent or enzyme. The endo H-released oligosaccharides from each of the two detergent-treated samples were analyzed by gel filtration chromatography (Fig. 3, A and C). The denatured protein, containing uncleaved oligosaccharides in the SDS and NP-40 samples or total virion oligosaccharides in the control sample, was treated with pronase and endo H and analyzed by gel filtration chromatography (Fig. 3, B, D, and E).

The composition of oligosaccharides released by endo H was identical for virions incubated in SDS or NP-40 (Fig. 3, A and C). Comparison of endo H-released and -resistant oligosaccharides in the two detergent-treated samples revealed that over 95% of total Man₉GlcNAc₃ chains were cleaved in the presence of detergent as compared to only 65% of the total Man₉GlcNAc₃ chains released by endo H under nondenaturing conditions (Fig. 1C). Furthermore, while less than 5% of high mannose oligosaccharides containing 6 to 9 mannose residues were cleaved under nondenaturing conditions (Fig. 1D), virtually all the Man₉GlcNAc₃ and Man₉GlcNAc₂, two-fifths of the Man₇GlcNAc₃, and one-fifth of the Man₆GlcNAc₂ oligosaccharides were cleaved by endo H in the presence of detergent (Fig. 3, B and D). Approximately 20% of the total oligosaccharide chains remained resistant to cleavage by endo H. As the gel filtration data in Fig. 3 show, the composition of uncleaved chains is biased toward Man₇GlcNAc₂ and Man₆GlcNAc₂ as well as oligosaccharides which are tentatively identified as glucose-containing high mannose structures. Since endo H retained over 90% of its initial activity following incubation of the enzyme in detergent under conditions used above, the incomplete release of oligosaccharides was not due to enzyme inactivation, and is probably attributable to incomplete denaturation of E1 and E2 glycoproteins.
at all four glycosylation sites and consisting almost exclusively of large, high mannose glycans is inaccessible to endo H under our conditions of detergent treatment.

**DISCUSSION**

Endo H was used to probe the relative accessibility of the four asparaginyl glycosylation sites of intact Sindbis virions. Clone 15B CHO cells were chosen as the host, since they are incapable of converting protein-linked Man\(_{5}\)GlcNAc\(_{2}\) to complex type glycans. Thus, sites which carry exclusively complex type oligosaccharides ("complex sites") in virus grown in wild type CHO cells have Man\(_{5}\)GlcNAc\(_{2}\) in virus from clone 15B cells; the oligosaccharide composition at a site which carries high mannose glycans ("high mannose site") is similar in virus from both hosts. Our analysis revealed that virtually all of the oligosaccharide chains released by endo H under non-denaturing conditions from intact clone 15B-grown Sindbis virions were either Man\(_{5}\)GlcNAc (90-93%) or Man\(_{5}\)GlcNAc (4-6%). Furthermore, the relative amount of endo H cleavage at each of the four glycosylation sites of the viral glycoproteins correlated with the extent of oligosaccharide processing observed for virus grown in wild type cells (20). Thus, only 15-16% of the oligosaccharides located at a high mannose site in E2 were cleaved by endo H, while 74-80% of the oligosaccharides at a complex site in E2 and 60-63% of the oligosaccharides at a complex site in E1 were cleaved. Endo H cleaved an intermediate number of oligosaccharide chains (23-25%) from the remaining E1 glycosylation site, which carries both high mannose and complex glycans in virus from wild type CHO cells. Interestingly, this "intermediate" site exhibits marked host-dependent variation of oligosaccharide composition; in virus grown in chicken embryo fibroblasts it carries exclusively high mannose glycans, while in virus grown in baby hamster kidney cells it carries predominantly complex glycans (20).

Preferential cleavage by endo H at complex glycosylation sites was abolished when the native conformations of E1 and E2 glycoproteins were disrupted. Thus, after digestion of virions with pronase, endo H released all oligosaccharides at the E2 glycosylation site, which carries both high mannose and complex glycans in virus from wild type CHO cells. Likewise, when virions were treated with pronase, endo H released all oligosaccharides remaining at E1 glycosylation site, which carries both high mannose and complex glycans in virus from wild type CHO cells. Thus, after digestion of cells it carries predominantly complex glycans (20).

Furthermore, the relative amount of endo H cleavage at each of the four glycosylation sites of the viral glycoproteins correlated with the extent of oligosaccharide processing observed for virus grown in wild type cells (20). Thus, only 15-16% of the oligosaccharides located at a high mannose site in E2 were cleaved by endo H, while 74-80% of the oligosaccharides at a complex site in E2 and 60-63% of the oligosaccharides at a complex site in E1 were cleaved. Endo H cleaved an intermediate number of oligosaccharide chains (23-25%) from the remaining E1 glycosylation site, which carries both high mannose and complex glycans in virus from wild type CHO cells. Interestingly, this "intermediate" site exhibits marked host-dependent variation of oligosaccharide composition; in virus grown in chicken embryo fibroblasts it carries exclusively high mannose glycans, while in virus grown in baby hamster kidney cells it carries predominantly complex glycans (20).

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