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Populations of Herpes Simplex Virus Glycoprotein gC with and without Affinity for the N-Acetyl-Galactosamine Specific Lectin of *Helix pomatia*

By

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With 8 Figures

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Summary

Two fractions of herpes simplex virus glycoprotein gC were isolated and characterized by means of immunosorbent-purification with monoclonal antibodies against gC and Helix pomatia lectin (HPA) affinity chromatography. About 25 per cent of the glycoprotein gC population demonstrated affinity for the lectin, compatible with presence of N-acetylgalactosamine as terminal sugar of the oligosaccharide. The HPA-binding populations of gC appeared as two electrophoretic bands with lower molecular weights than the non-binding gC.

The gC subfraction without affinity for the HPA was subjected to treatments aiming to desialylize the carbohydrate moiety. Only 5 per cent of the initially non-reactive fraction of gC became reactive to HPA after the treatments, suggesting that masking of penultimate N-acetylgalactosamine by sialic acid was not a main reason for lack of HPA affinity. Results of treatment with alkaline Na BH_4 demonstrated presence of oligosaccharide-peptide linkages sensitive to β-elimination suggesting O-glycosidic type of linkage.

The subfraction of gC demonstrating affinity for HPA as well as gC devoid of HPA binding capacity both revealed affinity for Con A. Therefore N-glycosidically as well as O-glycosidically linked oligosaccharides seemed to be present on the one and same glycoprotein.

On the basis of the results presented we assume that the glycosylation of HSV glycoprotein gC may lead to, at least, two populations of the glycoprotein gC, one with terminal N-acetylgalactosamine residues of oligosaccharides 0-glycosidically linked to the polypeptide and the other without affinity for HPA. However, both populations of gC contain similar proportions of oligosaccharides of the high

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mannose or complex types with N-glycosidic carbohydrate-peptide linkages as indicated by their affinity for Con A.

Introduction

All known glycoproteins of enveloped viruses contain high mannose or complex type oligosaccharides linked to the polypeptide via an N-glycosidic linkage between asparagine and N-acetylglucosamine (GlcNAc) (6). Under conditions of tunicamycin (TM) inhibition of glycosylation in virus-infected cells, aberrant polypeptides will be formed resulting in loss of viral infectivity (9, 10, 16, 17, 18, 25). According to prevailing concepts, the main function of N-glycosidic oligosaccharides is to stabilize the polypeptide conformation and the loss of infectivity is caused only indirectly by the absence of oligosaccharides due to such secondary events as increased sensitivity to intracellular proteolytic degradation or unspecific aggregation of underglycosylated viral glycoproteins (4).

We have found that the type-specific glycoprotein gC of herpes simplex virus type 1 (HSV-1) contains oligosaccharides with affinity for the N-acetylgalactosamine-binding Helix pomatia lectin (HPA). Most likely the oligosaccharide constituent of the HPA-binding HSV glycoprotein is linked to the peptide via an O-glycosidic bond between N-acetylgalactosamine (GalNAc) and a serine or threonine of the peptide (19, 20). Such O-glycosidic linkages of HSV (19), vaccinia (26) and coronavirus (7, 15) glycoproteins have been described recently. It is tempting to compare the HPA-binding HSV glycoproteins with several biologically highly active glycoconjugates such as blood group substances and lymphocyte differentiation antigens which also demonstrate HPA-binding activities (8, 11). For these types of antigens it is likely that the carbohydrate itself mediates the biological activity, a phenomenon which contrasts to the less specific biological significance of N-glycosidic oligosaccharides. Recently, the function of so called stage-specific differentiation antigens have been blocked by addition of competing carbohydrate haptens (5). Thus, the HPA-binding oligosaccharides of HSV glycoproteins might constitute a carbohydrate structure with a more defined biological significance, probably as a recognition structure. In the present study the distribution of HPA-binding activity among HSV glycoproteins was investigated, and it was found that only a subfraction of gC contains HPA-binding oligosaccharides while the other fraction contains 0-glycosidic oligosaccharide without affinity for HPA.

Materials and Methods

Virus Strains

The prototype virus HSV-1 (F) was propagated in VERO cells maintained as monolayer cultures in minimal essential medium (MEM) supplemented with 10 per cent foetal calf serum (FCS). The HSV-1 (HFEM) *ts* B 5 (12) was a generous gift from Dr. A. Buchan, University of Birmingham, England. This mutant, which was defective in gB at non-permissive temperature was used as a marker for gA and gB (12).

Monoclonal Antibodies

Hybridoma cells were produced as previously described (22) and ascites preparations containing monoclonal antibodies against glycoproteins gC (HC1), gA/gB (H 233) or gD (HD 1) were used.

Viral Infection and Labelling of Viral Glycoproteins

VERO monolayer cells grown in 150 cm^2 tissue culture flasks (2×10^7 cells) were infected with HSV-1 using a multiplicity of infection of 5 PFU/cell and a volume of 4 ml MEM containing 1 per cent FCS. After 1 hour of adsorption at 37° C the virus was aspirated and the cells overlayered from 5 to 18 hours postinfection by the addition of 10 ml MEM with 1 per cent FCS and 1.0 μCi/ml medium of D-(1-14-C)-glucosamine (specific activity 55.5 mCi/mMol, Amersham, England). At the end of the labelling period the cells were scraped off, washed once in PBS and collected as a cell pellet after centrifugation for 5 minutes at $500 \times g$.

Purification of Membrane Proteins and HPA Affinity Chromatography

The cell pellet from 1.2×10^8 glucosamine labelled HSV infected VERO cells was prepared as previously described (20). Briefly, packed cells were mixed with 0.025 M Tris-hydrochloride, pH 8.0, and homogenized in a tight fitting Dounce homogenizer. After centrifugation at $1500 \times g$ the membranes remaining in the supernatant were pelleted at $160{,}000 \times g$ for 1 hour. The pelleted membranes were suspended in 0.1 M glycine-NaOH buffer, pH 8.8, and centrifuged at $160{,}000 \times g$ for 1 hour. Finally, the washed membranes were suspended in a glycine-NaOH buffer containing 1 per cent Triton X-100. The suspension was homogenized and centrifuged at $160{,}000 \times g$ for 1 hour. The supernatant was used in HPA-affinity chromatography.

The Triton X-100 solubilized material was applied on columns (diameter 16 mm) with 8 ml of HPA coupled to Sepharose 6 MB (Pharmacia, Sweden). The gel was equilibrated with 0.02 M Tris buffered saline and 0.5 per cent Triton X-100 and was developed at 4 ml per hour with the same buffer. Proteins specifically bound were eluted with 0.02 M GalNAc.

Purification of gC on Immunosorbent

2×10^7 glucosamine labelled cells were suspended in 1 ml PBS supplemented with 0.5 per cent (v/v) Triton X-100. The cells were disrupted by sonication and insoluble material was sedimented by centrifugation at $100{,}000 \times g$ for 1 hour at 4° C in a SW 60 rotor. The soluble proteins were loaded on an immunosorbent column prepared as follows: HCl monoclonal antibodies against glycoprotein gC were coupled to CNBr activated Sepharose 4B (Pharmacia, Sweden) by adding 0.5 ml ascites (21 mg total protein per 1 g of Sepharose). The coupling was done for 2 hours at room temperature and the unbound antibodies were removed by extensive washing with the coupling buffer (0.1 M $NaHCO_3$ with 0.5 M NaCl). Remaining active groups on the Sepharose were blocked by incubation in ethanolamine, pH 8.5. The column was extensively washed in coupling buffer followed by wash on 0.1 M acetate buffer, pH 4.0, with 0.5 M NaCl. The column was equilibrated in PBS with 0.5 per cent Triton X-100. The soluble viral proteins in 2×10^7 cells were loaded (1.5 ml) on the immunosorbent column. The unbound protein was washed through the column with 0.5 per cent Triton X-100 in PBS. The specifically bound protein was eluted with 3 M KSCN in PBS with 0.5 per cent Triton X-100. The fractions of protein with or without affinity for the HCl antibodies were concentrated by precipitation in 10 per cent (w/v) TCA. The proteins were collected by centrifugation for 15 minutes at 15,000 rpm in s Sorwall RC-2 centrifuge and washed sequentially in 96 per cent ethanol and acetone. The dried protein was solubilized in 0.05 M Tris-HCl, pH 7.0 with 2 per cent (w/v) SDS, 5 per cent (v/v) 2-mercaptoethanol and 3 per cent (w/v) sucrose (disruption mixture), for SDS-polyacrylamide gel analysis (27). Both bound and unbound materials from the immunosorbent column were subjected to affinity chromatography on ConA-Sepharose (eluent 0.5 M α-methyl mannoside) in analogy with the procedure described above for HPA chromatography.

Immune Precipitation of HSV Glycoproteins

HSV-1 proteins with or without affinity for HPA were precipitated with TCA and washed in order to concentrate eluted protein. After wash the proteins were dried and

solubilized in buffer and reacted with specific monoclonal antibodies added in optimal amounts. The antigen-antibody complexes were bound to fixed staphylococci (strain cowan-I) (23). The immunoprecipitates were washed extensively in PBS supplemented with 0.5 M NaCl, 0.1 per cent (w/v) SDS and 0.4 per cent (v/v) Triton X-100. After further washing in the same buffer, supplemented with 1.0 M NaCl, the proteins were dissolved in disruption mixture for SDS-polyacrylamide gelelectrophoresis.

SDS-Polyacrylamide Gelelectrophoresis

SDS-polyacrylamide gelelectrophoresis was done according to Morse *et al.* (14). The separation gel was 9.25 percent (w/v) acrylamide cross-linked with 0.25 percent (w/v) dialyltartardiamide. The gels were stained and autoradiography was done on Kodak XRP-1 X-Omat film.

Desialylation

To remove sialic acid HSV glycoprotein gC without affinity for HPA was treated in amounts of less than 1 mg with of neuraminidase (1 unit) overnight at 37° C in 0.01 M acetate buffer, pH 5.5. Alternatively, aliquots of gC without affinity for HPA were subjected to treatment with 0.05 M H_2SO_4 at 85° C for 1 hour. The solution was neutralized with solid Na_2CO_3 and desalted by Sephadex G-25 gel filtration.

Treatment with Alkaline Borohydrid

The alkaline borohydride treatment was carried out as previously described (19). After fractionation by HPA chromatography the material was desalted on Sephadex G-25 and dissolved in 0.5 M NaOH and 0.5 M $NaBH_4$. This mixture was incubated for 48 hours at room temperature (21—23° C) under nitrogen in tefloncapped tubes. The reaction was stopped by adding glacial acetic acid and the results of the β-elimination reaction were assayed by Sephadex G-50 gel filtration.

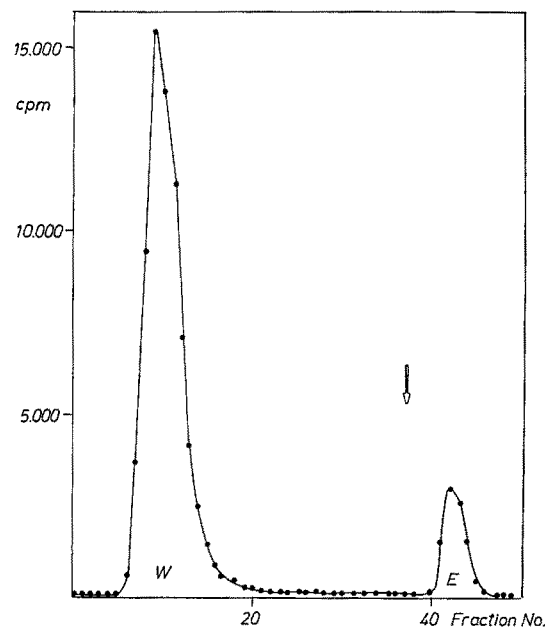

Fig. 1. HPA affinity chromatography of membrane glycoproteins from ^{14}C-glucosamine-labelled HSV-infected Vero cells. Addition of 0.02 M GalNAc is denoted by an arrow, bound *(E)* and unbound *(W)* fractions are indicated

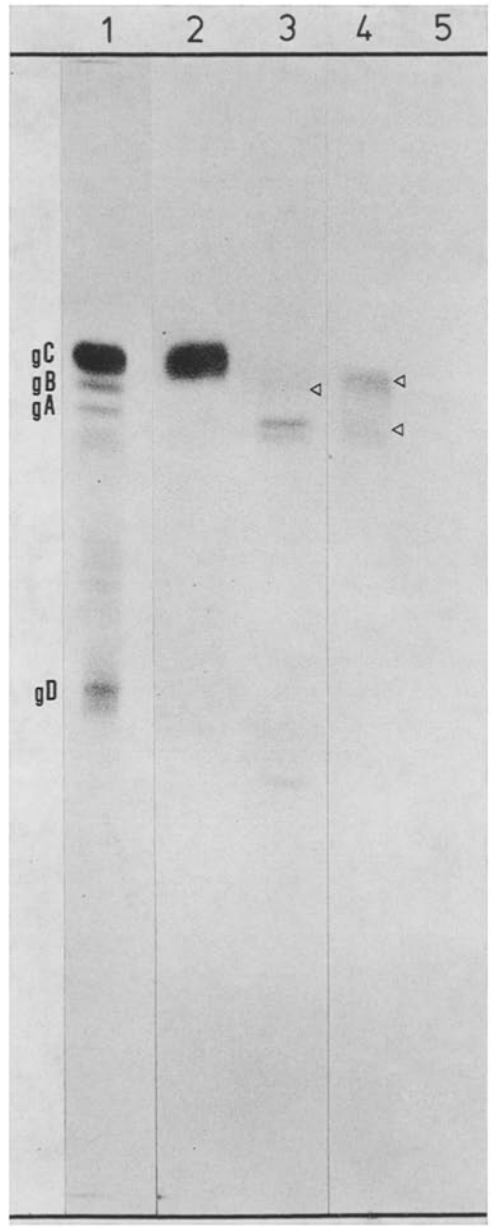

Fig. 2. The autoradiographic image of HPA purified glycoproteins identified by immunoprecipitation and separation on SDS polyacrylamide gel. The proteins were labelled with ^{14}C-glucosamine. Slot 1, total extract from HSV-1 (HFEM) *ts*B5 infected cells grown at 37° C and used as a reference. Slot 2 and 3, the unbound precipitated with monoclonal antibodies to gC and gA/gB respectively. Slots 4 and 5, the eluted proteins precipitated with monoclonal antibodies to gC and gA/gB respectively

Results

HSV Glycoproteins with and without Affinity for Helix Pomatia Lectin

Preparations of membrane proteins of HSV-1 infected Vero cells were subjected to HPA affinity chromatography (Fig. 1). Two fractions one with (bound) and the other without affinity (unbound) for the lectin were collected. The HPA binding material was eluted with 0.02 M GalNAc. Approximately 10 per cent of the HSV glycoprotein preparation demonstrated affinity for HPA. In order to identify the glycoproteins present both fractions were immunoprecipitated with monoclonal antibodies directed against gA/gB, gC or gD. Precipitated proteins were subsequently characterized in SDS-polyacrylamide gels.

The glycoprotein with affinity for HPA demonstrated gC specificity (Fig. 2, slot 4), but with a higher electrophoretic mobility than the nonbinding gC (Fig. 2, slot 2). The precursor to gC was also identified. Presence of glycoprotein gA/gB was not observed (Fig. 2, slot 5).

The glycoproteins without affinity for HPA contained gA/gB, gC and gD (Fig. 2, slots 2 and 3). The data on gD is not shown.

Purification of gC by Immunosorbent

Purification of gC was made possible by binding extracts of ^{14}C-glucosamine labelled infected cells on Sepharose immunosorbent columns containing monoclonal antibodies reactive with gC (Fig. 3). Material passing through the column and designated pool I was identified as a mixture of gA/gB, gD and their precursors as based on the electrophoretic mobilities observed. Pool II i.e. the

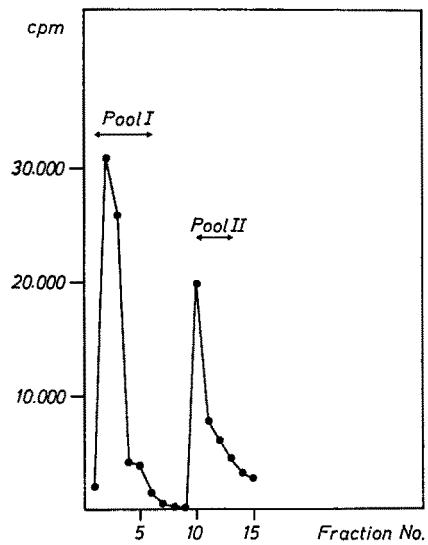

Fig. 3. Purification of gC by immunosorbent column. ^{14}C-glucosamine labelled cell extract from HSV-1 *(F)* infected cells were fractionated on the column. Pool I, the unbound material was isolated from the fractions 1—6, and pool II, the glycoproteins specifically eluted from the column were isolated from the pooled fractions 10—13

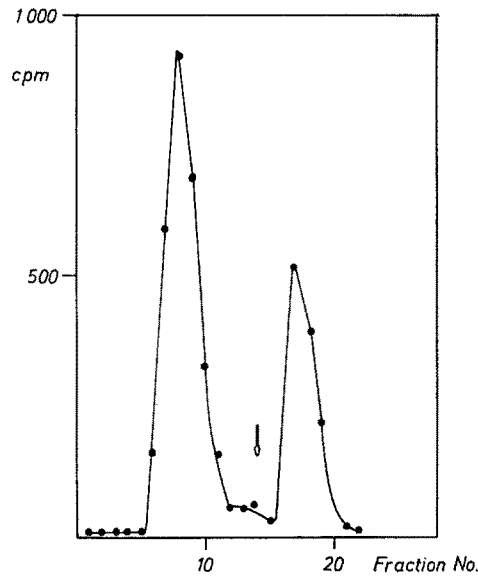

Fig. 4. HPA affinity chromatography of immunosorbent-purified gC. Addition of 0.02 M GalNAc is indicated by an arrow

specifically bound protein represented 30 to 40 per cent of the radioactive material loaded on the column, was identified as gC and precursors gC. Two additional polypeptide bands were detected corresponding to proteins with molecular weights of 76 K and 72 K (Fig. 5, slot 4).

HPA-Affinity or Immunosorbent Purified gC

The lectin affinity chromatography with immunosorbent-purified gC demonstrated that about 25 per cent of the isolated gC was biospecifically bound to HPA (Fig. 4). Both unbound and HPA-bound immunosorbent purified gC were subjected to SDS-PAGE. Two polypeptide bands with affinity for HPA were identified and both proteins had electrophoretic mobilities that were significantly higher than that of the major band identified without affinity for the lectin (Fig. 5, slots 2 and 3). These data together with those presented in Fig. 2 therefore indicate that among the HSV glycoproteins gA, gB, gC and gD only two subspecies of gC have affinity for HPA.

If the HPA-binding subfraction comprises an underglycosylated precursor to gC, one would expect that the non-binding fraction may consist of sialylized glycoproteins with masked HPA-binding oligosaccharides. For removal of such masking structures the gC fraction without HPA affinity was treated with neuraminidase or dilute H_2SO_4 before HPA chromatography (Fig. 6). However, only about 5 per cent of the originally HPA negative gC fraction became HPA positive after treatment with neuraminidase or H_2SO_4. Therefore the fraction of gC without affinity for the HPA seemed not to contain sialylized glycoprotein gC with GalNAc as penultimate sugars.

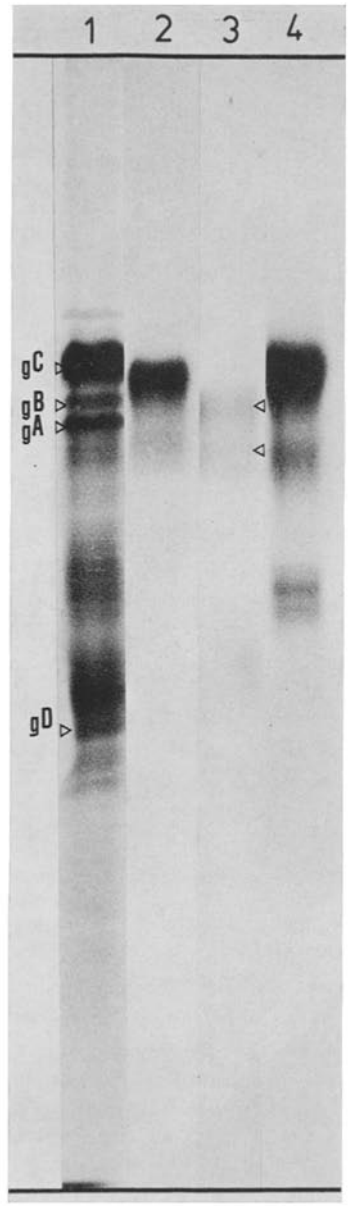

Fig. 5. The autoradiographic image of the immunosorbent purified, ^{14}C glucosamine labelled gC with affinity for HPA. Identified on SDS polyacrylamide gel. Slot 1, total extracts from HSV-1 (HEFM) *ts* B 5 infected cells grown at 34° C. Slot 2, gC isolated from pool W of an HPA column without a purification on immunosorbent. The protein was precipitated with monocloanl antibodies to gC (analogous to Fig. 2, slot 2). Slot 3, the fraction of immunosorbent purified gC which could bind to HPA. Slot 4, immunosorbent purified gC (analogous to Fig. 4, slot 3). Notice the difference in the electrophoretic mobility between gC isolated by immunosorbent chromatography (slot 4) and the sub-population of gC which had HPA affinity (slot 3, marked with arrows)

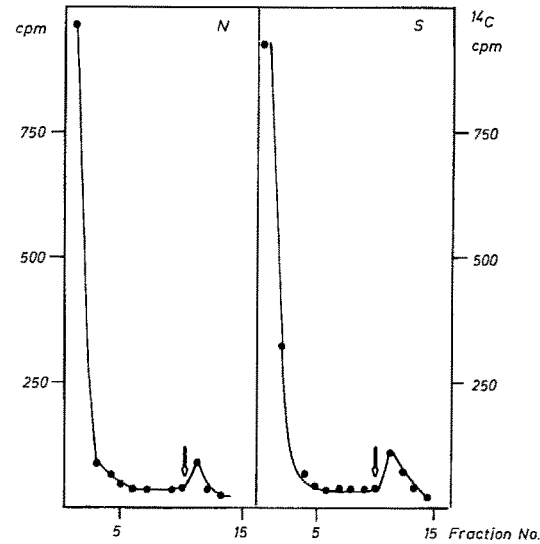

Fig. 6. Effect of treatment with neuraminidase *(N)* and 0.05 M H_2SO_4 *(S)* on gC fractions without affinity for HPA (corresponding to fractions 6—11 of Fig. 4). Additions of 0.02 M GalNAc indicated by arrows

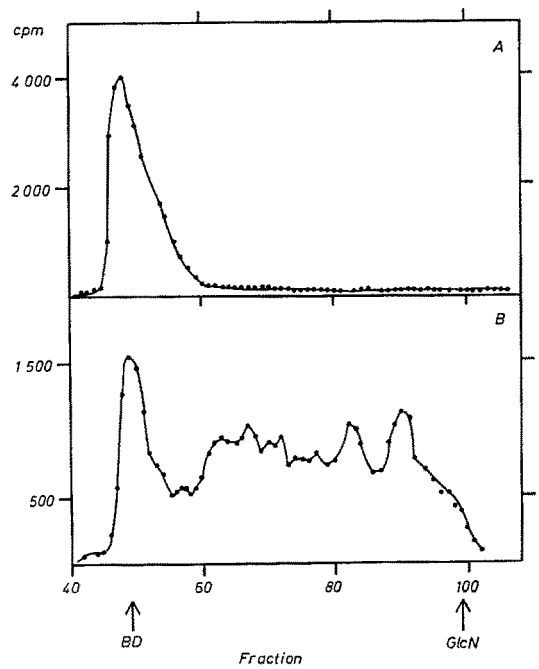

Fig. 7. Gelfiltration on Sephadex G 50 of reaction-mixtures from alkaline borohydride treatment of ^{14}C-glucosamine-labelled gC fraction without affinity for HPA. Glycoprotein were treated with TBS (mock-treatment, panel *A*) and 0.5 M $NaBH_4$ in 0.5 M NaOH *(B)*. Void volume and totally included volume were determined by Blue Dextran *(BD)* and glucosamine *(GlcN)* respectively. The oligosaccharide of the VSV Glycoprotein (about 3500 daltons) eluted between fractions 70 and 72

Oligosaccharide-Peptide Linkages of gC

Binding of gC to HPA suggests presence of terminal GalNAc and also presence of O-linked oligosaccharides. The finding that only a subfraction of gC contained HPA binding activity even after desialylation raised the question whether or not the subfraction which primarily did not bind to HPA contained oligosaccharides O-glycosidically linked to the peptide. Immunosorbent purified gC was therefore fractionated by HPA affinity chromatography and the subfraction not binding to HPA subsequently subjected to treatment with alkaline $NaBH_4$. Before and after treatment with $NaBH_4$ the fractions of ^{14}C-glucosamine labelled gC was studied on Sephadex G50. Untreated or mocktreated material was found in the void volume exclusively (panel A, Fig. 7). After treatment with alkaline $NaBH_4$ release of labelled material was demonstrated. The molecular weights of these structures corresponded to 3000 or more. The results were interpreted as β-elimination of O-linkages between oligosaccharides and peptides of gC. Experiments with pronase-digested underglycosylated HSV glycoprotein produced in the presence of tunicamycin indicate existence of at least two classes of relatively large tunicamycin-resistent oligosaccharides; with and without affinity for HPA (Olofsson *et al.*, submitted for publication). These data support the conclusion that more than one type of O-linked sugar may be attached to HSV glycoproteins.

Oligosaccharides with N-glycosidical linkages of the high mannose type or moderately branched complex type exhibit affinity to Con A (13). Testing of the HPA binding and non-binding fractions of gC with Con A affinity chromatography revealed approximately the same abundance of biospecific binding of both the gC fractions to Con A (Fig. 8). These data indicate that N-glycosidically linked

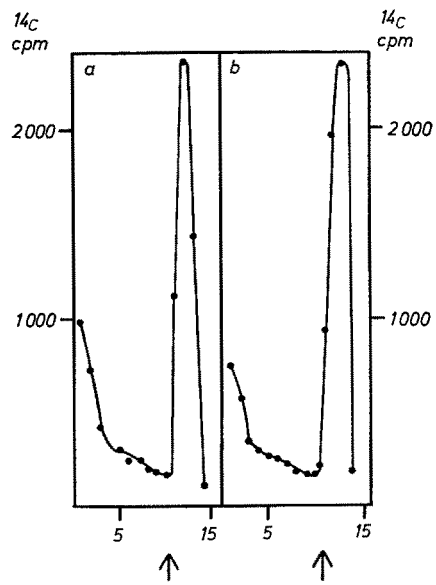

Fig. 8. Con A affinity chromatography of HPA-binding *(a)* and nonbinding *(b)* fractions of gC from the experiments of Fig. 5. Arrow denotes addition of 0.5 M α-methyl mannoside

oligosaccharides are prominent constituents in gC with affinity for HPA as well as in gC without binding capacity for HPA. N-glycosidically and O-glycosidically linked oligosaccharides are thus present on the one and same gC molecule.

Discussion

The viral glycoproteins (gA/gB, gC and gD) synthesized in HSV-1 infected cells are glycosylated in several discrete steps (2, 3, 27). We have found that the type-specific glycoprotein, gC, of HSV-1 contains oligosaccharides binding to HPA, a lectin with specific affinity for GalNAc (11). This sugar is generally absent in normal N-glycosidic oligosaccharides but is present in oligosaccharides linked O-glycosidically to a serine or a threonine residue of the polypeptide. Recent findings that glycoprotein gC is sensitive to weak alkaline borohydride treatment (19) and contains TM resistent oligosaccharides (Olofsson *et al.*, submitted for publication) further supports that gC, in addition to the N-glycosidic carbohydrate chains, contains O-glycosidically linked oligosaccharides.

In the present study the immunochemical specificity of the HSV glycoproteins without affinity for HPA and those with affinity for the lectin were identified by immunoprecipitation with monoclonal antibodies against HSV specified glycoproteins. The glycoproteins without affinity for HPA contained gA/gB, gD and the majority of gC, whereas only glycoproteins with the immunological specificity of gC were found in the fraction binding to HPA. The data thus emphasized that of the HSV-glycoproteins only gC contains oligosaccharides with affinity for HPA.

Using immunosorbent-purified glycoprotein it could be clearly demonstrated that glycoproteins with HPA-binding oligosaccharides constituted a subfraction comprising about 25 per cent of the glycosylated gC population (Fig. 4). However, our data also show that gC without affinity for HPA contains O-linked carbohydrates, and that O-glycosidic as well as N-glycosidic linkages exist on the one and same gC molecule (Figs. 7 and 8).

The electrophoretic analysis of the HPA-binding gC subfraction revealed two polypeptides, one with a molecular weight of 108 K which corresponds to the partially glycosylated precursor of gC (pgC) and one with a molecular weight of 121 K which is intermediate between that of the fully glycosylated gC and that of pgC. The low molecular weights of HPA-binding gC molecules relative to the bulk of glycoproteins with gC specificity suggest that gC with affinity for HPA constitute partially glycosylated gC intermediates. If we assume that the HPA-binding oligosaccharides represent such precursors to larger O-glycosidic oligosaccharides lacking HPA-affinity it might be suspected that HPA-binding sites were blocked by terminal sialic acids as observed for HPA-binding oligosaccharides of T lymphocytes (1, 8). However, our experiments with two different procedures for elimination of sialic acids only marginally increased the HPA-binding activity of gC, suggesting that sialylation of HPA-binding sites was not a main reason for absence of HPA binding properties. The HPA-binding glycoprotein might therefore represent a fully glycosylated fraction of gC, that is glycosylated differently than the bulk of gC.

One possible explanation would be that the biosynthesis of the gC-associated O-glycosidic oligosaccharides at some point is branched and follows different

pathways, one leading to HPA-binding oligosaccharides and the other to oligosaccharides with terminal structural properties without affinity for HPA. This diversity may be caused either solely by host cell glycosyl transferases or by one or more virus specified or virus modified enzymes acting in concert with the cell specified glycosyl transferases. We have previously pointed out the changes of kinetic properties of glycosyl transferases after HSV infection of cells (21). Presence of competing glycosyl transferases leading to biosynthetic branching of a core structure into different types of oligosaccharides with and without blood group A activity has been demonstrated with O-linked carbohydrates of the sheep submaxillary mucin (24).

In presence of TM only trace amounts of glycoproteins gA/gB and gC were demonstrable on the surface of the infected cells as shown by iodination of the intact cells (16) and by indirect immunofluorescence with monoclonal Ab HC-1 of fixed cells (unpublished data). In fact gC was the only glycoprotein transported to and inserted into the plasmamembrane in significant amounts in presence of TM. The gC exposed on the cell surface lacked reactivity in the antibody-dependent cell-mediated cytotoxicity test a finding which suggests that presence of N-glycosidic oligosaccharides were essential for the ADCC reaction (16). On the other hand the presence of O-linked carbohydrates seems sufficient for transport of glycoproteins to the plasmamembrane and different functions of the two classes of oligosaccharides on the HSV glycoprotein gC might thus be discerneable.

Acknowledgments

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