Demonstration and Mapping of Highly Carbohydrate-dependent Epitopes in the Herpes Simplex Virus Type 1-Specified Glycoprotein C

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SUMMARY

The carbohydrate dependence of epitopes in the herpes simplex virus type 1-specified glycoprotein C (gC) was studied using a new solid-phase assay procedure. Glycoprotein C, coated on 96-well microtitre plates, was treated with sialidase and increasing concentrations of periodate. A sequential removal of peripheral monosaccharides from the oligosaccharides of gC was ascertained by an enzyme-linked lectin assay. By using a panel of gC-specific monoclonal antibodies in ELISA, it was found that gC contained two types of epitopes differing in their dependence on terminal galactose and sialic acid for expression. Control experiments indicated that the carbohydrate-dependent epitopes were peptide structures and that the carbohydrates did not directly participate in the antibody-binding reaction. The carbohydrate-dependent epitopes were mapped to antigenic site II, according to the proposed nomenclature, whereas those expressed also in the absence of peripheral sugars were located mainly in antigenic site I. These results were compatible with the relative distribution of oligosaccharides in the gC molecule.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) specifies a number of antigenically distinct glycoproteins, designated gB [apparent molecular weight 118 000 (118K)], gC (130K), gD (59K), gE (70K to 80K) and gH (115K) (Spear, 1976, 1985; Baucke & Spear, 1979; Showalter et al., 1981; Buckmaster et al., 1984). Glycoprotein C differs from the other glycoproteins both with respect to its high carbohydrate content (Wenske & Courtney, 1983; Kikuchi et al., 1984; Spear, 1985), but also with respect to qualitative properties of some of its oligosaccharides, which lack counterparts in the other HSV-1-specified glycoproteins (Olofsson et al., 1981, 1983). Even a low estimate of the carbohydrate content of gC, based on differences in the electrophoretic mobilities between the fully glycosylated protein and a partially glycosylated precursor indicates that the sum of the oligosaccharide molecular weights is more than 30K (Wenske & Courtney, 1983; Kikuchi et al., 1984). From the nucleotide sequence of the gene encoding gC, as many as nine potential sites for N-glycosylation have been identified (Frink et al., 1983). In addition to these carbohydrates, gC contains at least two classes of O-glycosyl oligosaccharides (Olofsson et al., 1981, 1985), which seem to be arranged in Pronase-resistant arrays on the polypeptide as has been described by Dall’Olio et al. (1985). One of the qualitative differences between gC and other HSV-specified glycoproteins is that gC contains O-glycosyl oligosaccharides with affinity for the N-acetylgalactosamine-specific Helix pomatia lectin (HPA) (Olofsson et al., 1981, 1983).

Recently, two interesting biological activities have been attributed with gC. First it was shown that gC but no other HSV glycoprotein can act as a receptor for the C3b factor of the complement system (Cines et al., 1982; Friedman et al., 1984). This activity can be exerted not...
only by isolated and purified gC but also by the native glycoprotein, situated in the plasma membrane (Smiley & Friedman, 1985). It was also demonstrated that gC constitutes a virus attachment protein and, thus, is important for the initiation of a productive infection (Fuller & Spear, 1985; B. Svennerholm, personal communication).

The carbohydrate complement of a highly glycosylated protein, such as gC, might influence the antigenic and also the biological properties of the glycoprotein (Woodward et al., 1985). This may occur either because (i) the oligosaccharide contains a highly antigenic sugar sequence or because (ii) multiple oligosaccharides force the glycoprotein into a particular conformation essential for the expression of certain epitopes. In the present paper it is shown that gC contains epitopes which are highly dependent on carbohydrates for their expression and that (ii) rather than (i) above explains this phenomenon.

**METHODS**

**Virus and cells.** The HSV-1 strain F (kindly supplied by Dr B. Roizman) and the BHK-21 cell line were used throughout the study. The cells were infected at a multiplicity of 10 p.f.u./cell. Conditions for propagation and maintenance of cells have been published elsewhere (Olofsson et al., 1981, 1983).

**Preparation of antigens**

**Immunosorbent-purified gC.** The details for production of immunosorbent-purified gC have been reported previously (Olofsson et al., 1983). Briefly, a membrane fraction from HSV-infected cells was produced by the method of Springer et al. (1977). This fraction was solubilized in 0-1 m-glycine buffer pH 8-8 containing 1-0% (v/v) Triton X-100 and centrifuged at 100000g for 1 h. The solubilized glycoproteins were subjected to affinity chromatography using the gC-specific monoclonal antibody BIC1 coupled to CNBr-activated Sepharose, as previously outlined (Olofsson et al., 1983). The purified glycoprotein was subjected to SDS–polyacrylamide gel electrophoresis according to the procedure of Morse et al. (1978), and was found to migrate as a single band corresponding to an apparent molecular weight of 130K (Fig. 1).

**gC from tunicamycin-treated cells.** HSV-infected cells were propagated in the presence of 2μg/ml tunicamycin; in parallel, HSV-infected cells were cultured without the drug. A membrane fraction was produced, and this fraction was solubilized as described above. A gC fraction was prepared by means of lectin affinity chromatography on HPA as previously described (Olofsson et al., 1983). The gC fraction was eluted with 0-02 m-N-acetylgalactosamine (GalNAc).

**Antisera and monoclonal antibodies.** Characterization of the gC-specific monoclonal antibody BIC1 has been published previously (Olofsson et al., 1983; Sjögren-Jansson & Jeansson, 1985). In addition, gC-specific monoclonal antibodies, designated C1, C3, C8, C11 and C13, were used. These antibodies have been mapped and characterized in detail (Marlin et al., 1985).

**Polyclonal antiserum against gC (K642) was produced by immunization of rabbits with immunosorbent-purified glycoprotein as outlined elsewhere (Olofsson et al., 1983).**

**Periodate treatment and enzyme-linked assays.** Polystyrene 96-well microtitre plates (Nunc) were coated with purified preparations of gC (in 0-1 m-sodium carbonate buffer pH 9-6) by incubation for 2 h at 37 °C at 100 μl per well. In most experiments the coating concentration was 0-25 μg/ml of purified gC, but similar results were obtained with 0-5 μg/ml of protein. The wells were subsequently treated with 50 μl of sialidase (Behringwerke; 0-1 unit/ml at 37 °C for 2 h) and thereafter with serial dilutions of periodate, according to Woodward et al. (1985), as indicated in the legends to the figures. The plates were subsequently incubated with mouse monoclonal antibodies or the rabbit polyclonal monospecific serum against gC. The reactions were visualized as described below. All serum dilutions used in this assay were optimized by checkerboard titrations in which the antigen coating concentration and the antibody dilution were varied.

The effect of the combined sialidase–periodate treatment on the oligosaccharide side chains was determined by the use of biotinylated lectins from wheat germ (WGA), *Ricinus communis* (RCA) and HPA. The reactions were visualized by addition of horseradish peroxidase-conjugated avidin (lectin assays) and horseradish peroxidase-conjugated or alkaline phosphatase-conjugated goat antibodies against mouse or rabbit IgG as described by van der Schaal et al. (1984). The peroxidase-treated plates were read at 498 nm and the alkaline phosphatase-treated plates were read at 405 nm.

To rule out the possibility of chemical modification of the peptide portion caused by the periodate treatment, a control experiment described by Woodward et al. (1985), with a non-glycosylated protein, was carried out. Microtitre plates were coated with bovine serum albumin (BSA), and the plates were treated with periodate under the same conditions as described above for gC, except that the sialidase treatment was omitted. The plates were coated with two concentrations of BSA (10 μg/ml and 1 μg/ml) and four dilutions of polyclonal anti-BSA antibodies were tested. In all combinations tested, the absorbance values ranged between 1-2 and 0-9. None of the coating density/serum dilution combinations demonstrated any significant difference between untreated wells...
Carbohydrate-dependent epitopes of HSV gC

Fig. 1. SDS-polyacrylamide gel electrophoresis of immunosorbent-purified gC used in the sialidase-periodate experiments. About 30 μg of purified gC was precipitated with 10% trichloroacetic acid and subjected to electrophoresis on a 9.25% gel. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R. The positions of commercial protein molecular weight markers are indicated.

and those treated with the highest concentration, 50 mM, of periodate. These data indicated that the conditions used were not sufficient for producing modifications in the amino acid side chains of the peptide sequence, and did not result in a decrease in the antigenic activity of the protein.

Treatment with β-galactosidase. Digestion with β-galactosidase was carried out on purified gC, coated onto microtitre plates as described above. Prior to galactosidase treatment, terminal sialic acid was removed as described above. The wells were incubated with 100 μl Tris-buffered saline (supplemented with 1 mM-MgCl₂), containing 1, 10 or 100 units/ml of β-galactosidase (Sigma, grade X), for 4 h at 37 °C. Thereafter, the plates were washed and lectin or antibody affinities were assayed as described above.

RESULTS

Sialidase-periodate treatment and the carbohydrate complement of gC

The effects on purified gC of the combined sialidase-periodate treatment was determined by an enzyme-linked lectin assay (Fig. 2), in which the gradual removal of carbohydrates was monitored by the use of RCA and WGA. These lectins were chosen because they both bind to the peripheral sugars in complex-type N-glycosyl oligosaccharides as indicated in Fig. 3. Without any treatment gC bound WGA but not RCA. After sialidase treatment, the WGA affinity disappeared concomitantly with an increase in RCA-binding activity (Fig. 2a). This is consistent with the disappearance of distal sialic acid and exposure of subterminal galactose, occurring in N-glycosyl complex-type oligosaccharides. With increasing concentrations of periodate after the sialidase treatment, the RCA affinity of gC disappeared followed by an increase in the WGA affinity. Most likely this is due to the exposure of N-acetyl-D-glucosamine (GlcNAc) residues situated as indicated in Fig. 3. This WGA-binding activity was found to peak between 3 and 12 mM-periodate added after the sialidase treatment. The data thus indicated that the combined sialidase-periodate treatment removed at least two peripheral sugars of N-glycosyl oligosaccharides.

The finding that the treatment inhibited the HPA-binding activity of gC indicated that O-glycosyl oligosaccharides were also sensitive to periodate. The HPA-binding activity was, in contrast to the RCA-binding activity, not increased by sialidase treatment (Fig. 2), further supporting our previous conclusion that HPA-binding oligosaccharides of gC do not become sialylated (Olofsson et al., 1983).

Effect of the sialidase-periodate treatment on the antigenic reactivity

The sialidase-periodate treatment described above was used to (i) determine whether the presence of carbohydrates is essential for the antigenic reactivity of gC and (ii) if so, how much of the carbohydrate can be removed without any decrease in the antigenic reactivity. The reactions between two gC-specific monoclonal antibodies and gC were measured against increasing concentrations of periodate (Fig. 4). With one of these antibodies the sialidase-periodate treatment of gC significantly reduced the antibody binding as determined by ELISA. This inhibition took place after treatment with sialidase plus a periodate concentration of 1 mM or less, only removing the sialic acid and the penultimate galactose as determined by lectins in parallel experiments (dotted line in Fig. 4). The reactivity with the other monoclonal antibody,
on the other hand, was not reduced even by the highest concentration of periodate. Removal of galactose by an independent method (β-galactosidase digestion) demonstrated that the decrease in the B1C1-binding capacity of gC paralleled a decrease in the RCA-binding activity (Fig. 5), further indicating that terminal sialic acid and galactose are essential for the binding of this antibody. The data, therefore, suggested that gC contained at least two kinds of epitopes; those dependent on sialic acid and galactose for their expression and those expressed even in the absence of these peripheral sugars.
Carbohydrate-dependent epitopes of HSV gC-1

Fig. 4. Effects of the combined sialidase-periodate treatment on epitopes of gC. Purified gC was allowed to adsorb to microtitre plates which were treated with sialidase and periodate as indicated. The plates were incubated with a constant dilution of monoclonal antibodies directed against gC, i.e., B1C1 (▲, 1/6000) and C11 (■, 1/30000), or polyclonal gC-specific antiserum K642 (●, 1/800). The reactions were quantified by enzyme-conjugated goat antibodies against rabbit or mouse IgG in ELISA. A parallel experiment with biotinylated RCA is indicated by a dotted line.

Fig. 5. Influence of β-galactosidase treatment on carbohydrate-dependent epitopes of gC. Purified gC was coated on microtitre plates and the wells were treated with sialidase. Subsequently, the plates were treated with increasing concentrations of β-galactosidase as indicated. Thereafter, the plates were incubated with monoclonal antibody B1C1 (filled bars) or RCA (open bars) and subjected to ELISA (see Fig. 4). Means and standard errors of the mean of triplicate experiments are indicated.

A polyclonal antiserum monospecific for gC was also tested (Fig. 4). The reactivity of the gC-specific antiserum was decreased by the combined sialidase-periodate treatment of gC as observed for monoclonal antibody B1C1. These results suggested that carbohydrate-dependent epitopes, such as those recognized by B1C1, significantly contributed to the reactivity of gC.

Antigenic reactivity of gC produced in the presence of tunicamycin

In the next series of experiments the antigenic reactivity of gC produced in the presence of tunicamycin was compared with that of fully glycosylated gC. We and others have previously shown that in the presence of tunicamycin an underglycosylated gC, totally devoid of N-glycosyl oligosaccharides and with a molecular weight of about 100K, is produced (Olofsson et al., 1983; Wenske & Courtney, 1983). Despite the lack of N-glycosyl oligosaccharides the underglycosylated gC contains O-glycosyl oligosaccharides, of which one class is recognizable by its affinity for HPA (Olofsson et al., 1983). Two gC preparations, one produced in the presence of tunicamycin and the other in the absence of tunicamycin, were purified by means of HPA chromatography. Microtitre plates were coated with 2 μg/ml of the gC preparations mentioned and the reactivities with lectins and antibodies were tested (Table 1). The HPA reactivity was similar with untreated gC and gC produced in the presence of tunicamycin whereas the RCA reactivity of gC produced in the presence of tunicamycin was significantly reduced compared with the untreated gC. The results are consistent with the notion that the HPA-binding O-glycosyl oligosaccharides were not affected by tunicamycin, while most of the RCA-binding galactose is associated with N-glycosyl oligosaccharides.
Mapping of carbohydrate-dependent epitopes of gC. Monoclonal antibodies specific for different epitopes of gC (see Methods) were incubated with gC subjected to the combined sialidase-periodate treatment. The antibody dilutions were chosen to give an initial absorbance value of 1.0. The reactions were visualized as for Fig. 4. The following antibodies were tested: (a) C8 (○, dilution 1/200), C11 (■, 1/30000), C13 (▲, 1/10000); (b) C1 (■, 1/500), C3 (●, 1/10000). Similar results were obtained if (i) each antibody was tested at a dilution of 1/300 and (ii) if a gC coating concentration of 0.5 or 1.0 μg/ml was used.

Table 1. Effect of tunicamycin on the antigenic reactivity of gC*

<table>
<thead>
<tr>
<th>Antibody/lectin†</th>
<th>Absorbance value (mean ± S.E.M.) obtained with gC</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>- Tunicamycin</td>
<td>+ Tunicamycin</td>
</tr>
<tr>
<td>HPA</td>
<td>0.532 ± 0.005</td>
<td>0.527 ± 0.003</td>
</tr>
<tr>
<td>RCA</td>
<td>0.640 ± 0.007</td>
<td>0.195 ± 0.001</td>
</tr>
<tr>
<td>B1C1</td>
<td>1.275 ± 0.002</td>
<td>0.224 ± 0.002</td>
</tr>
<tr>
<td>C4H11</td>
<td>0.818 ± 0.004</td>
<td>0.252 ± 0.005</td>
</tr>
<tr>
<td>CI1</td>
<td>0.949 ± 0.003</td>
<td>0.355 ± 0.002</td>
</tr>
<tr>
<td>K642</td>
<td>1.571 ± 0.005</td>
<td>0.442 ± 0.003</td>
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</tbody>
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* Glycoprotein C from tunicamycin-treated cells and untreated cells was purified by HPA affinity chromatography. Microtitre plates were coated with similar concentrations of each gC preparation as described in Methods. The binding of biotinylated lectins and antibodies was measured as described in Methods.
† The lectins were used at 10 μg/ml and the monoclonal antibodies at a dilution of 1/200. The polyclonal gC antiserum was used at a dilution of 1/400.

When the binding of gC-specific antibodies and RCA was tested, we found that tunicamycin-treated gC reacted to a much lower extent than did untreated gC. It should be mentioned that the C11 monoclonal antibody, the binding of which was not inhibited by the sialidase-periodate treatment of gC, did not differ from the other gC-specific monoclonal antibodies in reactivity with tunicamycin-produced gC. These results indicated that (i) a total lack of N-glycosyl oligosaccharides resulted in a major decrease in the activity of most epitopes of gC and that (ii) the presence of O-glycosyl oligosaccharides alone was not sufficient for maintenance of these epitopes.

Mapping of carbohydrate-sensitive epitopes

The epitopes of gC have been mapped into two distinct sites, designated I and II (Marlin et al., 1985). Monoclonal antibodies reacting with each of these antigenic sites were used to investigate whether the sialidase-periodate-sensitive epitopes of gC belonged to one or both antigenic sites (Fig. 6). It was found that the reactivities of antibodies C3, C8 and C13, all
specific for epitopes situated in antigenic site II, were significantly decreased by the sialidase-periodate treatment. In contrast, the reactivity of antibody C11, reacting with antigenic site I, was not significantly decreased even by high concentrations of periodate. For the other antibody specific for site I, C1, a slightly different pattern was observed. Thus, the reactivity of this antibody was increased by treatment of gC with low to moderate concentrations of periodate. At the highest concentrations, a slight decrease in the activity was found. However, this decrease was much less pronounced than that observed for the reaction between gC and antibodies reacting with antigenic site II. It is probable that the initial increase in the reactivity of C1 was due to periodate-induced removal of peripheral sugars partially masking the specific epitope recognized by this antibody. Altogether, the results indicated that the carbohydrate-dependent epitopes of gC are clustered in antigenic site II, while at least two epitopes of site I were not inactivated by removal of peripheral sugars.

**Evidence for carbohydrate stabilization of peptide epitopes**

The finding that tunicamycin-produced gC reacts to a lesser extent with gC-specific antibodies than did untreated gC suggested that O-glycosyl oligosaccharides do not constitute the B1C1-binding epitope. To evaluate further whether carbohydrate sequences bind at all to the monoclonal antibodies, we incubated these antibodies with gC in the presence of competing sugars (Fig. 7). Of the monosaccharides tested none, even at 25 mM, inhibited the binding between gC and B1C1. In contrast, the binding between HPA and gC was significantly reduced at a concentration as low as 1 mM of the relevant monosaccharide, GalNAc. Similar results have also been obtained for (i) the binding of gC and RCA and (ii) for the binding between gC and the other carbohydrate-dependent monoclonal antibodies used in the present study (data not shown). Altogether, our data support the conclusion that the peptide sequences rather than carbohydrate structures constitute the epitopes for the monoclonal antibodies studied.
In the present paper it is shown that the HSV-1-specified glycoprotein C contains some epitopes that are highly dependent on carbohydrates for their expression. So far, these epitopes seem to be unique among viral glycoproteins as their reactivity is lost after treatments removing only a small fraction of the carbohydrate content. Carbohydrate-dependent epitopes have been demonstrated previously for glycoproteins of other viruses such as Newcastle disease virus (Merz et al., 1981; Long et al., 1986), Semliki Forest virus (Kaluza et al., 1980) and influenza virus (Gitelman et al., 1981; Hongo et al., 1986). However, the carbohydrate dependence of these epitopes was detected in glycoprotein produced in the presence of tunicamycin, resulting in a completely non-glycosylated protein (Long et al., 1986; Kaluza et al., 1980) or in extensively glycosidase-treated glycoproteins (Gitelman et al., 1981).

Our results indicate that the removal of as few as one or two peripheral sugars from gC has a dramatic effect on the reactivity of its carbohydrate-dependent epitopes. One explanation may be that the binding determinants in fact are identical with the terminal sugar sequences in analogy with the blood group antigens. However, several independent lines of evidence in the present paper clearly show that the epitopes studied are of a peptide nature, and that carbohydrates, although they are necessary for reactivity, only indirectly contribute to the stability of the epitope.

Two major antigenic sites in gC, designated I and II, have been identified (Marlin et al., 1985). The results of the present paper show that the carbohydrate-dependent epitopes are clustered in site II, while the epitopes resistant to the periodate-sialidase treatment are located in antigenic site I. Mapping studies indicate that antigenic site II probably is located in the amino-terminal half of gC (Holland et al., 1984; Marlin et al., 1985) which also contains the majority of the carbohydrates. Thus, eight of the nine potential sites for N-glycosylation are situated in this latter region of gC (Frink et al., 1983). In addition, most of the O-glycosidic oligosaccharides are clustered in the amino-terminal part of gC (Dall'Olio et al., 1985; Campadelli-Fiume & Serafini-Cessi, 1985; Olofsson et al., 1986). Evidence for the extremely high carbohydrate content of this region was further strengthened by the finding that the N-terminal CNBr fragment of gC, with a theoretical peptide mol. wt. of 15K in fact appeared as a 63K glycopeptide in gel filtration (Kikuchi et al., 1984). Due to this high number of oligosaccharides in the proximity of site II it is evident that removal of one or two terminal monosaccharides from each oligosaccharide would have a prominent effect on the total hydrophilicity of this domain, which may lead to changes in the polypeptide folding and disappearance of the epitopes recognized by site II-specific monoclonal antibodies.

The most probable location of antigenic site I is between amino acids 297 and 360 of gC (Homa et al., 1986), and it seems to contain very little carbohydrate. Thus, only one potential site for N-glycosylation was recognized (Frink et al., 1983), and it seems unlikely that this region contains any O-glycosyl oligosaccharides (Dall'Olio et al., 1985; Olofsson et al., 1986). The hydrophilicity of this scarcely glycosylated part of gC is little affected by removal of the peripheral sugars of this single oligosaccharide, explaining why the epitopes of site I are also expressed in the absence of peripheral monosaccharides.

The hypothesis that non-specific physical properties, such as the degree of hydrophilicity, of the oligosaccharides are sufficient to maintain at least one carbohydrate-dependent epitope is supported by the finding that pgC, a partially processed precursor to gC with large high-mannose oligosaccharides instead of the complex-type oligosaccharides (Spear, 1985; Campadelli-Fiume & Serafini-Cessi, 1985), is also recognized by antibody B1C1 (Olofsson et al., 1983). However, there are other gC-specific monoclonal antibodies that recognize only the fully processed form of gC (Holland et al., 1983). The observation that the polyclonal antibody was inhibited by sialidase-periodate treatment of gC suggested that carbohydrate-dependent epitopes significantly contribute to the overall antigenicity of gC.

The results in the present paper have several implications. Purified gC has been used as an antigen in ELISA for the detection of HSV-1 type-specific antibodies, but the method is hampered by findings that the HSV type specificity of the antigen is highly variable and very batch-dependent (Suchankova et al., 1984; Svennerholm et al., 1984). These previously
unexplained variations might very well have been due to carbohydrate-dependent variations in the epitopes, similar to those described in the present paper. At present the biological significance of this carbohydrate-induced modulation is not fully understood. However, if gC's secondary and tertiary structure varies depending on the carbohydrate content, it is clear that these variations in glycosylation might also interfere with the ability of gC to absorb to permissive cells, or with the C3b receptor activity. Evidence supporting at least the latter statement is that the C3b receptor activity is inhibited by sialidase treatment of gC (Smiley & Friedman, 1985).

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