Glycoprotein C of Herpes Simplex Virus Type 1: Characterization of O-linked Oligosaccharides

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SUMMARY

In contrast to other viral glycoproteins, the herpes simplex virus (HSV) glycoprotein C (gC) binds to the N-acetylgalactosamine-specific Helix pomatia lectin (HPA). In the present paper gC was purified by affinity chromatography with monospecific antibodies and the purified glycoprotein was subjected to protease digestion. HPA-binding protease-resistant glycopeptides were isolated by lectin affinity chromatography. The isolated structures did not bind to concanavalin A and seemed to lack charged groups as determined by ion-exchange chromatography. In gel filtration, the glycopeptides appeared in two peaks with molecular weights higher than 4000. The HPA-binding structures of gC were synthesized in the presence of tunicamycin, indicating that they belong to the O-glycosyl class of oligosaccharides. In addition to HPA-binding oligosaccharides, synthesis of tunicamycin-resistant wheat germ lectin-binding gC oligosaccharides was demonstrable. These were sensitive to sialidase and apparently unrelated to the HPA-binding oligosaccharides.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) specifies five major glycoproteins designated gA to gE (Spear, 1976; Bauke & Spear, 1979), of which gA and gB are antigenically closely related (Eberle & Courtney, 1980; Haffey & Spear, 1980). At least some of the glycoproteins are sulphated (Hope et al., 1982). As is the case for most viral glycoproteins, it is well established that HSV-induced glycoproteins contain N-glycosyl oligosaccharides of the high-mannose or complex type (Campadelli-Fiume et al., 1982; Wenske et al., 1982). Results obtained in our (Olofsson et al., 1981a, b) and other (Norrild & Pedersen, 1982; Wenske et al., 1982; Johnson & Spear, 1982, 1983) laboratories suggest in addition the occurrence of HSV glycoproteins with oligosaccharides O-glycosidically linked to the polypeptide. This other major class of carbohydrates of membrane glycoproteins has also been observed in vaccinia virus-infected (Shida & Dales, 1981) and mouse hepatitis virus-infected cells (Holmes et al., 1981; Niemann & Klenk, 1981). The O-glycosyl and N-glycosyl classes of carbohydrates differ not only in the type of sugar–protein linkage but also in monosaccharide composition (Kornfeld & Kornfeld, 1976), mode of biosynthesis (Li et al., 1978; Strous, 1979) and resistance to glycosylation inhibitors such as tunicamycin (Heifetz et al., 1979; Gahmberg et al., 1980).

Previously, we have demonstrated that HSV glycoprotein C (gC) contains oligosaccharides with affinity for the N-acetylgalactosamine (GalNAc)-specific lectins of Helix pomatia (HPA) and soybean (SBA), suggesting that it is gC that contains the HSV carbohydrate structures belonging to the O-glycosidic class of oligosaccharides (Olofsson et al., 1981b). N-linked oligosaccharides, with the exception of the poly(glycosyl) chains of erythrocytes, are not known to contain GalNAc (Kornfeld & Kornfeld, 1976; Kusius et al., 1978). An affinity for HPA and SBA of viral glycoproteins has not been reported previously, and, since the total amount of glycoprotein demonstrating affinity for GalNAc-binding lectins increases five to tenfold during
the course of HSV infection, an active role for HSV gene products in the induction of the HPA-binding structures seems possible (Olofsson et al., 1981b).

The present paper describes properties of the HPA-binding carbohydrates isolated from protease-digested [3H]glucosamine-labelled gC. We also show that HPA-binding oligosaccharides of gC are synthesized in the presence of tunicamycin, further supporting the assumption that gC oligosaccharides are O-glycosidically linked to the polypeptide.

METHODOLOGY

Virus and cells. The HSV-1 strain F and an established line of green monkey kidney cells (GMK AH-1) were used throughout the studies. Eagle's minimal essential medium supplemented with 10% newborn calf serum and antibiotics was used for the cultivation of cells. For maintenance of cell cultures, the same medium was used without serum.

Chemicals, radiochemicals and chromatographic media. Proteases, from various sources, including Pronase from Streptomyces griseus, were obtained from Sigma. d-[1-3H]Glucosamine (GlcN; 3 Ci/mmol) was purchased from Amersham International. All organic solvents were of reagent grade and obtained from Merck. Helix pomatia Lectin—Sepharose 6MB (HPA), concanavalin A—Sepharose (Con A), Wheat germ Lectin—Sepharose 6MB (WGA), Protein A—Sepharose CL-4B, Sephadex G-50 Superfine and DEAE-Sephacel were all obtained from Pharmacia. Neuraminidase (Vibrio cholerae) was purchased from Hoechst (Frankfurt, F.R.G.). Tunicamycin (TM) was a kind gift of Dr R. L. Hamill of Eli Lilly Research Laboratories (Indianapolis, Ind., U.S.A.) TM was used at a concentration of 2 µg/ml.

Solubilization of HSV-infected cells and purification of gC. HSV-infected cells were solubilized as previously described (Jeansson et al., 1982). Briefly, 10⁶ GMK cells in 50 mm Petri dishes were infected with HSV-1 (strain F, 10 p.f.u./cell) and 25 µCi of labelled glucosamine was added at 5 h post-infection. After 18 h at 37 °C, the cells were harvested and solubilized in TBS (0.15 M-NaCl, 0.02 M-Tris-HCl pH 7.5) with 10 mg/ml Triton X-100. Insoluble material was pelleted by centrifugation at 100 000 g for 1 h. The solubilized radiolabelled glycoproteins were subjected to immunosorbent affinity chromatography by one of the two following methods.

(i) Monoclonal antibodies were produced by the method of Fazekas de St Groth & Scheidegger (1980). Briefly, FO cells were fused with spleen cells from BALB/c mice immunized with a sodium deoxycholate-solubilized HSV-1 membrane antigen obtained from infected homologous embryonic cells (Jeansson et al., 1982). An HSV-1 gC-specific monoclonal antibody (subclass IgG₁) designated B1C1 was characterized by radioimmunoprecipitation and subsequent SDS-polyacrylamide gel electrophoresis (Fig. 1d). In this type of test as well as in all other tests performed, the B1C1 antibody showed a reaction pattern identical to that of the gC-specific monoclonal antibody designated HC1 and characterized by Pereira et al. (1980). Culture fluids from cells of clone B1C1 were concentrated by ammonium sulphate precipitation, dissolved and coupled to cyanogen bromide-activated Sepharose at a concentration of 2 mg IgG per ml gel. HSV glycoproteins from 10⁶ cells solubilized as described above were fractionated on a 16 x 16 mm column containing B1C1 monoclonal antibodies equilibrated with a 0.1 M-glycine–NaOH buffer pH 8.8 plus 0.1% bovine serum albumin and 0.5% (w/v) Tween 80. The gel was washed with 20 ml buffer (15 ml/h) and adsorbed material was eluted with 3 ml of 0.1 M-glycine–HCl pH 2.5, containing 0.5% Tween 80 and 0.1% bovine serum albumin. The isolated eluted glycoprotein was desalted on Sephadex G-25 equilibrated with TBS prior to further experimentation.

(ii) A rabbit hyperimmune serum containing antibodies against gC and gA/gB was allowed to adsorb to Protein A-Sepharose for 20 min at room temperature (200 µl of serum to 1 ml of gel in a minicolumn, inner diam. 6 mm). The gels were washed with 10 × 1 ml of a buffer containing 0.02 M-Tris–HCl, 0.5 M-NaCl and 0.5% (w/v) Tween 80. Thereafter, 500 µl of radiolabelled glycoprotein was allowed to adsorb for 20 min. After another washing with 10 × 1 ml of this Tris buffer, the antibody–glycoprotein complexes were eluted with 1 M-acetic acid and the eluate was desalted on a 10 × 1 cm Sephadex G-25 column, equilibrated with TBS. The desalted eluates were characterized by SDS–polyacrylamide gel electrophoresis (Fig. 1c). Based on the same procedure, an immunosorbent was prepared using a polyclonal but monospecific antiserum against gC (Fig. 1b). This antiserum was obtained by immunization of a rabbit with antigen purified by the immunosorbent technique using the B1C1 monoclonal antibody described above.

Enzymic digestion. Immunosorbent-purified glycoproteins were subjected to protease digestion according to Spiro (1965). The reaction mixture consisted of 0.1 ml Pronase (10 mg/ml, sterile-filtered) in TBS and 1 ml solubilized cell material. The protease was allowed to digest for 5 days at 37 °C. The digest was then examined by gel filtration and lectin affinity chromatography. Although Pronase treatment may not quantitatively remove all peptide material, Pronase-resistant glucosamine-labelled structures included in Sephadex G-50 gels are, in what follows, referred to as oligosaccharides. In cases where remaining peptide material might possibly influence results, this has been commented on in the text.

Sialidase treatment was performed as previously described (Olofsson et al., 1983).
Fig. 1. SDS–PAGE of [3H]glucosamine-labelled HSV glycoproteins. (a) Solubilized glycoproteins from HSV-infected cells. (b) HSV glycoproteins precipitated with gC-specific polyclonal antibody. (c) HSV glycoproteins precipitated with gA/gB/gC-specific antibody. (d) HSV glycoproteins precipitated with B1C1 monoclonal antibodies, specific for gC. All lanes were run on the same gel.

*SDS–polyacrylamide gel electrophoresis (SDS–PAGE).* Isolated glycoproteins were precipitated in 10% ice-cold trichloroacetic acid and subjected to SDS–PAGE according to Morse et al. (1978). Gels were immersed in Enlightning (New England Nuclear), dried and subjected to fluorography on Kodak XRP X-Omat film at −70 °C. The HSV-1 tsB5 mutant (a gift from Dr A. Buchan, University of Birmingham, U.K.) is conditionally defective in gB and was used as a marker for gA and gB (Manservigi et al., 1977) in control experiments (data not shown). Heavy chains of immunoglobulin G (mol. wt. 50000), bovine serum albumin (mol. wt. 68000) and β-galactosidase (mol. wt. 130000) were used as molecular weight markers.

*Chromatographic procedures.* A micromethod for gel-bound lectin affinity chromatography was used (Olofsson & Blomberg, 1977). Two-hundred μl samples of [3H]glucosamine-labelled lysates or protease digests were applied to 1 ml of gel-bound lectin in 6 mm-wide minicolumns. The materials were allowed to adsorb for 15 min at room temperature. After adsorption, the gels were washed with 10 bed vol. of 1% Triton X-100 in TBS. The adsorbed material was eluted by addition of appropriate sugar solutions. Fractions of 1.5 ml were collected and subjected to liquid scintillation counting. Columns were eluted with the following sugars in the same TBS buffer: 5 mM-GalNAc (for HPA), 100 mM-α-methylmannoside (for Con A) and 50 to 200 mM-GlcNAc (for WGA) as indicated in legends to figures.

Gel filtration was carried out on Sephadex G-50 equilibrated with TBS, in 90 × 9 mm columns (1.5 ml/h at 4 °C). Fractions (0.5 ml) were analysed for radioactivity. Blue dextran, phenol red, and oligosaccharides from fetuin (mol. wt. 4100), vesicular stomatitis virus glycoprotein (mol. wt. 3500) and ovalbumin (mol. wt. 1550) were used as markers.

Ion-exchange chromatography was carried out on DEAE-Sephael equilibrated with 1 mM-phosphate buffer pH 7.0. Aliquots of protease-resistant glycopeptides (corresponding to 1000 to 2000 ct/min) were dissolved in the phosphate buffer and added to the gel in a 9 × 50 mm column. The columns were washed with several bed volumes of sample buffer and developed by a stepwise increase of sodium phosphate buffer (25 ml/h) to 200 mM-phosphate and 1.0 ml fractions were collected.

*Fractionation in organic solvents.* Glycolipids were isolated from HSV-infected cells by the method of Svennerholm & Fredman (1980) for quantitative ganglioside isolation. Glucosamine-labelled HSV-infected cells were washed five times with TBS. An amount of cells that corresponded to 10 mg of protein, according to a modified Lowry method (Dulley & Grieve, 1975), was suspended in 2 ml TBS. The suspensions were subjected to
four cycles of freeze–thawing and subsequent disruption by ultrasonication in a Raytheon sonicator at 1.25 A for 1 min. The lipid extraction was carried out with 1.8 ml of the cell homogenate, using volumes of 5.4 ml methanol and 2.7 ml chloroform. Addition of the solvents was followed by thorough mixing in a Vortex shaker and the mixture was clarified by centrifugation for 10 min at 800 g. The supernatant was saved and the pellet was dissolved in 1.8 ml of water and re-extracted with the same volumes of methanol and chloroform as described above. Both chloroform/methanol-soluble fractions were pooled and the pool was regarded as the lipid fraction. The protein pellet was extensively digested with protease as described above and analysed by lectin affinity chromatography.

RESULTS

HPA-binding oligosaccharides of HSV-1 glycoprotein C

To obtain information about the relative amounts of HPA-binding and non-binding oligosaccharides, immunosorbent-purified glucosamine-labelled gC from HSV-infected cells was subjected to extensive Pronase digestion followed by HPA affinity chromatography. The gC-specific immunosorbent adsorbed about 30% of the total glucosamine label. Eluted material was subjected to SDS-PAGE and the two glycosylated polypeptides appearing were identified as gC and its precursor (pgC) (Fig. 1 d). HPA affinity chromatography demonstrated that about 30% of immunosorbent-purified gC adsorbed to the lectin (Fig. 2a). About 70% of gC lacked HPA-binding structures. After Pronase digestion, only about 2% of [3H]glucosamine-labelled structures derived from immunosorbent-purified gC demonstrated affinity for HPA (Fig. 2b). Thus, a subfraction of about 6% of the glucosamine label of HPA-binding gC seemed to be located on HPA-binding oligosaccharides. These findings are consistent with reports that gC may contain N-linked as well as O-linked oligosaccharides (Olofsson et al., 1983; Johnson & Spear, 1983).

Lipid extraction was then performed to exclude the possibility that glycolipids present in preparations of detergent-extracted HSV glycoproteins contributed to the HPA-binding activity observed. Since classical Folch partition (Folch et al., 1951) would not completely remove higher glycosphingolipids, we used a method developed by Svennerholm & Fredman (1980) for quantitative isolation of this kind of glycoconjugates.

More than 75% of the Pronase-resistant HPA-binding activity was found in the insoluble protein fraction after solvent fractionation (Table 1), indicating that HPA-binding activity of HSV-infected cells was not associated with glycolipids.

Size distribution of HPA-binding oligosaccharides

Pronase-resistant 3H-labelled oligosaccharides of immunosorbent-purified gC were fractionated by HPA affinity chromatography and GalNAc-eluted fractions (Fig. 2b) were gel-filtered on Sephadex G-50 (Fig. 3). It was found that the HPA-binding oligosaccharides eluted in one large peak (I) immediately after the void volume and in a second, less prominent peak (II) around fraction 48. Judging from control experiments with carbohydrate markers, pool II comprised material of mol. wt. 4000 to 5000 and pool I contained material of mol. wt. 7500 or more. The possibility that structures of this size contain polypeptide segments with multiple oligosaccharides arranged in protease-resistant clusters (Honess & Roizman, 1975) cannot be excluded.

Lectin affinity of oligosaccharides

The binding of oligosaccharides of preparations I and II to HPA, WGA and Con A was studied. Lectin-binding properties of the pool I and II preparations were compared with those of protease-resistant carbohydrates, that passed through an HPA column without demonstrable affinity (Table 2). As expected, the majority of both peak I and II oligosaccharides adsorbed to the HPA upon re-chromatography. However, the enrichment of HPA-binding structures was accompanied by a total loss of Con A-binding material, whereas the proportion of WGA-binding material in preparations I and II did not differ from that of the bulk of oligosaccharides that lacked HPA-binding properties. No HPA-binding activity was detected after re-
Fig. 2. HPA affinity chromatography of [3H]glucosamine-labelled immunosorbent-purified gC before (a) and after (b) extensive Pronase digestion. Arrow denotes addition of 5 mM-GalNAc.

Fig. 3. Sephadex G-50 gel chromatography of Pronase-resistant HSV glycoproteins with affinity for HPA. The sample corresponds to fractions 11 to 13 of Fig. 2(b). BD and GlcN indicate positions of markers blue dextran and [3H]glucosamine, respectively.

Table 1. **HPA-binding activity before and after solvent* extraction of 10⁶ HSV-infected cells**

<table>
<thead>
<tr>
<th>[3H]Glucosamine</th>
<th>Before extraction†</th>
<th>After extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total ct/min × 10⁻⁶</td>
<td>8.5</td>
<td>6.2</td>
</tr>
<tr>
<td>HPA-binding‡, ct/min × 10⁻⁴</td>
<td>9.3</td>
<td>8.2</td>
</tr>
<tr>
<td>HPA-binding, % of total</td>
<td>1.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

* Chloroform/methanol/water (4:8:2:7).
† Prior to HPA chromatography and assay of ct/min the extracts were digested with Pronase.
‡ Eluted with 0.01 M-GalNAc.
### Table 2. Lectin affinity chromatography of gC oligosaccharides separated on HPA-Sepharose

<table>
<thead>
<tr>
<th>Lectin/eluting sugar</th>
<th>Oligosaccharides without affinity for HPA</th>
<th>Oligosaccharides with affinity for HPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPA/GalNAc</td>
<td>≤ 0.5*</td>
<td>83.5</td>
</tr>
<tr>
<td>Con A/Man</td>
<td>15.3</td>
<td>≤ 0.5</td>
</tr>
<tr>
<td>WGA/GlcNAc</td>
<td>5.0</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* Percentage of label bound to and eluted from the lectin. About 10⁴ cts/min of radiolabelled oligosaccharide was added in each experiment.

chromatography of protease-resistant oligosaccharides initially lacking affinity for HPA, suggesting that the preparative HPA chromatography almost completely removed oligosaccharides with HPA affinity. Elution of peak I and II materials from the HPA column was achieved with 0.5 to 1 mM-GalNAc, the same concentration range required for eluting HPA-bound, native non-digested gC (Olofsson *et al.*, 1981b).

**HPA-binding oligosaccharides lack sialic acid**

Glycoprotein C oligosaccharides with affinity for HPA almost all passed through a DEAE-Sephacel column, indicating that they essentially comprised neutral sugar structures (Fig. 4b, c). In contrast, a high proportion of the oligosaccharides without affinity for HPA adsorbed to the DEAE-Sephacel (Fig. 4a) and were eluted with high concentrations of phosphate buffer. The results thus suggest that the HPA-binding oligosaccharides of HSV-infected cells were mainly devoid of negatively charged groups such as sialic acids.

**Tunicamycin-resistant HPA-binding oligosaccharides**

Glycoproteins with O-glycosidically linked oligosaccharides are produced in the presence of TM, an efficient inhibitor of N-linked sugar production (Heifetz *et al.*, 1979). HSV glycoproteins produced by infection of cells in the presence of TM therefore essentially represent glycoconjugates of the O-glycosidic linkage type. When the immunosorbent technique was applied to the purification of gC, two observations were made: the B1C1 antibody-binding epitope of gC was not present in glycoproteins produced in the presence of TM, but gC with HPA-binding capacity was synthesized.

The latter statement was supported by the following findings. HSV glycoproteins synthesized in the presence (2 μg/ml) or absence of TM were subjected to immunosorbent fractionation using two immunosorbents, one made with a hyperimmune serum with specificities against gC and gA/gB, and another based on a monospecific anti-gC serum. Immunosorbent-processed fractions were studied by SDS-PAGE. In the presence of TM, about 5% of the total cytoplasmic soluble glucosamine label bound to both the immunosorbents used; the label appeared in SDS-PAGE as one band corresponding to about 100K mol. wt. The radioactivity was assayed by liquid scintillation, on 2 mm strips of a cut gel (data not shown). The immunosorbent-enriched fractions (produced either in the presence or absence of TM) were also extensively digested with Pronase and subsequently studied by lectin affinity chromatography (Con A and HPA). Fig. 5 demonstrates that about 10% of the [³H]glucosamine label from the immunosorbents was recovered in Con A-binding material, i.e. presumably the N-linked high-mannose and moderately branched complex type of oligosaccharides. In the presence of TM, these carbohydrates with Con A affinity were reduced at least 100-fold, revealing an almost complete block of the synthesis of the N-glycosyl oligosaccharide-containing glycoproteins. For the HPA-binding structures, a different pattern was observed. As expected, about 2%, of the glucosamine-labelled glycopeptides produced in the absence of TM demonstrated affinity for HPA. However, the relative amount of HPA-binding glycopeptides in material derived from TM-treated cells is at least twice that in corresponding material from untreated HSV-infected cells (Fig. 5). In addition, the total amount of radiolabelled HPA-binding structures was as much as
O-linked carbohydrates of HSV gC

Fig. 4. DEAE-Sephacel chromatography of HPA-binding oligosaccharides of immunosorbent-purified gC. (a) Oligosaccharides without affinity for HPA, corresponding to fractions 1 and 2 of Fig. 2(b). (b, c) HPA-binding oligosaccharides of preparations I (b) and II (c) from Fig. 3. Glucosamine radiolabel (●) and concentration of eluting phosphate buffer (...) are indicated. Identical elution conditions were used in all experiments, but the discontinuous phosphate concentration gradient is indicated only in (a).

Fig. 5. Lectin affinity chromatography of tunicamycin-resistant gC oligosaccharides. Glycoprotein C-enriched [3H]glucosamine-labelled glycoproteins produced in the presence (c, d) or absence (a, b) of tunicamycin (2 μg/ml) were Pronase-digested and subjected to lectin affinity chromatography. Glycopeptides bound to Con A (a, c) or HPA (b, d) were eluted with α-methylmannoside (100 mM) or GalNAc (5 mM), respectively. Each culture was radiolabelled with 25 μCi per ml of glucosamine and 100 μl from each protease digest was subjected to lectin affinity chromatography.

50% of that in untreated cells (1200 ct/min versus 2400 ct/min per 100 μl). Altogether, the data indicate that the HPA-binding carbohydrates of gC are O-glycosyl oligosaccharides.

Different classes of tunicamycin-resistant oligosaccharides

From Fig. 5, it is clear that HSV glycoproteins produced in the presence of TM contain glucosamine-labelled material both with and without affinity for HPA. In the next series of experiments these classes of carbohydrates were characterized.

Gel filtration on Sephadex G-50 revealed that the pattern of oligosaccharides demonstrating HPA affinity and synthesized in the presence of TM was similar to that reported above for HPA-binding carbohydrates produced without TM-induced blockage of glycosylation (Fig. 3 and 6a). Thus, the TM-induced block of glycosylation had no major influence on size distribution of HPA-binding oligosaccharides.
In Sephadex G-50 gel filtration, TM-resistant oligosaccharides without affinity were eluted as one peak at a position corresponding to that of peak I (Fig. 3), i.e. the largest of the HPA-binding oligosaccharides (Fig. 6b). At the position of peak II, chromatography revealed a relatively broad shoulder. Fractions were pooled as indicated in Fig. 6 and subjected to WGA affinity chromatography (Fig. 7). Whereas most of the pool (a) material and about 30% of the pool (b) oligosaccharides bound to WGA, pools (c) and (d) showed no affinity for this lectin, suggesting the occurrence of at least two affinity classes of TM-resistant oligosaccharides, one having its main affinity for HPA and one lacking the capacity to react with HPA but with affinity for WGA. The oligosaccharides of pools (c) and (d) were not further characterized, but they may represent intermediates in the synthesis of HPA- and WGA-binding TM-resistant oligosaccharides.

**TM-resistant WGA-binding oligosaccharides**

WGA affinity of carbohydrates is mainly due to the presence of β-linked GlcNAc or sialic acid (Peters et al., 1979; Goldstein & Hayes, 1978). WGA-binding properties of TM-resistant gC oligosaccharides may therefore be attributed to sialic acid linked to and perhaps blocking an
O-linked carbohydrates of HSV gC

Fig. 7. WGA lectin affinity chromatography of pooled tunicamycin-resistant oligosaccharides without affinity for HPA. Parts (a) to (d) represent the pools (I to IV) of fractions depicted in Fig. 6(b). Elution was performed with 100 mM-GlcNAc (arrow).

HPA-binding penultimate GalNAc. This hypothesis was tested on WGA-binding oligosaccharides from Pronase-treated, immunosorbent-purified glycoproteins produced in the presence of TM. The oligosaccharides were subjected to sialidase treatment and subsequent Sephadex G-25 separation of remaining oligosaccharides and low mol. wt. material. Finally, the oligosaccharides were studied by HPA and WGA affinity chromatography (Fig. 8). The following results were obtained. (i) The G-25 gel filtration yielded a peak of included material, indicating that WGA-binding carbohydrates were sialylated. (ii) Sialidase-treated oligosaccharides did not bind to WGA upon re-chromatography (Fig. 8d), suggesting that a terminal sialic acid group was the mediator of WGA binding. (iii) Sialidase treatment did not uncover HPA-binding activity (Fig. 8e), indicating that a direct masking of HPA-binding structures by sialic acid was not occurring.

DISCUSSION

In previous reports (Olofsson et al., 1981b, 1983), we have demonstrated that some HSV-specified glycoproteins contain unusual oligosaccharides not described earlier for glycoproteins of enveloped animal viruses. The presence of terminal non-reducing GalNAc was made plausible by the affinities of HSV glycoproteins for SBA and HPA. These characteristics distinguish certain gC oligosaccharides involved from those of the N-glycosidic, high-mannose and complex types (Kobata, 1979) and suggest structural similarities with oligosaccharides of glycoconjugates such as those constituting several blood group substances and the Forssman antigens (Donald et al., 1982; Hakomori et al., 1977). In the present paper, glycoproteins of HSV-infected cells were purified by immunosorbent techniques and HPA affinity chromatography of the protease-digested glycoproteins. The protease-digested lectin-reactive glycopeptides (referred to as oligosaccharides) were analysed with respect to their molecular size, lectin-binding spectrum and content of negatively charged groups.

We and others have reported that HSV-specified glycoproteins contain oligosaccharides linked to the polypeptide via an O-glycosidic bond (Olofsson et al., 1981a; Norrild & Pedersen, 1982; Wenske et al., 1982; Johnson & Spear, 1982, 1983). The fact that gC binds to HPA (Olofsson et al., 1983) is in agreement with such a structural arrangement. The O-linked oligosaccharides are, in contrast to N-linked ones, synthesized via a dolichol-independent
Fig. 8. Sialidase treatment of WGA-binding oligosaccharides. (a) Sephadex G-25 gel filtration of reaction mixture after sialidase treatment of WGA-binding oligosaccharides from tunicamycin-treated HSV-infected cells. Arrows in (a) indicate elution positions of blue dextran (BD) and glucosamine (GlcN). Re-chromatography was on WGA (b) and HPA (c) prior to sialidase treatment, or on WGA (d) and HPA (e) after sialidase treatment. HPA columns were eluted with 5 mM-GalNAc and WGA columns were eluted sequentially with 50 mM- and 200 mM-GlcNAc (see arrows).

pathway and are therefore insensitive to blockage of glycosylation induced by TM (Strous, 1979; Gahmberg et al., 1980). Moreover, results reported in the present paper demonstrating formation of HPA-binding structures in the presence of TM are consistent with the occurrence of O-glycosidically linked oligosaccharides in gC. In contrast to these observations, Kumarasamy & Blough (1982) were unable to demonstrate a presence of O-linked glycans in gC.

Johnson & Spear (1983) recently reported O-linked sialylated HSV oligosaccharides ranging from 1500 to 3000 mol. wt., i.e. of a size apparently smaller than those reported by us. However, Johnson & Spear used a gel matrix in their chromatography less suited to the resolution of larger glycopeptides and, moreover, used a different cell line (Hep-2) for production of HSV glycoproteins. The HPA-binding oligosaccharides we observed were large also in comparison with oligosaccharides observed in other types of viral glycoproteins (Etchison et al., 1977; Pesonen & Renkonen, 1977; Schwartz et al., 1978; Burke & Keegstra, 1979; Hunt et al., 1979). The minimal number of monosaccharide residues of the smaller oligosaccharides we observed may be estimated to be about 20. This number is considerably larger than for example that of the complex-type oligosaccharide of glycoprotein G of vesicular stomatitis virus; it has a mol. wt. of about 3400 and contains 12 to 14 monosaccharide units (Etchison et al., 1977). Estimations of
<table>
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<th>Population</th>
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<td>Lectin affinity: HPA</td>
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<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Con A</td>
<td>$-^*$</td>
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<td>$-$</td>
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<td>DEAE-Sephacel affinity</td>
<td>Yes, partly</td>
<td>No</td>
<td>ND</td>
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</table>

$^*$ $-$, Absence of lectin-binding property; $+$, part of the population (5 to 30%) binds to the lectin; $++$, the whole population binds to the lectin.

$^*$ ND, Not done.

The size of the HPA-binding structures are subject to some uncertainty, since gel chromatographic positions of carbohydrates are not affected by molecular weights only but also by factors such as the degree of oligosaccharide branching (Kainuma et al., 1976). Moreover, it is possible that peptide material remaining on Pronase-digested structures may influence the size determination. However, the occurrence of large glucosamine-labelled oligosaccharides in virions and membranes of HSV-infected cells has been reported by Honess & Roizman (1975). Interestingly, large oligosaccharides with affinity for GalNAc-binding lectins have been observed in glycoproteins of mouse teratocarcinoma and embryocarcinoma cells, and were shown to contain Dolichos biflorus lectin-binding oligosaccharides, eluting near the void fraction in Sephadex G-50 gel filtration (Muramatsu et al., 1981).

The observation that a minority of HPA-binding HSV oligosaccharides adsorbed to WGA, might indicate the presence of sialic acid or GlcNAc (Goldstein & Hayes, 1978; Peters et al., 1979). However, since our results of sialidase treatment and DEAE-Sephacel chromatography were not consistent with such an assumption, the binding to WGA seems rather to be due to internal $\beta$-linked GlcNAc residues. Although structural inferences based on lectin affinity chromatography must be interpreted cautiously, the lack of affinity for Con A strongly suggests an absence of repetitive units of $\alpha$-mannose (So & Goldstein, 1968; Goldstein & Hayes, 1978); a finding which in turn excludes resemblance between the HPA-binding oligosaccharides of gC and derivatives of neutral, $N$-glycosidically linked, high-mannose type oligosaccharides, present in several viral glycoproteins (Burke & Keegstra, 1979; Kobata, 1979).

Glycoprotein C has been demonstrated to exist in two populations differing in their HPA-binding properties and it is plausible that completely glycosylated gC occurs either with or without affinity for HPA (Olofsson et al., 1983). The hypothesis that HPA-binding oligosaccharides of gC are end-products rather than intermediates in the formation of glycosylated structures is supported by our present results. One of the two preparations of large, TM-resistant carbohydrates of the enriched gC demonstrated WGA affinity. It may be that in some oligosaccharides the WGA affinity represented sialic acid blocking the HPA-binding structures. Such a masking has been described for HPA-binding glycoproteins of T lymphocytes (Hammarström et al., 1973). However, we found that although the WGA affinity seemed to be attributable to a terminal sialic acid, an almost complete removal of sialic acid did not induce the oligosaccharides to bind to HPA. Therefore, if we assume that the HPA-binding oligosaccharides of gC comprise intermediates, we have to postulate that at least two monosaccharide units must be added to the HPA-binding oligosaccharide in order to form a completely glycosylated WGA-binding structure. This seems unlikely, considering the striking similarities in gel filtration elution patterns of WGA- and HPA-binding TM-resistant structures.

We consider that the HPA-binding gC belongs to a population of wholly glycosylated glycoproteins of HSV and these have an $O$-glycosidic linkage between oligosaccharide and peptide. Based on the reported findings, four populations of gC oligosaccharides are discernible (Table 3). Of these, one would represent the various $N$-linked complex and high-mannose oligosaccharides (population 1 of Table 3). This very large class of oligosaccharides is not discussed in the present paper. The other three populations are synthesized in the presence of TM and presumably are of the $O$-linked class but differ in their terminal structures. The HPA-
reactive population probably represents those oligosaccharides with a terminal GalNAc. Of the
two populations without affinity for HPA, the WGA-binding one probably contains sialic acid,
a statement compatible with the neuraminidase sensitivity of the WGA-binding capacity.
Finally, the fourth population, reacting with neither HPA nor WGA may comprise intermediates which, when wholly glycosylated, would be found among oligosaccharides of populations 2 and 3. Of course, it is possible that the fourth population may include complete oligosaccharides with terminal structures other than those discussed.

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REFERENCES
O-linked carbohydrates of HSV gC


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